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Caractérisation de la fonction vasculaire dans les vaisseaux sanguins isolés en réponse au glucose élevé et de l'artère mammaire interne de patients diabétiques

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1. Cyril Auger, Amissi Said, **Phuong Nga Nguyen**, Noureddine Idris-Khodja, Valérie B. Schini-Kerth**.** Potential of food and natural products to promote endothelial and vascular health. *(Journal of Cardiovascular Pharamacology, 2016.).*

2. Sherzad Rashid, Noureddine Idris-Khodja, Cyril Auger, **Phuong Nga Nguyen**, Claire Kevers, Joel Pincemail, Mahmoud Alhosin, Nelly Boehm, Monique Oswald-Mammosser and Valérie B. Schini-Kerth. Anthocyanin-rich blackcurrant juice prevents ED in the mesenteric artery of cirrhotic rats with portal hypertension: Role of oxidative stress and the angiotensin system. *(Journal of Functional Food, submitted, 2016.).*

II. Conference

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ABBREVIATIONS

ACh	: acetylcholine
AGEs	: Advanced Glycation End-products
Ang II	: angiotensin II
ANOVA	: analysis of variance
AT1R	: AT1 receptor of angiotensin II
AT2R	: AT2 receptor of angiotensin II
ATP	: adenosine triphosphate
BH_4	: tetrahydro – L – biopterin
BK	: bradykinin
BMI	: body mass index
CABG	: coronary artery bypass grafting
CAD	: coronary arterial disease
cAMP	: cyclic adenosine monophosphate
cGMP	: cyclic guanosine monophosphate
CVD	: cardiovascular disease
DAG	: diacylglycerol
DHE	: dihydroethidine
DM	: diabetes mellitus
EDCF	: endothelium-derived constricting factor
EDHF	: endothelium-derived hyperpolarizing factor
EDRF	: endothelium-derived relaxing factor
eNOS	: endothelial nitric oxide synthase
GTP	: guanosine triphosphate
HbA1c	: glycated hemoglobin
HDL	: high-density lipoproteins
HG	: high glucose
IDF	: International Diabetes Federation
IMA	: internal mammary artery
LDL	: low-density lipoproteins
mins	: minutes
NAD+	: nicotinamide adenine dinucleotide
NADH	: reduced nicotinamide adenine dinucleotide
NADPH	: nicotinamide adeninedinucleotide phosphate
NADPH Ox	: nicotinamide adeninedinucleotide phosphate oxidase
NF-κB	: transcription factor nuclear factor-kappa B
NO	: nitric oxide
OADs	: orally administered antidiabetic drugs
$ONOO^-$	: peroxynitrite
OR	: Odds ratio
PCA	: porcine coronary artery
PE	: phenylephrine
pEC_{50}	: negative logarithm of the half maximal effective concentration
PKC	: protein kinase C
PLC	: phospholipase C
PLD	: phospholipase D
RAGE	: Advanced Glycation End-products Receptors

ROS	: reactive oxygen species
sAC	: soluble adenylyl cyclase
S.E.M	: the standard error of the mean
sGC	: soluble guanylyl cyclase
SGLTs	: sodium-glucose co-transporters
SMC	: smooth muscle cells
SNP	: sodium nitroprusside
STZ	: streptozotocin
T1D	: type 1 diabetes
T2D	: type 2 diabetes
TX	: the thromboxane, prostanoid receptors
vs	: *versus*
VSMC	: vascular smooth muscle cells
WHO	: World Health Organization

Résumé

Le diabète sucré (diabètes mellitus, DM) est un désordre métabolique d'origine multiple, caractérisé par une hyperglycémie chronique, résultant d'un défaut de sécrétion d'insuline et/ou d'un défaut de son activité. En 2015, 415 millions de personnes étaient atteintes du diabète, la projection pour 2040 est estimée à 642 millions (ce chiffre ne tient pas compte du nombre de personnes diabétiques non diagnostiqués). Il existe principalement deux types de diabète: le diabète de type 1 (DT1) et le diabète de type 2 (DT2). DT1 se caractérise par la destruction auto-immune des cellules β pancréatiques conduisant à un déficit de la sécrétion d'insuline. DT1 représente 10% des cas, il est le plus souvent diagnostiqué chez les enfants et les jeunes adultes (Patterson, Dahlquist et al. 2009). Les patients atteints de DT1 ont une faible production d'insuline endogène, par conséquent, l'injection d'insuline est nécessaire afin de compenser la perte de sécrétion d'insuline par le pancréas. Cependant, la glycémie doit être fréquemment contrôlée afin d'éviter un risque d'hypoglycémie. Le DT2 est le type de diabète le plus fréquent (environ 90% des diabètes connus). Il est dû à une résistance à l'insuline. Les cellules beta pancréatiques produisent davantage d'insuline jusqu'à ce qu'elles ne puissent plus répondre et finissent par s'épuiser, la production d'insuline devient alors insuffisante, le glucose ne pouvant pas entrer dans les tissus périphériques tels que les muscles squelettiques et le tissu adipeux, s'accumule dans le sang conduisant à une hyperglycémie. De plus, le stockage du glucose sous forme de glycogène dans le foie est diminué, par conséquent, le niveau de glucose sanguin s'élève. En outre, la lipolyse est régulée positivement entraînant l'augmentation du niveau d'acides gras, substrat potentiel de la néoglucogenèse (Guyton, Hall 2006). D'autre part, l'insuline stimule les hépatocytes qui synthétisent les triglycérides et facilite leur stockage dans le tissu adipeux. De ce fait, l'absence d'insuline provoque une hypertriglycéridémie. Par ailleurs, la carence en insuline augmente la protéolyse conduisant à des concentrations élevées d'acides aminés dans le plasma (Charlton et Nair 1998). Etant donné que tous les acides aminés, à l'exception de la lysine et la leucine, sont des acides aminés glucogènes, précurseurs de la gluconéogenèse hépatique et rénale, ils contribuent davantage à l'hyperglycémie (Raju et Raju 2010). Le DT2 est souvent diagnostiqué fortuitement car la première période du développement de la maladie est asymptomatique, il est donc souvent diagnostiqué chez les adultes d'âge moyen. Le risque du DT2 augmente proportionnellement avec l'âge, et est favorisé par l'obésité.

Il a été démontré que le DM augmente le risque de maladies cardiovasculaires (cardiovascular diseases, CVD) avec une incidence deux à six fois supérieure chez les patients diabétiques, par rapport aux patients non-diabétiques. (Coutinho, Gerstein et al. 1999; Buyken, von Eckardstein et al. 2007). De plus, les maladies cardiovasculaires représentent près de 80 % du taux de la mortalité chez les patients diabétiques (Winer et Sowers 2004). En dépit d'une nette association entre le diabète et les maladies cardiovasculaires, les mécanismes sous-jacents qui relient les deux maladies ne sont pas encore bien compris. La littérature récente a indiqué l'importance des facteurs non-glycémiques, tels que l'âge, le sexe, l'obesité, l'hypertension et la dyslipidémie dans l'augmentions du risque cardiovasculaire. Ces facteurs peuvent altérer la fonction de plusieurs types cellulaires, notamment, les cellules endothéliales, les cellules musculaires lisses (smooth muscle cells, SMC) et les plaquettes, conduisant à des complications microvasculaires et macrovasculaires chez les patients diabètiques (Beckman, Creager et al. 2002). Les complications macrovasculaires incluent la maladie coronarienne, la maladie artérielle périphérique et l'accident vasculaire cérébral, tandis que les complications microvasculaires comprennent la néphropathie diabétique, la neuropathie diabétique et la rétinopathie diabétique. Le principal mécanisme des complications macrovasculaires est l'athérosclérose. L'athérosclérose est une maladie inflammatoire chronique initiée dans l'endothélium, suite à l'interaction entre les lipoprotéines oxydées, notamment lipoprotéines de basse densité (LDL), les lymphocytes T, les macrophages dérivés des monocytes et les autres constituants de la paroi artérielle. Après l'accumulation des lipides oxydés dans l'endothélium, les monocytes infiltrent la paroi artérielle et se différencient en macrophages, qui ingurgitent les lipides oxydés pour former les cellules spumeuses. Une fois formé, les cellules spumeuses stimulent la prolifération des macrophages et des lymphocytes T. Les lymphocytes T, à leur tour, induisent la prolifération des cellules musculaires et la production de matrice extra cellulaire dans la paroi artèrielle. Le résultat essentiel de ce processus complexe est la formation d'une plaque athérosclérose riche en lipides avec une chope fibreuse. Les complications microvasculaires du diabète sont les résultats d'une altération de la microvascularisation dans la rétine, les reins et les neurones. Les caractéristiques physiopathologiques dans le développement des complications microvasculaires sont similaires dans ces différents organes (Brownlee 2005). Les cellules endommagées par une hyperglycémie comprennent des cellules endothéliales capillaires dans la rétine, des cellules mésangiales dans le glomérule rénal et les neurones et les cellules de Schwann des nerfs périphériques. Ces cellules présentent un risque particulièrement élevé de

dommages parce qu'elles sont incapables de réguler l'absorption du glucose efficacement pendant l'hyperglycémie (Kaiser, Sasson et al. 1993; Heilig, Concepcion et al. 1995).

Sur la base des preuves convaincantes des études épidémiologiques et physiopathologiques, il est maintenant admis que l'hyperglycémie chronique est en grande partie responsable de la progression de la maladie cardiovasculaire dans le diabète (Pistrosch, Natali et al. 2011). Il a été suggéré que l'hyperglycémie via le stress oxydant modifie la fonction de nombreux types de cellules incluant les cellules endothéliales, les SMC et les plaquettes et modifie en conséquence la structure et la fonction des vaisseaux sanguins. La dysfonction endothéliale, qui est impliquée dans le développement de nombreuses maladies cardiovasculaires a été observée chez les patients diabètiques. Le mecanisme initial par lequel l'hyperglycémie modifie la fonction endothéliale est l'induction d'un déséquilibre entre la formation de monoxyde d'azote (NO•) et les espèces réactives de l'oxygène (reactive oxygen species, ROS). En effet, les concentrations du glucose intracellulaire élevées conduisent à l'activation de la protéine kinase C (PKC) et ainsi génèrent la formation des ROS par la NADPH oxydase. Par ailleurs, la réaction du NO• avec l'anion superoxyde ($O_2^{\bullet -}$), entraîne la formation de l'ion peroxynitrite ($ONOO^-$), un puissant oxydant, qui traverse facilement les membranes phospholipidiques et joue un rôle important dans la nitrosation des protéines inhibant ainsi leurs fonctions, en effet, la nitrosylation de la prostacycline synthase altère son activité induisant la diminution de la synthèse prostacycline (PGI_2), un puissant agent vasorelaxant. La disponibilité réduite du NO est également due à une diminution de l'expression et de l'activité de la eNOS, en effet, l'hyperglycemie diminue la phosphorylation du site activateur de la eNOS (Ser1177) et induit la formation des ROS par la eNOS découplée. De plus, l'activation de la PKC induite par l'hyperglycémie provoque la synthèse accrue d'endothéline-1 (ET-1), des prostaglandines (PGH_2) et de thromboxane (TXA_2) favorisant la vasoconstriction. En outre, la dysfonction endothéliale dans le diabète est également liée à une production accrue des ROS par les mitochondries, l'oxydation de l'ADN nucléaire par les ROS active la poly (ADP-ribose) polymérase (PARP). Une fois activée, PARP procède à la synthèse des polymères d'ADP-ribose, qui altèrent sur la glyceraldehyde- 3 phosphate déshydrogénase (GAPDH), une enzyme glycolytique clé, réduisant son activité. Ce phénomène perturbe différentes voies métaboliques telles que : (1) l'activation de la voie des polyols, qui provoque l'accumulation de sorbitol et de fructose; (2) l'activation de la voie hexosamine, (3) l'activation de la PKC, (4) la formation accrue des produits avancés de glycation (AGEs), ainsi que la surexpression de leurs récepteurs

(RAGEs). Toutes ces voies participent à l'accumulation des ROS, conduisant à la dysfonction endothéliale. En outre, l'accumulation de ROS déclenche également une régulation positive des facteurs pro-inflammatoires, tels que la protéine chimio-attractive monocytaire-1 (MCP-1), les molécules d'adhésion des cellules vasculaires (VCAM-1) et les molécules d'adhésion intercellulaires 1 (ICAM-1), sous la dépendance du facteur de transcription (NF-kβ). Ces événements conduisent à l'adhérence des monocytes, la formation des cellules spumeuses, qui, à leur tour, libèrent un certain nombre de cytokines inflammatoires pour maintenir l'inflammation vasculaire, ainsi que la prolifération des cellules musculaires lisses, ce qui accélère le processus athérosclérotique. Pris ensemble, ces évènements induisent la diminution des facteurs vasorelaxants (NO, PGI_2) et l'augmentation des facteurs vasocontracturants (ET-1, PGH_2, TXA_2), l'inflammation et le remodelage vasculaire, favorisant ainsi l'apparition des maladies cardiovasculaires (Paneni, Beckman et al. 2013).

Il a été rapporté qu'un mauvais contrôle de la glycémie semble jouer un rôle important dans le développement de maladies cardio-vasculaires dans le diabète. (Lehto, Ronnemaa et al. 1997). Depuis plusieurs décennies, de nombreux médicaments antidiabétiques sont sur le marché afin d'obtenir un contrôle glycémique *optimal* dans la gestion du diabète. Parmi lesquels, les Biguanides qui inhibent la production hépatique du glucose, les sulfonylurées et glinides qui sont des agents *insulino-sécrétagogues*, les thiazolidinediones qui sont des *insulino-sensibilisateurs*, les Incrétinomimétiques (*analogue du glucagon-like peptide-1 (GLP-1)* et les inhibiteurs de la dipeptidyl peptidase-4 (DPP-4)) qui stimulent la sécrétion *d'insuline* de façon *glucose-dépendante* et diminuent la sécrétion de glucagon. Cependant, parmi ces médicaments, seules la metformine (biguanide) et le liraglutide, un *analogue* du GLP-1, ont montré une réduction du risque cardiovasculaire chez le diabétique. Récemment, une nouvelle classe d'antidiabétiques a été mise sur le marché, les inhibiteurs des co-transporteurs sodium-glucose (SGLT) ou gliflozines, qui ont un effet protecteur prononcé sur le système cardiovasculaire en comparaison avec d'autres antidiabétiques oraux. Les Gliflozines réduisent l'hyperglycémie chez les patients diabétiques en diminuant la réabsorption rénale du glucose. Les SGLT2 sont situés dans les segments S1 et S2 du tubule contourné proximal du néphron, ils sont responsables de la réabsorption de 90% du glucose filtré. Ils ont une grande capacité de transport mais une faible affinité pour le glucose, contrairement aux SGLT1 qui ont une haute affinité, mais une faible capacité de transport. Les SGLTs 1 sont situés dans le segment S3 du tubule proximal et filtrent les 10% restant du glucose rénal. Les SGLT1/2 transportent le glucose et le sodium dans les cellules en utilisant

un gradient de sodium créé par les pompes Na^+/K^+ ATPase, contrairement aux transporteurs GLUTs, qui transportent le glucose par diffusion passive.

De manière intéressante, l'étude clinique EMPA-REG, a montré que le traitement des patients diabétiques avec des antécédents cardiovasculaires, par l'empagliflozine (Jardiance®, Boehringer Ingelheim/Eli Lilly), réduit la mortalité cardiovasculaire de 38%, qui est 2 fois plus élevée par rapport à la metformine – le traitement de première intention dans la prise en charge du diabète. Ces résultats suggèrent que l'empagliflozine offre une meilleure protection cardiovasculaire en comparaison avec les autres antidiabétiques oraux. Toutefois, les mécanismes par lesquels l'inhibition des SGLT2 réduit la mortalité cardiovasculaire ne sont pas bien compris. Plusieurs mécanismes potentiels ont été proposés afin d'expliquer les effets bénéfiques de l'empagliflozine dans l'étude de l'EMPA-REG. L'amélioration du contrôle glycémique n'explique probablement pas l'effet bénéfique de l'empagliflozine, de part la faible réduction de l'hemoglobine glyquée (HbA1c) dans le bras traité (0,4% à 0,6%). Cette réduction modeste de l'HbA1c est similaire à celle obtenue par l'inhibition du DPP-4, qui n'a pas réussi à mettre en évidence un bénéfice cardiovasculaire. De plus, la metformine qui réduit l'HbA1c d'environ 1,5%, montre une réduction du risque cardiovasculaire inferieure à celle de l'empagliflozine. Autres mécanismes probables contribuant à expliquer l'effet cardioprotecteur de l'empagliflozine dans l'étude EMPA-REG, sont la réduction significative du poids corporel, de la pression artérielle sans augmenter le rythme cardiaque et même une augmentation significative du niveau de cholestérol des lipoprotéines de haute densité. Nous émettons l'hypothèse que l'empagliflozine aurait un effet protecteur de la fonction vasculaire, cependant, peu d'informations existent quant à l'expression des SGLTs dans la paroi vasculaire.

Pour cette raison, le but de la première étude a été d'établir des modèles de dysfonction endothéliale induite par des concentrations élevées en glucose dans des artères isolées de rats Wistar et de porc. Nous avons émis l'hypothèse que les transporteurs SGLT1/2 pourraient être présents dans les membranes des cellules endothéliales et ainsi contribuer au transport du glucose menant au stress oxydant. Par conséquent, SGLT1/2 pourrait contribuer à l'augmentation du niveau de stress oxydant induit par le glucose élevé (high glucose, HG) dans les cellules endothéliales dans les vaisseaux sanguins des patients diabétiques. Un tel concept expliquerait que les inhibiteurs de SGLT1/2 ont un effet direct sur les vaisseaux

sanguins en diminuant la dysfonction endothéliale induite par des concentrations élevées en glucose menant finalement à une réduction de risque cardiovasculaire.

Dans la première partie de cette étude, nous avons évalué par des études de la réactivité vasculaire la fonction vasculaire d'artères de rat Wistar soumis à un niveau de glucose élevé pour induire une dysfonction endothéliale. Une étude antérieure par Ling-Bo Qian *et al.* a montré qu'après 30 min ou 1 h d'incubation d'anneaux d'aorte dans un milieu riche en glucose (44 mM), l'acétylcholine (ACh) induit encore une relaxation normale, avec une E_{max} atteignant respectivement 80,5 ± 4,4% et 78,2 ± 3,6%; à l'inverse après 2, 3 ou 4 h d'exposition à une forte concentration de glucose, la E_{max} chute à 70,1 ± 11%, 55 ± 5,6% et 51,2 ± 5,1%, respectivement. Une autre étude indique également qu'après l'incubation pendant 6 h d'anneaux aortiques dans un milieu riche en glucose (44 mM), la E_{max} chute à 43,7 ± 16,1% tandis que les anneaux soumis à une concentration normale de glucose (11 mM) ont atteint 88,4 ± 12,3% de relaxation. Par conséquent, nous avons sélectionné les deux temps d'incubation de 4 h et 6 h dans nos expériences. Nous avons ainsi incubé des anneaux aortiques en présence une concentration élevée en glucose (44 mM) pendant 4 h et 6 h dans les chambres à organes isolée contenant une solution de Krebs. Cependant, nos résultats montrent que 4 h ou 6 h d'incubation d'anneaux aortiques avec 44 mM de D-glucose n'a pas affecté la réponse de relaxation dépendant de l'endothélium à l'ACh.

En outre, nous avons également évalué l'effet d'une forte concentration en glucose sur la relaxation dépendante de l'endothélium en réponse à l'ACh dans d'autres lits artériels du rat Wistar comme l'artère mésentérique principale, l'artère rénale, l'artère carotide et l'artère fémorale. Il a été rapporté que l'exposition d'artères mésentériques de troisième ordre de rat Wistar femelle à HG (45 mM) pendant 2 h, induit une dysfonction endothéliale (Taylor and Poston 1994). Selon les protocoles publiés dans la littérature, nous avons incubé les anneaux de l'artère mésentérique supérieure, l'artère rénale, l'artère carotide et l'artère fémorale dans 25 mM D-glucose pendant 3 h dans des bains d'organes. De façon similaire aux résultats obtenus sur les anneaux d'aorte, les concentrations élevées en glucose n'induisent aucune altération de la relaxation dépendante de l'endothélium dans ces 3 types d'artères.

Par conséquent, nos résultats contrastent avec les résultats, qui ont été publiés dans la littérature. Ceci pourrait être lié à la différence de la souche et le sexe des rats utilisés dans chaque étude: nous avons étudié l'aorte et l'artère mésentérique des rats mâles Wistar, et les études publiées ont utilisé les anneaux aortiques des rats mâles Sprague-Dawley et des anneaux artériels mésentériques des rats femelles Wistar.

En outre, étant donné que le stress oxydant est considéré comme le principal contributeur de la dysfonction endothéliale induite par l'hyperglycémie; par conséquent, nous avons soulevé la question de savoir si le stress oxydant induit la dysfonction endothéliale dans les anneaux aortiques de rat Wistar. Afin de préciser si le stress oxydant induit une dysfonction endothéliale dans les anneaux aortiques, nous avons utilisé le composé pharmacologique - pyrogallol, une source d'anion superoxyde. Des anneaux aortiques ont été incubés en présence du pyrogallol aux concentrations de 10 µM et 30 µM pendant 30 min. Nous avons observé que les anneaux aortiques exposés à pyrogallol (10 et 30 µM) pendant 30 min montrent que la contractilité à la phényléphrine (PE, 3.10^{-8} à 10^{-5} M) est significativement plus élevée par rapport aux anneaux dans le groupe contrôle. En outre, l'incubation avec les deux concentrations du pyrogallol (10 et 30 µM) pendant 30 min a induit une réduction de la relaxation dépendante de l'endothélium en réponse à l'ACh par rapport au groupe contrôle. E_{max} à l'ACh était significativement diminué, passant de 91,5 ± 3,6% (dans le groupe contrôle) à 67,6 ± 3,9% et 34,3 ± 5,1% en présence du pyrogallol (10 et 30 µM), respectivement. A l'inverse, il convient de noter que pyrogallol n'a pas affecté la relaxation indépendante de l'endothélium en réponse à nitroprussiate de sodium (SNP). E_{max} par SNP était inchangé en présence du pyrogallol; E_{max} sont 101,8 ± 1,4%, 102,6 ± 1,3%, 100,5 ± 2,2% dans le groupe contrôle et anneaux incubés avec du pyrogallol (10 et 30 µM), respectivement. Nos résultats sont en ligne avec les études publiées dans la littérature concernant l'effet de pyrogallol sur la relaxation dépendante de l'endothélium en réponse à l'ACh dans les anneaux aortiques (Salheen, Panchapakesan et al. 2015; Demirci, McKeown et al. 2008). De ce point, le stress oxydant est capable d'induire une dysfonction endothéliale. Ainsi, l'absence de l'effet d'HG dans la fonction endothéliale dans les anneaux aortiques avec nos conditions expérimentales pourrait être du à un faible niveau de stress oxydant induit par HG dans les anneaux aortiques.

Par conséquent, dans l'étape suivante, nous avons évalué l'effet de glucose élevé sur la fonction endothéliale dans les anneaux aortiques présentant une dysfonction endothéliale partielle induite par une faible concentration du pyrogallol. Des anneaux aortiques ont été incubés dans HG (25 mM) en présence d'une faible concentration de pyrogallol (3 µM) pendant 6 h dans une solution de Krebs dans le bain d'organe. Nous avons observé que l'incubation des anneaux aortiques avec du pyrogallol (3 µM) pendant 6 h a légèrement réduit la relaxation dépendante de l'endothélium en réponse à l'ACh par rapport aux anneaux dans le contrôle. E_{max} à l'ACh est tombé de 85,7 ± 3,6% à 75,0 ± 7,2% dans le contrôle et les

anneaux incubées avec du pyrogallol, respectivement. Cependant, la co-incubation d'anneaux aortiques avec HG (25 mM) et du pyrogallol (3 µM) pendant 6 h n'a pas affecté la relaxation dépendante de l'endothélium en réponse à l'ACh par rapport aux anneaux incubés en présence du pyrogallol seul. Nous suggérons que l'absence d'effet d'une forte concentration en glucose sur la relaxation dépendante de l'endothélium dans ces anneaux aortiques pourrait être liée au fait que la combinaison du glucose et pyrogallol pourrait induire la formation accrue du peroxyde d'hydrogène (H_2O_2) (Peiro, Lafuente et al. 2001; Ho, Liu et al. 2000; Qian, Fu et al. 2012), qui a été montré comme un facteur hyperpolarisant dérivé de l'endothélium (endothelium-derived hyperpolarizing factor, EDHF) (Shimokawa and Matoba 2004; Graier and Hecker 2008; Edwards, Li et al. 2008). Il a été observé une relaxation dépendante de l'endothélium induite par H_2O_2 dans l'aorte de rat (Yang, Zhang et al. 1999; Mian and Martin), dans l'aorte de lapin (Zembowicz, Hatchett et al. 1993), dans l'artère mésentérique de lapin (Itoh, Kajikuri et al. 2003), dans l'aorte des rats diabétiques STZ (Pieper and Gross 1988) et dans les artérioles coronaires de souris db/db (Park, Capobianco et al. 2008). Sur la base de ces résultats, nous suggérons que H_2O_2 peut agir comme un facteur hyperpolarisant endothélial (EDHF) pour compenser l'effet d'une forte concentration en glucose à atténuer la relaxation dépendante de l'endothélium en réponse à l'ACh. Ces effets pourraient expliquer pourquoi, on n'a pas observé d'altération de la fonction endothéliale dans notre modèle *ex vivo*. Ainsi, d'autres études sont nécessaires en utilisant la catalase (CAT) et l'analogue perméant membranaire de la la CAT (PEG-CAT) pour bloquer l'effet de H_2O_2 extra- et intracellulaire sur les réponses de relaxation afin d'évaluer l'effet d'une forte concentration en glucose sur la relaxation dépendante de l'endothélium médiée par NO dans les artères isolées des rats.

En outre, le court temps d'incubation de l'artère en présence d'une concentration de glucose élevée pourrait expliquer l'absence d'effet. Pour cette raison, nous avons évalué l'effet de HG sur la fonction endothéliale dans une incubation à long terme. Nous avons ensuite incubé des anneaux aortiques pendant des temps longs de 15 h et 22 h dans le milieu DMEM en présence de pénicilline (100 U/ml), streptomycine (100 U/ml), fungizone (250 µg/ml) et polymixine B (10 µg/ml) afin d'éviter une contamination dans la période d'incubation à long terme. Malheureusement, nous avons été incapable d'évaluer l'effet de glucose élevé sur la fonction endothéliale après une incubation de longue durée en raison que les anneaux ne relâchent plus en réponse à l'ACh, et les réponses contractiles sont aussi diminuées

probablement en raison d'une augmentation de l'expression de la NOS inductible et de cyclooxygenase en réponse aux cytokines pro-inflammatoires.

Dans la deuxième partie de l'étude, nous avons choisi d'utiliser l'artère coronaire du porc (ACP) car la relaxation dépendante de l'endothélium de l'ACP peut persister même après une incubation à long terme. Du fait le nombre limité de publications rapportant l'effet d'une forte concentration de glucose sur les relaxations dépendantes de l'endothélium de l'ACP, nous avons testé l'effet de l'HG à court et long termes. Dans un premier temps, des anneaux d'ACP ont été incubés dans la cuve à organe isolé pendant 6 h en présence d'une concentration normal (11 mM) ou élevé (44 mM) en glucose. Nos résultats n'ont pas montré de différence concernant entre la relaxation dépendante de l'endothélium en réponse à la bradykinine (BK) dans les anneaux incubés avec du glucose normal (11 mM) et ceux incubés avec une concentration en glucose élevée (44 mM) pendant 6 h. Dans un second temps, les anneaux d'ACP ont été incubés en présence du glucose des concentrations 25 mM, 30 mM et 44 mM pendant 24 h dans le milieu RPMI 1640 supplémenté en polymixine B (10 µg/ml), pénicilline (100 U/ml), streptomycine (100 U/ml) et fungizone (250 µg/ml). En comparaison avec le milieu DMEM, le milieu RPMI 1640 est un milieu moins riche en nutriments et contenant moins de calcium et plus de phosphate, ce qui peut diminuer la libération de facteurs pro-inflammatoires. Une atténuation faible de la relaxation dépendante de l'endothélium en réponse à la BK a été observée seulement dans les anneaux incubés en présence du glucose de 44 mM par rapport au groupe contrôle (11 mM). En outre, il y a eu un décalage significatif vers la droite dans la courbe concentration-réponse à la BK à 3.10^{-9} M: les anneaux du groupe contrôle (11 mM) ont atteint environ 72,6 ± 2,91% de relaxation alors que les anneaux incubés avec une concentration de glucose élevé (44 mM) ont atteint autour de 36,9 ± 3,51% ($p < 0.05$). Toutefois, les E_{max} des anneaux du groupe contrôle et du groupe HG étaient semblables (95,8 ± 3,01% et 93.61 ± 2.28%, respectivement). Il convient également de noter que les plus faibles concentrations du glucose (25 et 30 mM) n'ont pas affecté la relaxation dépendante de l'endothélium en réponse à la BK. En outre, 24 h d'incubation des anneaux de l'APC à 44 mM n'a pas affecté la relaxation indépendante de l'endothélium en réponse au sodium nitroprusside (SNP). Ces résultats indiquent que l'incubation des APC avec HG (44 mM) pendant 24 h n'induit qu'une atténuation de la relaxation dépendante de l'endothélium en réponse à la BK. De plus, nous avons évalué l'effet du SGLT2 inhibiteur (gliflozine) sur la dysfonction endothéliale induite par HG (44 mM). Malheureusement, la gliflozine à 10^{-7} M n'a pas affecté la relaxation dépendante de

l’endothélium des anneaux d’APC incubés avec HG (44 mM). Il serait peut-être lié au fait que le la dysfonction endothéliale induite par HG dans APC était trop petite, par conséquent, nous avons été incapables d'évaluer l'effet de gliflozine.

Dans l’ensemble, nos résultats contrastent avec les résultats précédents décrits dans la littérature. Bien que, la littérature a également démontré la fluctuation de la dysfonction endothéliale induite par HG dans différents types d'artère. Certains groupes ont constaté que l'incubation 6 h avec 44 mM D-glucose induit environ 20% de l’atténuation de la relaxation dépendante de l’endothélium dans les anneaux aortiques chez le rat mâle Sprague Dawley (Qian, Wang et al. 2006; Zhang, Yang et al. 2004). Tandis que d'autres groupes ont observés à environ 40% ceux dans les mêmes conditions d’incubation (Wang, Xiong et al. 2005). En ce qui concerne l'artère mésentérique, une variation similaire a également été observée dans la littérature. Taylor et ses collègues ont rapporté une atténuation de la relaxation dépendante de l’endothélium en réponse à l'ACh (environ 30%) observée dans les artères mésentériques de troisième ordre incubées dans 45 mM de D-glucose pendant 2 h chez les rats femelles Wistar (Taylor and Poston 1994). Par contre, chez les rats mâles Wistar, ils ont observé qu'environ 10% et 20% d’une atténuation de la relaxation endothélium, respectivement, dans les artères mésentériques de troisième et de second ordre, avec le même temps d'exposition (2 h) avec 40 mM D-glucose (Salheen, Panchapakesan et al. 2015; Brouwers, Niessen et al. 2010). Plusieurs possibilités existent pour expliquer les résultats discordants des études publiées dans la littérature et de notre étude. Tout d'abord, cette différence peut être liée à des différences de sexe des animaux utilisés dans chaque étude. En effet, il a été démontré que la différence entre le sexe peut contribuer différemment à l’atténuation de la relaxation dépendante de l’endothélium induite par HG dans l'aorte des rats et des lapins (Goel, Zhang et al. 2007; Goel, Thor et al. 2008). Goel et ses collègues ont rapporté que 3 h d'incubation avec un niveau élevé de D-glucose (44 mM) réduit la relaxation dépendante de l’endothélium en réponse à l'ACh seulement dans les anneaux aortiques des rats femelles Sprague Dawley: le E_{max} à l'ACh est tombé de 77,8 ± 4,6% à 56,2 ± 4,3% dans le groupe contrôle et groupe d’HG, respectivement. En revanche, la relaxation induite par l'ACh n'a pas été affectée par HG dans les anneaux aortiques prélevés sur des rats mâles Sprague Dawley (Goel, Zhang et al. 2007). Dans une autre étude, il a été observé que l’HG induit une atténuation de la relaxation dépendante de l’endothélium plus élevée dans l’aorte des lapins femelles que des lapins mâles: dans l’aorte des mâles E_{max} est tombé de 53,60 ± 3,42% à 45,84 ± 2,84% et dans l’aorte des femelles E_{max} est tombé de 57,8 ± 3,39% à 43,74 ± 3,74% dans les anneaux du

groupe contrôle et du groupe HG, respectivement (Goel, Thor et al. 2008). Par conséquent, l'effet des hormones sexuelles peut être affecté différemment la réponse des artères au HG.

Deuxièmement, la variation des résultats peut être due à la différence des souches et des espèces des animaux étudiés et à leurs sensibilités variables au diabète. La littérature a montré les différents effets d'HG sur la relaxation dépendante de l'endothélium en réponse à l'ACh chez les rats Sprague-Dawley et Wistar. Le groupe de Dhar a constaté que l'incubation avec 25 mM de D-glucose pendant 2 h a réduit d'environ 50% de la relaxation maximale en réponse à l'ACh dans les anneaux aortiques de rats Sprague-Dawley mâles (Dhar, Dhar et al. 2010). En revanche, dans une autre étude avec des rats de même âge Wistar mâles: la même durée d'exposition (2 h) à une concentration élevée de D-glucose (44 mM) a diminué significative la relaxation en réponse à l'ACh, mais avec un effet plus faible d'environ 20% (valeurs E_{max} de contrôle: 85.01 ± 2.90 et HG: 64,68 ± 2,71%) (Rama Krishna Chaitanya and Chris 2013). Par conséquent, les souches et les espèces sont des facteurs importants lors du choix d'un modèle. Idéalement, is devrait être étudié plus d'une espèce ou des souches différentes.

Troisièmement, la durée de l'hyperglycémie pourrait également affecter une atténuation dans la relaxation dépendante de l'endothélium. En effet que, 6 h d'incubation d'APC avec 44 mM de D-glucose n'a pas affecté la relaxation dépendante de l'endothélium en réponse à la BK. Par contre, on a observé que 24 h d'incubation dans les APC avec la même concentration du D-glucose a induit une petite atténuation de la relaxation dépendante de l'endothélium en réponse à la BK. Donc, une incubation à long terme (24 h) avec HG a induit une petite atténuation de la relaxation dépendante de l'endothélium, ce qui n'est pas observé suite à une l'incubation à court terme (6 h). Ces résultats suggèrent que le temps d'incubation avec HG affecte différemment la relaxation dépendante de l'endothélium en réponse à la BK. Nous supposons que le niveau de stress oxydant n'est pas le même après 6 h et 24 h. De même, les différents effets d'HG de la relaxation dépendante de l'endothélium entre le court terme et le long terme ont été rapportés dans l'étude *in vivo*. Il a été montré une réponse différente de la fonction endothéliale à différents stades du diabète dansn un modèle animaul du diabète induit par la streptozotocine. La relaxation dépendante de l'endothélium en réponse à l'ACh a été augmentée à 24 h après l'injection de streptozotocine par rapport au contrôle, reste stable après 1 et 2 semaines de la maladie, puis a diminuée à partir de la 8ème semaine de la maladie (Pieper 1999). Cependant, dans nôtre étude, 24 h d'incubation d'APC avec 44 mM D-glucose induit seulement une petite diminution de la relaxation dépendante de

l’endothélium en réponse à la BK; par conséquent, d'autres expériences doivent être effectuées, comme augmenter le temps d'incubation avec d’HG pour clarifier l'effet d’HG sur la fonction endothéliale dans les APC.

En bref, nous concluons que:

- L'auto-oxydation de pyrogallol (10 et 30 µM) induit un stress oxydant résultant en une augmentation significative du contraction en réponse à la PE et une atténuation significative de la relaxation dépendante de l’endothélium en réponse à l'ACh dans les anneaux aortiques de rats mâles Wistar.
- L'incubation à court terme avec HG (44 mM) n'a pas modifié la relaxation dépendante de l’endothélium en réponse à l'ACh dans les anneaux aortiques de rats mâles Wistar. Il peut éventuellement être lié à certains facteurs, tels qu'un sexe, l'âge, la souche, l’espèce animale étudiée, et les conditions de logement des animaux. D'autres études sont nécessaires pour clarifier l'effet d’HG sur la relaxation dépendante de l'endothélium en réponse à ACh dans nôtre modèle *ex vivo*.
- Le long terme l'incubation des anneaux aortiques de rats mâles Wistar avec d’HG (44 mM) a déprimé la réponse contractile à la PE.
- L'incubation à long terme des APC avec HG (44 mM) induit une petite atténuation de la relaxation dépendante de l’endothélium en réponse à la BK. Bien que cette atténuation soit trop faible, par conséquent, nous n’avons pas pu examiner l'effet protecteur de gliflozine sur la dysfonction endothéliale induite par HG.

Nous suggérons que:

- D'évaluer l'effet de HG sur la fonction endothéliale dans les anneaux aortiques prélevés sur le rat mâle Sprague Dawley parce que la littérature a montré que une dysfonction endothéliale dans les anneaux aortiques de rat mâle Sprague Dawley induite par HG supeneure à celles chez les rats mâles Wistar;
- D'ajouter une combinaison de 1400W et l'indométacine (inhibiteurs de NOS inductible et COX2, respectivement) dans RPMI 1640 pour évaluer l'effet de 24 h d'incubation avec HG (44 mM) sur la fonction endothéliale dans les anneaux aortiques.
- D’utiliser la catalase et de polyéthylène glycol catalase (l’analogue perméant membrane de la catalase) pour empêcher l'effet de H_2O_2 sur la relaxation dépendante

de l’endothélium et d'évaluer l'effet de HG seulement sur la relaxation dépendante de l’endothélium médiée par NO dans les vaisseaux isolés.

- D'augmenter le temps d'incubation dans le modèle de l’APC de 24 h à 36 et 48 h pour vérifier si l'incubation plus longue de l’APC avec HG (44 mM) serait capable d'induire une altération importante de la relaxation dépendante de l’endothélium en réponse à la BK.
- D'utiliser des modèles diabétiques *in vivo*: Les modèles les plus classiques sont: administration de faibles doses répétées de streptozotocine (STZ), ce qui induit DT1, modèle combinant un régime alimentaire riche en graisses et une faible dose unique de STZ pour DT2 et des modèles diabétiques génétiquement dérivés tels que y compris les rats Zucker diabétiques obèses, les souris ob/ob et db/db. Ces modèles génétiquement diabétiques sont induits par une mutation dans le gène de la leptine ou des récepteurs de la leptine. Cependant, les modèles diabétiques *in vivo* ont des limites. STZ est une toxine des cellules β pancréatiques, ce qui conduit à une destruction rapide de la β-cellules résultant en l'hyperglycémie. Parce que STZ pénètre dans les cellules par les transporteurs du glucose (GLUT2), par conséquent, son action est non seulement spécifique aux cellules β pancréatiques et peut causer des dommages à d'autres tissus, y compris le foie et le rein. Il a été démontré que le taux de mortalité des rats est très élevé après l'injection de STZ. En outre, les modèles génétiquement diabétiques actuels sont associés à des syndromes métaboliques tels que une tolérance altérée au glucose, l'hyperinsulinémie, la résistance à l'insuline, les dyslipidémies et l'obésité, ce qui peut accélérer l'hyperglycémie induite la dysfonction endothéliale.

Le but de la seconde étude a été de caractériser les modifications vasculaires survenant dans des fragments d’artère mammaire interne humaine (internal mammary artery, IMA) issus de patients atteints des certaines pathologies cardiovasculaires, tels que le diabète et l'hypertension. Nous avons divisé les patients eu 4 groupes : non-diabétiques et normotendus (Control), hypertendus (H), diabétiques (DM) et diabétiques et hypertendus (DM & HT). Nous avons évalué le niveau de stress oxydant, ainsi que le niveau d'expression de la eNOS, des SGLTs et des composants du système de l’angiotensine. Les segments d’IMA ont été prélevés à partir de 58 patients, ayant subi un pontage coronarien (coronary artery bypass

graft, CABG) entre juillet 2013 et janvier 2016 au sein du Nouvel Hôpital Civil de Strasbourg.

En premier lieu, une augmentation du niveau de stress oxydant a été observée chez les patients diabétiques et hypertendus et seulement hypertendus. Nous avons utilisé du dihydroéthidium (DHE), une sonde fluorescente redox sensible, afin de déterminer la formation des espèces réactives de l'oxygène (reactive oxygen species, ROS). Nous avons trouvé que la formation des ROS est fortement augmentée dans les segments d'IMA de patients hypertendus ou de patients diabétiques souffrant d'hypertension, en comparaison avec des patients non-diabétiques et normotendus. Pour déterminer la nature des ROS formés, les segments d'IMA ont été prétraités avec différents antioxydants avant la coloration au DHE. Les résultats ont montré que le signal de fluorescence du DHE a été nettement réduit en présence de la superoxyde dismutase (SOD), de la catalase (CAT) et d'analogues perméants membranaires de la SOD et de la CAT (PEG-SOD et PEG-CAT), indiquant l'implication des anions superoxydes et du peroxyde d'hydrogène. De plus, nous avons cherché à identifier la source enzymatique de ces ROS en prétraitant les segments d'IMA avec différents inhibiteurs avant la coloration au DHE. Le signal de fluorescence a été aussi significativement réduit en présence d'inhibiteurs de la NADPH oxydase (VAS-2870), de la NO synthase endothéliale (L-NA), du cytochrome P450 (sulfaphenazol), des cyclooxygénases (indométacine) et par une combinaison d'inhibiteurs de la chaîne respiratoire mitochondriale (KCN, myxothiazol et roténone). Ces résultats indiquent ainsi l'implication de la NADPH oxydase, de la eNOS découplée, des cyclooxygénases, du cytochrome P450 et de la chaîne respiratoire mitochondriale dans la formation des ROS au niveau de la paroi vasculaire des segments d'IMA.

En outre, nous avons déterminé le niveau de stress oxydant grâce à la coloration par la nitrotyrosine. Nitrotyrosine est un produit final du peroxynitrite ($ONOO^-$), qui est un puissant oxydant formé à partir de NO et de $O_2^{\bullet -}$. Or, la littérature rapporte que l'oxydation par le $ONOO^-$ de la tétrahydrobioptérine, un cofacteur essentiel de la eNOS, peut représenter une cause pathogène de formation de eNOS découplée, conduisant à la production d'$O_2^{\bullet -}$ à la place de NO (Forstermann and Munzel 2006; Pacher, Beckman et al. 2007). Par conséquent, nitrotyrosine est également considéré comme un indicateur du stress oxydant. Une augmentation significative du niveau de nitrotyrosine a été observée dans les segments d'IMA de patients hypertendus ou de patients diabétiques souffrant d'hypertension, en comparaison avec les non-diabétiques et normotendus. Ces résultats confirment l'implication de l'oxydant

$ONOO^-$ dans l'augmentation plus prononcée de stress oxydant dans les segments d'IMA des patients hypertendus et les patients diabétiques souffrant d'hypertension. Nos résultats sont en adéquation avec la littérature existante. En effet, le groupe de Guzik a mis en évidence du rôle de la NADPH oxydase et de la eNOS découplée dans l'augmentation de la production de $O_2^{\bullet -}$ à la place de NO, résultant en l'augmentation de la formation de $ONOO^-$ et l'induction d'une dysfonction endothéliale dans les segments d'IMA de patients diabétiques (Guzik, Mussa et al. 2002; Guzik, West et al. 2002).

De plus, nous avons évalué les modifications du niveau d'expression de la eNOS en l'impact des deux facteurs de risque cardiovasculaires: le diabète et l'hypertension, en association ou non. Les résultats de l'immunofluorescence du niveau d'expression de la eNOS dans les segments d'IMA des différents patients ont donné une indication de la localisation de la eNOS majorairement dans l'endothélium. Nous avons trouvé un niveau d'expression significativement accru de la eNOS dans les segments d'IMA des patients hypertendus et les patients diabétiques souffrant d'hypertension, par rapport aux patients non-diabétiques et normotendus. Il convient de noter que, de manière inattendue, le signal de la eNOS est également observée dans les cellules musculaires lisses (smooth muscle cells, SMC) des segments d'IMA chez certains patients. La littérature a montré l'expression de la eNOS dans les cellules endotheliales des segments d'IMA humaines, cependant, les données concernant l'expression de cette enzyme dans SMC des segments d'IMA humaines a été rarement décrite. Gaudino et ses collègues ont fourni la preuve que la eNOS est fortement exprimée dans SMC des segments d'IMA humaines, mais pas dans la veine saphène (un autre type de greffe en CABG). Aucune information sur le rôle fonctionnel de la eNOS dans SMC des segments d'IMA humaines a été décrite. En outre, nous avons également évalué le niveau d'expression de la eNOS dans les segments d'IMA humaines par Western blot. Cependant, ces résultats ont montré une fluctuation élevée, par conséquent, nous n'avons pas pu confirmer le niveau d'expression accrue de la eNOS observé par immunofluorescence. Nous supposons que ces résultats sont liés au fait que les expériences d'immunofluorescence et de Western blot n'ont pas été réalisées avec les même patients. En effet, la taille des segments d'IMA obtenus à partir des patients était particulièrement limitée. De plus, l'immunofluorescence a été réalisée sur des segments d'IMA obtenus en 2014, et seulement en 2015 pour le Western blot. Or, les variations d'âge, de sexe, de stade d'avancement de la maladie et des traitements de chaque patient pourraient être la cause de résultats différents. Par conséquent, d'autres expériences

doivent être effectuées avec un plus grand nombre de patients pour préciser le niveau d'expression de la eNOS dans les segments IMA humaine.

En outre, le stress oxydant a été démontré être induit par le système d'angiotensine local dans les cellules endothélials (Pueyo, Arnal et al. 1998; Sohn, Raff et al. 2000; Nguyen Dinh Cat, Montezano et al. 2013; Touyz 2004). Nous avons donc cherché à déterminer le niveau d'expression de l'angiotensine II (Ang II), ainsi que de ses 2 récepteurs (AT1 et AT2) afin d'évaluer l'implication du le système d'angiotensine local dans l'induction du stress oxydant dans les segments d'IMA de patients ayant une ou l'autre des deux les principaux facteurs de risque cardiovasculaire. Nos études d'immunofluorescence ont montré une augmentation significative de l'expression d'Ang II et de ses récepteurs AT_1 et AT_2 dans la paroi des segments d'IMA chez les patients hypertendus et les patients diabétiques souffrant d'hypertension en comparaison avec les patients non diabétiques et normotendus. Ceci suggère une association entre le diabète et la surexpression du système local d'angiotensine dans les segments d'IMA. Nos résultats sont en accord avec la littérature (Berry, Hamilton et al. 2000; Giacchetti, Sechi et al. 2005). En effet, l'augmentation du niveau de Ang II et de récepteur AT1 dans les segments d'IMA de patients diabétiques et/ou hypertendus a été rapportée (Nguyen Dinh Cat, Montezano et al. 2013, Touyz 2004). De plus, nos résultats ont également montré le niveau d'expression du récepteur AT2 est prononcé dans les segments d'IMA des patients hypertendus et les patients diabétiques souffrant d'hypertension. Il a été rapporté que l'angiotensine II active le récepteur AT2, ce qui entraîne une production augmentée de NO et BK (Gohlke, Pees et al. 1998, Siragy, Inagami et al. 1999). Le NO, à son tour, pourrait réagir avec le $O_2^{\bullet -}$ conduisant à la production $ONOO^-$, qui accentue le niveau de stress oxydant, comme mentionné ci-dessus. De plus, van de Wal et ses collègues ont montré que l'ARNm du récepteur AT2 est présent dans l'IMA humaine, mais que, la stimulation des récepteurs AT2 n'induit pas la vasodilatation dans des artères (van de Wal, van der Harst et al. 2007). Pris ensemble, nos résultats confirment une augmentation significative du système local d'angiotensine dans les segments d'IMA de patients hypertendus et de patients diabétiques souffrant d'hypertension. Il convient de noter que l'expression du système local d'angiotensine a été augmentée à la fois dans l'endothélium et dans SMC. Notre observation est cohérente avec d'autres études in vitro (Griendling, Minieri et al. 1994), ainsi que chez l'homme (Berry, Hamilton et al. 2000). Ceci suggère que les SMC vasculaires pourraient être une source importante de $O_2^{\bullet -}$ génération, en parallèle de la eNOS découplée dans l'endothelium.

Certaines études ont montré l'effet des inhibiteurs de SGLT2 sur la réduction significativement des événements cardiovasculaires chez les patients diabétiques (Zinman, Wanner et al. 2015; Vasilakou, Karagiannis et al. 2013; Dziuba, Alperin et al. 2014; Neal, Perkovic et al. 2013). Cependant, les mécanismes sous-jacents de l'effet cardioprotecteur ne sont pas encore bien compris. Nous émettons l'hypothèse que les inhibiteurs de SGLT peuvent avoir un effet direct sur les vaisseaux sanguins en diminuant la dysfonction endothéliale induite par HG, aboutissant finalement à la réduction du risque cardiovasculaire. Toutefois, les études concernant l'expression de SGLT1/2 sur les vaisseaux sanguins sont rares. Han et ses collègues ont examiné le niveau d'expression de l'ARNm des sous-types de SGLT dans les cellules endothéliales ainsi que les SMC des artères pulmonaires et coronaires de la souris et l'homme (Han, Cho et al. 2015). Ils ont constaté que comme prévu, le niveau d'expression de SGLT1 ARNm a été détecté fortement dans l'intestin de la souris et chez l'homme. Cependant, SGLT1 ARNm est moins exprimé dans les cellules endothéliales et les cellules musculaires lisses des artères pulmonaires et coronaires de la souris et de l'homme, par rapport à l'intestin. En ce qui concerne l'expression de SGLT2 ARNm, elle a été détectée comme prévu principalement dans les reins de la souris et d'hommes, cependant, pas dans les cellules endothéliales et les cellules musculaires lisses des artères pulmonaires et coronaires chez la souris et ainsi que chez l'homme. Un autre groupe a montré que SGLT1 est exprimé dans les cellules endothéliales humaines de veine ombilicale (HUVEC) et l'endothélium vasculaire de l'aorte thoracique isolée, où SGLT1 joue le rôle de transporteur de la delphinidine-3-glucoside (Dp) dans les cellules endothéliales (Jin, Yi et al. 2013). En revanche, les informations relatives à l'expression de SGLT2 dans les vaisseaux sanguins ne sont pas disponibles. Pour cette raison, nous avons étudié le niveau d'expression des protéines SGLT1/2 dans les segments d'IMA des patients diabétiques, en association ou non avec de l'hypertension en utilisant la technique du Western Blot afin de clarifier la présence de SGLT1/2 dans les segments d'IMA humaines. Nos résultats ont montré que le SGLT1 a été détecté des segments d'IMA humaines à un niveau de poids moléculaire inférieur à celui de SGLT1, du rein de rat comme contrôle positif. Le poids moléculaire inférieur de SGLT1 observé pourrait s'expliquer soit par la dissociation et la dégradation de la protéine, soit par l'anticorps utilisé non spécifique de la protéine humaine. Ceci n'était pas le cas avec SGLT2, où le même poids moléculaire a été observé entre les segments d'IMA humains et ceux de reins de rat. Cependant, le niveau d'expression de SGLT2 est assez élevé dans les segments d'IMA humains, ce qui est en opposition avec les informations trouvées dans la litérature. Par

ailleurs, les résultats ont montré une forte fluctuation du niveau d'expression des protéines de SGLT1 et SGLT2 chez les patients. A partir des données que nous avons obtenues, il n'est pas possible de tirer une conclusion sur le niveau d'expression de SGLT1/2 dans les segments d'IMA humains. Les raisons pour lesquelles le niveau de poids moléculaire de SGLT1 est inférieur et l'expression de SGLT2 est élevée restent en effet peu claires. Des expériences supplémentaires devront être effectuées, notamment en utilisant un autre contrôle positif provenant de rein humain, afin de clarifier l'expression de ces protéines dans les vaisseaux sanguins humains. Pour approfondir cette étude, nous suggérons également d'évaluer le niveau d'expression de l'ARNm des SGLT1/2 dans les segments d'IMA humains afin de préciser le niveau d'expression de ces transporteurs du glucose au niveau moléculaire.

En résumé, notre étude sur les segments d'IMA humains a mis en évidence que:

- Le profil clinique des patients montre que le diabète et l'hypertension surviennent fréquemment de manière conjointe.
- Le stress oxydant est plus prononcé chez les patients avec un ou deux des principaux facteurs de risque cardiovasculaire (diabète et hypertension). En outre, plusieurs sources, comprenant la eNOS découplée, le COX, la NADPH oxydase, le cytochrome P450 et la chaîne respiratoire mitochondriale, sont impliquées dans la formation accrue de ROS dans les segments d'IMA de patients hypertendus ou de patients diabétiques et hypertendus.
- Les résultats en immunofluorescence ont indiqué une augmentation significative de l'expression de la eNOS et du système local de l'angiotensine dans les segments d'IMA humains avec des facteurs de risque cardiovasculaire. Cependant, des études supplémentaires seront nécessaires pour confirmer ces résultats, notamment par une autre méthode telle que l'examen du niveau d'expression de ces protéines par Western blot.
- Les niveaux d'expression de SGLT1 et SGLT2 ne sont pas claires.

L'étude des segments d'IMA humains a présenté certaines limites:

1. La difficulté de la collecte d'échantillons et leur petite taille. Seulement une petite quantité de protéines, provenant des segments d'IMA humains, a été obtenue. Par conséquent, tous les marqueurs intéressants n'ont pas pu être étudiés.
2. Un petit nombre de sujets dans le groupe de patients non diabétiques et normotendus soulève les difficultés de conclure de manière précise.

3. La difficulté dans le contrôle de l'intégrité de l'endothélium, il est difficile de distinguer si les résultats sont liés à l'association avec le diabète ou de la perte de l'intégrité de l'endothélium. Être plus précis, nous devons évaluer les échantillons dans les deux conditions: avec et sans endothélium (l'endothélium pourrait être dénudé mécaniquement). Cependant, la taille limitée des échantillons reçus est un facteur limitation.
4. La forte fluctuation parmi les patients. La fluctuation entre les patients peut être due à des caractéristiques cliniques hétérogènes des patients. Ces patients ont des différences génétiques, dans les facteurs de risque d'athérosclérose, de la durée de la maladie et de la gravité, et des traitements médicamenteux.

Face à ces limites, nous suggérons :

- D'augmenter le nombre de patients
- D'effectuer une étude de réactivité vasculaire afin de clarifier le rôle de la eNOS dans l'endothélium des segments d'IMA de patients ayant subi un pontage.
- De déterminer le niveau d'expression des acteurs du système local de l'angiotensine par Western Blot afin de confirmer les résultats obtenus par immunofluorescence
- D'utiliser un autre contrôle positif comme le SGLT1/2 provenant de rein humain afin de clarifier le poids moléculaire du SGLT1/2 des segments d'IMA humains. Et plus précisément, de déterminer le niveau d'expression de l'ARNm d'SGLT pour mettre en évidence leur présence dans les segments d'IMA humains.

CHAPTER 1

REVIEW OF LITERATURE

1.1. Diabetes mellitus

The term diabetes is the shortened version of the full name **"Diabetes mellitus"**. It was known since the 17th century that the word **"Diabetes"** comes from a Greek word, which means "to siphon" or "passing though", referring to the excessive urination associated with the disease. The word "**Mellitus**" derives from a Latin word, which means sweet like honey; due to the reason that there is an excess sugar found in blood as well as urine of diabetic patients. Nowadays, diabetes mellitus represents an increasingly heavy health burden in our society and has acquired epidemic dimensions. From 2012 to 2015, diabetes was estimated to have resulted in **1.5 to 5.0** million deaths each year worldwide, every seven seconds a person dies from diabetes (Update 2015, IDF). Following the latest data from World Health Organization (WHO), all over the world there are **415** million people with diabetes in 2015; by 2040 this number will rise to **642** million (still not counting the number of undiagnosed diabetic people). In Europe, the number of people with diabetes is estimated to be 59.8 million (9% of the population aged 20-79) including 23.5 million undiagnosed cases. It has also been estimated that diabetes was responsible for 9% of total health expenditure in the Europe (IDF Diabetes Atlas, 7 ed). In order to reduce the global burden of diabetes and its effects on lives and economies worldwide, it is necessary to have an improved understanding of its etiology, pathogenesis and pathophysiology to focus therapeutic and research efforts appropriately.

1.1.1. Definition of diabetes

Diabetes mellitus is a group of disorders characterized by chronic or sustained abnormally high blood glucose levels resulting either from insulin deficiency and/or from insulin resistance (Taylor, 1999). The acceleration of the diabetes is naturally also accompanied by an increase in diabetic vascular complications, which are the major causes of heart disease, blindness, renal failure and lower limb amputation.

1.1.2. Pathophysiology of diabetes

T1D and T2D are common cases of diabetes. T1D is characterized by the autoimmune destruction of pancreatic β-cells leading to a deficiency of insulin secretion. In addition to the

loss of insulin secretion, it has been also found that the glucagon secretion is not suppressed by hyperglycemia in patients with T1D. Consequently, the metabolic defects are exacerbated in T1D patients. Unlike T1D, patients with T2D have detectable levels of circulating insulin but they are often resistant to the action of insulin. Beside the fact that insulin resistance is the primary cause of T2D, the level of insulin is also found declined in patients with advanced T2D. Most T2D patients have both insulin resistance and insulin deficiency (Raju and Raju, 2010).

Normally, insulin is released into the blood by pancreatic β-cells, in response to rising levels of blood glucose. Insulin regulates blood glucose levels by stimulating glucose uptake in peripheral tissues, increasing the storage of glucose as glycogen through glycogenesis in liver and inhibiting hepatic glucoce production (Aronoff, Berkowitz et al. 2004). In addition, insulin also inhibits lipolysis in adipose tissues (Bailey, Gross et al. 2010) and has an effect on increasing the rate of protein synthesis and decreasing the rate of protein degradation (Raju and Raju, 2010). Due to the lack of insulin secretion and insulin resistance, the metabolism of all the main foodstuffs is altered. Firstly, the uptake of glucose is impaired in peripheral tissues like skeletal muscle and adipose tissues (**Figure 1**). Reduced glucose uptake by peripheral tissues, in turn, leads to a reduced rate of glucose metabolism. In addition, diabetic conditions with deficiency of insulin or insulin resistance lead to decrease the expression level of glucokinase in hepatocytes (Haeusler, Camastra et al. 2015). Glucosekinase is the enzyme catalyzes glucose phosphorylation, which is an initial event in glucose metabolism by the liver (Ferre, Pujol et al. 1996). Therefore, a reduced rate of glucose phosphorylation in hepatocytes leads to increased level of glucose in the blood. Secondly, storage of glucose as glycogen in the liver is reduced; consequently, blood glucose levels will become elevated. Thirdly, lipolysis is upregulated resulting in the increased levels of fatty acids (Guyton, Hall 2006). Besides, insulin stimulates hepatocytes to synthesize and store triglycerides in adipose tissue therefore the absence of insulin results in hypertriglyceridemia. Moreover, in hepatocytes under low or absent insulin, the majority of the fatty acyl-CoA is metabolized into ketone bodies (acetoacetate and β-hydroxybutyrate) (Laffel 1999; Stojanovic and Ihle 2011). Production of ketone bodies in excess of the body's ability to utilize them will lead to ketoacidosis, which is more pronounced in patients with T1D. Forthly, insulin deficiency will lead to increased rate of proteolysis resulting in elevated concentrations of amino acids in plasma (Charlton and Nair, 1998). Since all of the amino acids except lysine and leucine are glucogenic amino acids, which are precursors for hepatic and renal gluconeogenesis further

contributing to the hyperglycaemia (Raju and Raju, 2010). In all, as the glucose concentration exceeds the renal reabsorption capacity, dehydration develops via glucosuria and osmotic diuresis and these cause the first typical symptoms of diabetes: polyuria and thirst.

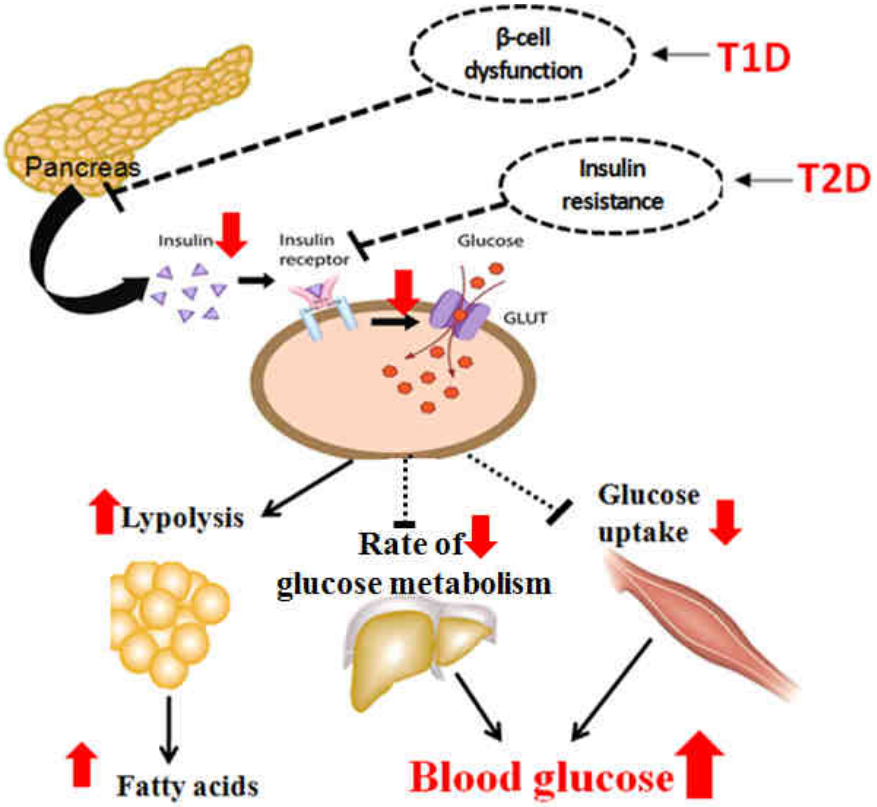

Figure 1. ***Metabolic defects induced by insulin deficiency and insulin resistance in diabetes.*** In normal conditions, blood glucose levels are controlled by insulin-stimulated glucose uptake and the suppression of hepatic glucose production. Insulin also inhibits lipolysis in adipose tissues. In diabetic patients, insulin deficiency and insulin resistance induce hyperglycemia leading to augmented blood glucose levels and increase levels of circulating fatty acids. T1D: type 1 diabetes; T2D: type 2 diabetes; GLUT: glucose transporter. Modified from (Stumvoll, Goldstein et al. 2005).

1.1.3. Classification of diabetes

Diabetes mellitus can be classified into the following general categories:

a) Type 1 diabetes (T1D) also called insulin dependent diabetes mellitus (IDDM) is caused by a pancreatic β-cell destruction, usually leading to absolute insulin deficiency, representing around 5–10% of all cases affecting approximately 20 million people worlwide;

b) Type 2 diabetes (T2D) also called non-insulin dependent diabetes mellitus (NIDDM) is caused by a progressive insulin secretory defect on the background of

insulin resistance (decreased sensitivity of target tissues to insulin). NIDDM represents 85–95% of all cases of diabetes, and its incidence is rapidly expanding, concomitant with the epidemic of obesity;

c) Gestational diabetes mellitus: diabetes diagnosed in the second or third trimester of pregnancy;
d) Specific types of diabetes due to other causes, e.g., monogenic diabetes syndromes such as neonatal diabetes and maturity-onset diabetes of the young; diseases of the exocrine pancreas such as cystic fibrosis; and drug- or chemical-induced diabetes such as in the treatment of HIV/AIDS or after organ transplantation (Raju and Raju, 2010).

1.2. Association between diabetes and cardiovascular disease

Diabetes can lead to several complications including vascular complications. It has been well established that cardiovascular disease (CVD) is the principal cause of death and disability in patients with diabetes (Nesto, 2004; Ceriello, 2005). People with diabetes have a 2- to 4-fold greater risk for having a CVD event than people without diabetes after controlling for traditional risk factors for CVD, such as obesity, tobacco use, dyslipidemia, and hypertension (Coutinho, Gerstein et al. 1999; Buyken, von Eckardstein et al. 2007). It has been estimated that CVD account up to nearly 80% of mortality in diabetic patients (Winer and Sowers 2004). It has also been shown that patients with prediabetic conditions, such as impaired fasting glucose levels, impaired postprandial glucose levels (DECODE, 2001), are at increased risk of CVD as well (Kirpichnikov and Sowers, 2001). Several studies have suggested that impaired glucose tolerance increases the risk for macrovascular disease by approximately 2-fold (Ford, Zhao et al. 2010). Despite a clear association between diabetes and CVD, the underlying mechanisms that link the two diseases are still not fully understood. Recent literature has indicated the importance of both non-glycemic factors, such as age, gender, insulin resistance, hypertension and dyslipidemia, and hyperglycemia in increasing CVD (Stern, Williams et al. 2004; Nathan, Cleary et al. 2005; Stratton, Adler et al. 2000).

1.2.1. Hyperglycemia - an independent cardiovascular risk factor in diabetic patient

1.2.1.1. Diagnosis hyperglycemia in diabetes

The diagnosis of diabetes is based on the criteria recommended by the World Health Organization (Table 1; WHO, 2005). Diagnostic cut-off points for diabetes are fasting plasma glucose (FPG) ≥ 7.0 mmol/l or 2-hour plasma glucose concentration (2-h PG) ≥ 11.1 mmol/l

in an oral glucose tolerance test. Besides, impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) represent intermediate states of abnormal glucose regulation that exist between normal glucose homeostasis and diabetes. IFG is now defined by an elevated FPG concentration (≥ 6.1 and < 6.9 mmol/l) and an elevated 2-h PG concentration (< 7.8 mmol/l). IGT is defined by an elevated 2-h PG concentration (≥ 7.8 and < 11 mmol/l) with the presence of FPG concentration (< 7 mmol/l). This state is also called pre-diabetes, since it precedes full-blown disease in T2D patients (WHO, 2005). Current estimates indicate that most individuals (perhaps up to 70%) with these pre-diabetic states eventually develop diabetes (Tabak, Herder et al. 2012).

Table 1. ***Diagnostic criteria for diabetes and intermediate hyperglycemia (IFG and IGT)*** (WHO 2005).

Diagnostic criteria for diabetes and intermediate hyperglycemia (impaired fasting glucose, IFG and impaired glucose tolerance, IGT)							
	Fasting glucose (mmol/l)				2-hour post-glucose load (mmol/l)		
	Plasma (venous)	Wholeblood			Plasma (venous)	Wholeblood	
		Venous	Capillary			Venous	Capillary
Normal	< 6.1	< 5.6	< 5.6	AND	< 7.8	< 6.7	< 7.8
IFG	6.1 - 6.9	5.6 - 6.0	5.6 - 6.0	AND	< 7.8	< 6.7	< 7.8
IGT	< 7.0	< 6.1	< 6.1	AND	7.8 - 11.0	6.7 - 9.9	7.8 - 11.0
Diabetes	≥ 7.0	≥ 6.1	≥ 6.1	OR	≥ 11.1	≥ 10.0	≥ 11.1

1.2.1.2. Relationship between hyperglycemia and cardiovascular diseases

People with diabetes (particularly, T2D) frequently have many traditional risk factors for CVD, including age, insulin resistance, central adiposity, dyslipidemia, hyperglycemia, chronic inflammation and hypertension. These factors act cumulatively over time and can alter the function of multiple cell types, including endothelial cells, smooth muscle cells (SMC) and platelets, leading to macrovasculopathy and microvasculopathy in people with diabetes (Beckman, Creager et al. 2002). While the role of non-glycemic factors, such as insulin resistance, hypertension, dyslipidemia, and others, has been clearly documented in association with CVD, hyperglycemia remains a primary focus linking diabetes to vascular disease (**Figure 2**).

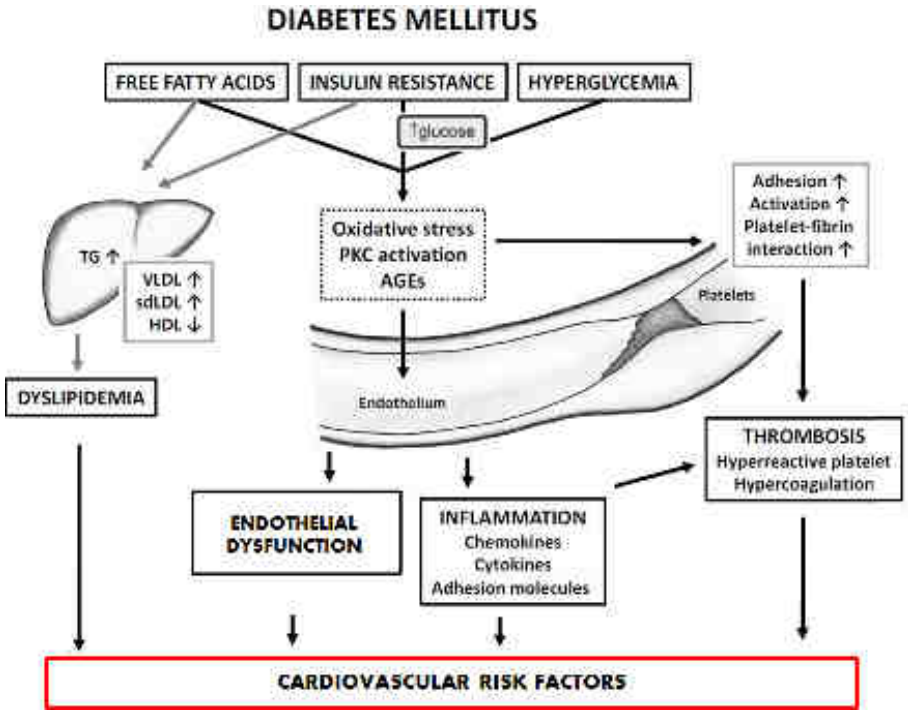

Figure 2. ***Association between diabetes and CVDs***. In diabetes, hyperglycemia, excess free fatty acid and insulin resistance can cumulatively alter the function of multiple cell types leading to augment cardiovascular risk factors. VLDL: very low density lipoprotein; sdLDL: small dense low density lipoprotein; HDL: high density lipoprotein; PKC: protein kinase C; AGEs: advanced glycation end-products. Modified from (Creager, Luscher et al. 2003).

A number of epidemiological studies have suggested that the effect of hyperglycemia on cardiovascular risk is independent of other known risk factors. The epidemiological analysis from the UK Prospective Diabetes Study (UKPDS) in patients with T2D within the range of glycated hemoglobin (HbA_{1c}) studied (5.5-10.5%), suggested that the 6.5% as the threshold above which there is a significant increase risk in cardiovascular events and death in diabetes patients: every 1% higher HbA_{1c} level was associated with a 38% higher risk of a macrovascular event and a 40% higher risk of a microvascular event (Zoungas, Chalmers et al. 2012). Another epidemiological analysis of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study in patients with T2D also showed that 1% (11 mmol/mol) increase in average HbA_{1c} during 3.4 years' duration of the study was associated with 22% increase in mortality (Riddle, Ambrosius et al. 2010). A meta-analysis of observational studies, in which involved three studies in patients with T1D (n = 1688) and 10 studies in patients with T2D (n = 7435), reported the association between HbA_{1c} and CVD (Selvin, Marinopoulos et al. 2004). This study showed that the pooled relative risk for CVD was 1.18;

this represented a 1% point increase in HbA_{1c} level (95% confidence interval, 1.10 to 1.26) in T2D patients. Results in patients with T1D were similar but had a wider confidence interval (pooled relative risk, 1.15; 95% confidence interval, 0.92 to 1.43). Moreover, in another study, Selvin and her colleagues have reported that HbA_{1c} was not only associated with a risk of diabetes but also strongly associated with the risk of CVD and death from any cause as compared with fasting glucose in 15,792 middle-aged non-diabetic adults from four U.S. communities (Selvin, Steffes et al. 2010). After 15-year follow-up, an increase in HbA_{1c} of 1% (11 mmol/mol) was associated with hazard ratio (HR) of 1.19 (1.11–1.27) for coronary heart disease and 1.34 (1.22–1.48) for stroke. Taken together, chronic hyperglycemia can be considered as an independent risk factor in the development and progression of CVD.

In contrast to chronic hyperglycemia, which is well known to impair cardiovascular function, the role of acute hyperglycemia is still less clear. Acute hyperglycemia is contributed by fasting plasma glucose, postprandial glucose levels and glucose variability. Recently, existing evidences demonstrate that acute hyperglycemia can add to the effect of chronic hyperglycemia in inducing cardiovascular risk factors (Milicevic, Raz et al. 2008). In the DECODE (Diabetes Epidemiology: Collaborative analysis of Diagnostic criteria in Europe) group has reported that the acute hyperglycemia correlates with cardiovascular risk in 10 prospective European cohort studies including 15 388 men and 7126 women aged 30 to 89 years, with a median follow-up of 8.8 years (2001). Hazards ratios (95% confidence intervals) in subjects with diabetes on 2 h-blood glucose were 1.73 (1.45-2.06) for all causes, 1.40 (1.02-1.92) for CVD, 1.56 (1.03-2.36) for coronary heart disease, and 1.29 (0.66-2.54) for stroke mortality, compared with the normal 2 h-blood glucose group. Whereas, the corresponding hazards ratios in subjects with diabetes on fasting blood glucose were 1.21 (1.01-1.44), 1.20 (0.88-1.64), 1.09 (0.71-1.67), and 1.64 (0.88-3.07), respectively, in comparison with the normal fasting blood glucose group. Thess findings suggest that the 2 h-blood glucose level is a better predictor of deaths from all causes and CVD than is fasting blood glucose. A number of other authors have also reported that postprandial blood glucose levels correlate more strongly with cardiovascular risk than fasting blood glucose levels in polulations without known diabetes (de Vegt, Dekker et al. 1999; Meigs, Nathan et al. 2002). Besides, several studies performed in patients with T2D have shown a significant positive association between glucose variability and the development or progression of diabetic retinopathy, CVD, and mortality (Nalysnyk, Hernandez-Medina et al. 2010). However,

further studies are required to clarify the role of glucose variability as a potential additional component in the network of vascular complications of diabetes.

1.2.1.3. Role of hyperglycemia in development of cardiovascular disease in diabetic patients

Based on convincing evidences from epidemiological and pathophysiological studies, it is now concluded that chronic hyperglycemia is largely responsible for the progression of the CVD in diabetes (Pistrosch, Natali et al. 2011). It has been suggested that hyperglycemia through oxidative stress alters the function of multiple cell types including endothelial cells, SMC and platelets, and consequently alters the structure and function of blood vessels (**Figure 2**). The main biochemical mechanisms to explain how hyperglycemia may lead to vascular damage is thought through increased production of oxygen free radicals, which will be discussed the more details in the later part of the thesis 1.2.4. Briefly, these pathways include the increased formation of advanced glycation end-products, the activation of protein kinase C-beta (PKCβ), and increased activation of the polyol and hexosamine pathways (Brownlee, 2001). Overall, hyperglycemia through these pathways induces oxidative stress, which, in turn, causes profound alterations in the endothelium. The endothelial alterations include the down-regulation of the major endogenous vasodilator nitric oxide (NO) which, coupled with increased endothelin-1 and angiotensin II formation, causes vasoconstriction, the activation of nuclear factor kappa B (NFκB) and inflammatory gene expression, leading to the increased expression of leucocyte-attracting chemokines, inflammatory cytokines, and cellular adhesion molecules, and the enhanced production of tissue factor and plasminogen activator inhibitor-1, thereby promoting coagulation and decreasing fibrinolysis (Creager, Luscher et al. 2003). Taken together, all these alterations can lead to decreased vasodilation, increased vasoconstriction, enhanced cell proliferation, increased coagulability, augmented matrix formation, decreased fibrinolysis, and increased vascular permeability; all of which are known to be involved in the formation of atherogenesis in CVD.

In addition to its effects on endothelial cells, hyperglycaemia has been shown to alter the function of vascular SMC. Hyperglycemia induces also oxidative stress in vascular SMC by activation of PKC, receptors for advanced glycation end-products, and NFκB. During the development of the fatty streak, SMC migrate from the arterial media to the intima, where they proliferate under the influence of growth factors and lay down a complex extracellular matrix that contributes to the progression of the fatty streak into the advanced atherosclerotic plaque. Lastly,

diabetes is associated with increased platelet activation, which, in conjunction with pro-coagulant changes and impaired fibrinolysis, yields to a state that favours thrombus formation (Kakouros, Rade et al. 2011; Eckel, Wassef et al. 2002). Taken together, hyperglycaemia through several pathological effects enhances the atherogenic environment characterised by oxidative stress, endothelial and vascular dysfunctions, inflammation, and pro-thrombotic changes.

1.2.2. Vascular complications in diabetes

Diabetes is not only a metabolic disease but also considered as a vascular disease, which is characterized by chronic hyperglycemia inducing alterations of cellular homeostasis, leading to diffuse vascular complications. Generally, diabetic vascular complications are separated into macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) (**Figure 3**). Diabetic macrovascular complications mostly involving large arteries and microvascular complications involving small vessels, such as capillaries have similar etiologic characteristics (Mokini and Chiarelli, 2006).

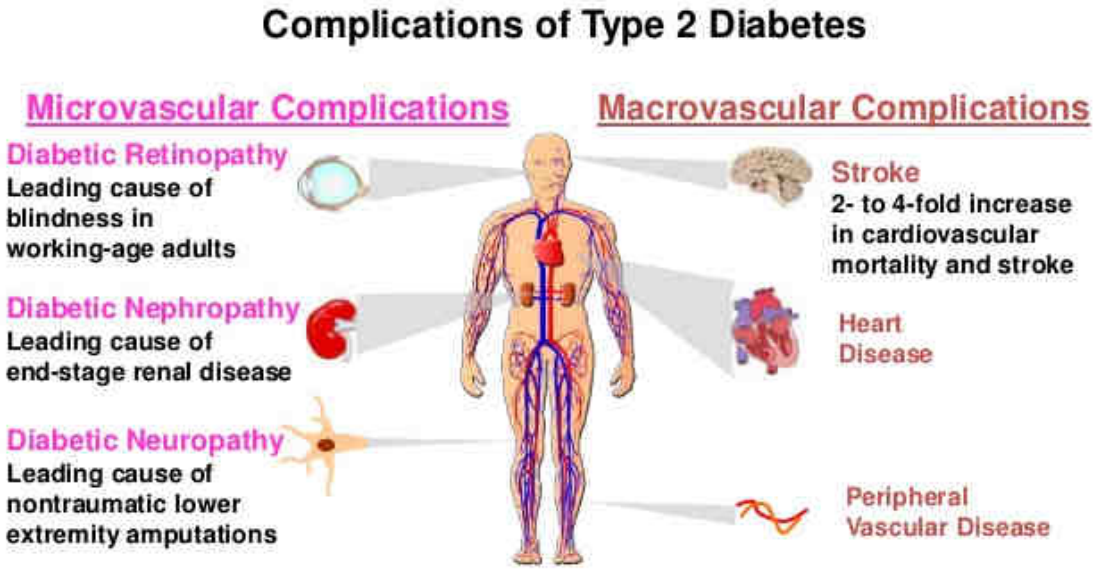

Figure 3. ***Vascular complication of diabetes*.** Adapted from http://www.diabetes.org.

1.2.2.1. Microvascular complications

The microvascular complications of diabetes, resulting from a damage of the microvasculature of retina, kidneys and neurons, include diabetic retinopathy, nephropathy and neuropathy. Despite the differences in target organs, the main pathophysiological characteristics in the development of microvascular complications are similar. Hyperglycemia

induces tissue damage through mitochondrial superoxide anion production (Details of the mechanism will be diccussed in the next part of the thesis 1.2.4.4). Briefly, hyperglycemia initiates diabetic vascular complications through many metabolic and structural derangements, including elevated production of reactive oxygen species (ROS), the production of advanced glycation end products (AGEs), abnormal activation of signaling cascades such as protein kinase C, and abnormal stimulation of hemodynamic regulating systems such as the renin-angiotensin system (Brownlee, 2005). Cells damaged by hyperglycemia include capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves. These cells are at particularly high risk for damage because they are unable to regulate glucose uptake effectively during hyperglycemia (Kaiser, Sasson et al. 1993; Heilig, Concepcion et al. 1995).

- **Diabetic retinopathy** is diabetic complication that affects the eyes. It is one of the leading causes of blindness in people because microvascular complications can affect the peripheral retina, the macula, or both. Clinically, diabetic retinopathy is seperated into non-proliferative and proliferative disease stages. In the early stages, hyperglycemia can lead to intramural pericyte death and thickening of the basement membrane, which contribute to changes in the integrity of blood vessels within the retina, altering the blood-retinal barrier and vascular permeability (Frank, 2004). Further, the increased inflammatory cell adhesion to retinal blood vessels, and capillary blockage can result in hypoxia in the retina (Kohner, Patel et al. 1995). These progressive events contribute to the disease into the proliferative phase where neovascularization and accumulation of fluid within the retina, termed macula edema, contribute to visual impairment. The most significant factor in the development and progression of diabetic retinopathy is the poor glycemic control (Henricsson, Nilsson et al. 1997). Besides, other factors like hypertension, hyperlipidemia, the duration of diabetes and smoking also take a part in the development of diabetic retinopathy. In studies including people with both T1D and T2D, after 30 years of diabetes, most patients had some form of diabetic retinopathy; people with type 1 diabetes and taking insulin had the highest prevalence of diabetic retinopathy, and people with type 2 diabetes diagnosed after age 30 had the lowest prevalence of diabetic retinopathy (Kempen, O'Colmain et al. 2004).
- **Diabetic nephropathy** is diabetic complication that affects the kidneys. The first manifestation of diabetic nephropathy is typically microalbuminuria, which progresses

to overt albuminuria (ie, increased albumin levels in the urine, indicating more severe renal dysfunction) and eventually to renal failure and is the leading cause of end-stage renal disease (Drummond and Mauer, 2002). Characteristic features of diabetic nephropathy are thickening of glomerular basement membranes, glomerular hyperfiltration and podocyte loss leading to mesangial (central part of the renal glomerulus) extracellular matrix expansion and further increases in urinary albumin excretion resulting in microalbuminuria and macroalbuminuria. Other features of diabetic nephropathy include increased serum creatinine, increased blood pressure progressing to glomerular and tubular sclerosis and renal failure (van Dijk and Berl, 2004). The main risk factors for development of diabetic nephropathy include hyperglycemia, hypertension, dyslipidemia, smoking and genetic risk factors. It has been published that about 20–30% of patients with T1D or T2D develop evidence of nephropathy, but in type 2 diabetes, a considerably smaller fraction of these progress to end-stage renal disease (Molitch, DeFronzo et al. 2004).

- **Diabetic (peripheral) neuropathy** is characterized by the dysfunction of the microvasculature supplying the nerves. Neuronal microvasculature is impaired because of the increased oxidative stress, endothelial dysfunction, which potentially leads to neuronal dysfunction (Feldman, 2003). Neuronal dysfunction includes demyelination, axonal degeneration, basement membrane thickening, pericyte loss, loss of microfilaments (ie, cytoskeletal filaments comprising actin and myosin), and decreased capillary blood flow, leading to decreased nerve perfusion and neuronal hypoxia (Dyck and Giannini, 1996). Like those for diabetic nephropathy, the risk factors for peripheral neuropathy include poor glycemic control (ie, elevated glycation hemoglobin levels and impaired glucose tolerance), age, duration of diabetes, tobacco use, dyslipidemia, and hypertension (especially diastolic) (Tesfaye and Selvarajah, 2012). It was estimated that more than half of all individuals with diabetes eventually will develop neuropathy (Tesfaye, 2011).

1.2.2.2. Macrovascular complications

Macrovascular complications include cardiovascular disease, cerebrovascular disease (stroke), and peripheral arterial disease. The main mechanism of marcovascular complications is the atherosclerosis.

- **Cardiovascular disease** (coronary artery disease and myocardial infarction): CVD is a major cause of morbidity and mortality among patients with diabetes mellitus. It was reported that ≈ 70 % of people age 65 or older with diabetes die from some form of heart disease (Retrieved from http://www.americanheart.org/). People with diabetes have a 5-fold-greater risk for a first myocardial infarction (MI) and a 2- to 4-fold-greater risk of developing coronary artery disease (Haffner, Lehto et al. 1998). The central pathological mechanism in CVD is the process of atherosclerosis. Atherosclerosis is a chronic inflammatory condition initiated in the endothelium in response to injury and maintained through the interactions between oxidized lipoproteins, particularly low-density lipoprotein (LDL), T lymphocytes, monocyte-derived macrophages, and other constituents of the arterial wall. After the accumulation of oxidized lipids from LDL particles in the endothelium, monocytes then infiltrate the arterial wall and differentiate into macrophages, which accumulate oxidized lipids to form foam cells. Once formed, foam cells stimulate macrophage proliferation and attraction of T-lymphocytes. T-lymphocytes, in turn, induce smooth muscle proliferation in the arterial walls and collagen accumulation. The central result of the process is the formation of a lipid-rich atherosclerotic plaque with a fibrous cap. Some of the diseases that may develop as a result of atherosclerosis include coronary heart disease, angina (chest pain), and carotid artery disease. In coronary artery disease, atherosclerotic plaques lead to narrowing the diameter of coronary arteries and consequently increased coronary arterial tension (Roberts 1998; Ishida and Sakuma, 2014). Further, complex atherosclerotic plaques may then destabilize and rupture, resulting in myocardial infarction, unstable angina (Boyle 2007). Hyperglycemia is now recognized a major factor in the pathogenesis of atherosclerosis in diabetes. Hyperglycemia induces a large number of alterations at the cellular level of vascular tissue that potentially accelerate the atherosclerotic process (Aronson and Rayfield, 2002).
- **Cerebrovascular disease (stroke)**: Approximately 20% of patients with diabetes die as a result of stroke, the leading disease of death among diabetic patients (Phipps, Jastreboff et al. 2012). The cerebrovascular disease includes the ischemic stroke and the hemorrhagic stroke. From a clinical perspective, diabetes increases the risk of ischemic stroke more than hemorrhagic stroke, resulting in a greater ischemic to hemorrhagic stroke ratio in people with diabetes compared with the general population

(Cheng, 2013). It has been estimated that in patients with T2D range from a 2- to 3-fold increase in men and a 2- to 5-fold increase in women for ischaemic stroke (Lehto, Ronnemaa et al. 1996; Karapanayiotides, Piechowski-Jozwiak et al. 2004).The high stroke risk in diabetes may be due to the complex interplay between the various hemodynamic and metabolic components of the diabetes syndrome. Experimental stroke models have revealed that chronic hyperglycemia leads to deficits in cerebrovascular structure and function that may explain some of the clinical observations. The most consistent mechanisms of the deleterious effects of hyperglycemia on the brain is acidosis, excitatory amino acids release, edema formation, blood-brain barrier injury, hemorrhagic transformation of the infarct and carotid plaque (Kagansky, Levy et al. 2001). Besides, microvessel structural and functional changes due to acute and chronic hyperglycemia also lead to increased incidence and worsened outcomes from stroke in diabetic patients (Ergul, Kelly-Cobbs et al. 2012).

- **Peripheral artery disease**: Peripheral artery disease is characterized by atherosclerotic occlusion of the lower extremity arteries (Kullo, Bailey et al. 2003), which can cause intermittent claudication and pain, especially upon exercise and activity, and which can result in functional impairments and disability (Schainfeld 2001; Vogt, Cauley et al. 1994). Peripheral arterial disease affects approximately 12 million people in the U.S.; approximately 20% to 30% of these patients have diabetes (Marso and Hiatt 2006). People with diabetes have worse arterial disease and a poorer outcome than non-diabetic patients (Jude, Oyibo et al. 2001). The pathophysiology of peripheral arterial disease in the diabetic population is similar to that in the non-diabetic population. However, the distribution of peripheral atherosclerosis in patients with peripheral arterial disease and diabetes is often more distal than in patients without diabetes (Haltmayer, Mueller et al. 2001). The abnormal metabolic state that accompanies diabetes directly contributes to the development of atherosclerosis; pro-atherogenic changes include increases in vascular inflammation and alterations in multiple cell types. In addition, elevated levels of C-reactive protein (CRP) are strongly associated with the development of PAD (Ridker, Cushman et al. 1998). Furthermore, CRP has procoagulant effects related to its ability to enhance expression of tissue factor (Cermak, Key et al. 1993). CRP can increase production of plasminogen activator inhibitor-1, which inhibits the formation of fibrinolytic plasmin

from plasminogen (Chen, Nan et al. 2008; Devaraj, Xu et al. 2003). In addition, CRP also inhibits endothelial cell nitric oxide (NO) synthase (Signorelli, Fiore et al. 2014; Venugopal, Devaraj et al. 2002), resulting in abnormal regulation of vascular tone. Overall, all of these factors enhance the susceptibility of vascular wall to the formation of atherosclerosis in peripheral arteries of diabetic patients.

1.2.3. Endothelial dysfunction – a hallmark of cardiovascular disease

During the last two decades, it has been well established that endothelial dysfunction represents as a key early step in the development of atherosclerosis and is also involved in the plaque progression and the occurrence of atherosclerotic complications in patients suffering CVD (Anderson, Gerhard et al. 1995).

1.2.3.1. Key role of the endothelium in the control of vascular homeostasis

The endothelium is formed by a monolayer of endothelial cells lining the surface of a blood vessels (**Figure 4**). For many years, this cell layer was thought to be simply a physical barrier between circulating blood and the underlying tissues. It is now recognized to be a predominant endocrine organ, releases a number of substances in order to maintain the vascular tone, blood fluidity and platelet aggregation as well as to control the regulation of immunology, inflammation and angiogenesis.

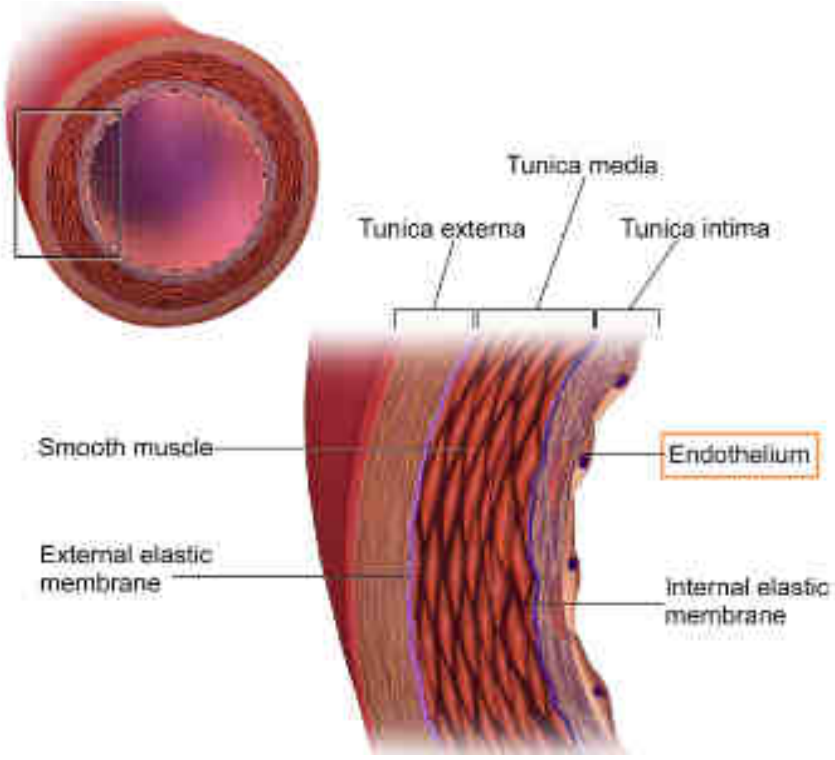

Figure 4. ***The structure of blood vessels***. The arterial wall has three layers: the intima, the media, and the adventitia. Intima is the most inner layer consisting of the endothelium lining the luminal surface. Adapted from Blausen.com staff.

Under physiological conditions, the endothelium plays a pivotal role in maintaining cardiovascular homeostasis by releasing a number of vasodilator and vasoconstrictor substances, which act to regulate the vascular tone and hence to limit the increase in blood pressure, consequently controlling tissue blood flow and maintaining blood fluidity. Besides, endothelium also releases a number of substances, which have antiproliferative, antithrombotic and antiplatelet effects to regulate the antiproliferation, inflammatory cell adhension and the platelet aggregation (**Table 2**). Moreover, endothelial cells also regulate the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) involved in oxidation and nitrosylation of proteins and lipids, and growth factors promoting cell growth (Galley and Webster, 2004). **In pathological conditions**, there is a shift toward vasoconstrictors, proliferative and inflammatory substances. Thus, the endothelial dysfunction is characterized by impaired endothelium-dependent vasodilatation and a prothrombotic and proinflammatory state of endothelial cells. The alterations of the endothelial function have been consistently found in hypertension, atherosclerosis, coronary heart disease, diabetes, sepsis, obesity and aging (Widlansky, Gokce et al. 2003). Thus, endothelial dysfunction is an early and independent predictor of poor prognosis in most forms of CVDs (Endemann and Schiffrin, 2004).

Table 2. ***Summary the main endothelial-derived substances.***

Funtions	**Substances released from endothelium**
Vasodilatation	NO, prostacyclin, endothelium-derived hyperpolarizing factor, bradykinin, adrenomedullin, C-natriuretic peptide
Vasoconstriction	ET-1, angiotensin-II, thromboxane A_2, oxidant radicals, prostaglandin H_2
Inhibition of proliferation	NO, prostacyclin, transforming growth factor-β, heparan sulfate
Proliferation	ET-1, angiotensin-II, oxidant radicals, platelet-derived growth factor, basic fibroblast growth factor, insulin-like

	growth factor, interleukin 1, 2, 3, 6, 11
Inhibition of thrombosis	NO, prostacyclin, plasminogen activator, protein C, tissue factor inhibitor, von Willebrand factor
Stimulation of thrombosis	ET-1, oxidant radicals, plasminogen-activator inhibitor-1, thromboxane A_2, fibrinogen, tissue factor
Inflammatory responses	P- and E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intracellular cell adhesion molecule-1 (ICAM-1), chemokines, nuclear factor κ-B
Permeability	Receptor for advanced glycosylation end-products
Angiogenesis	Vascular endothelial growth factor

As the principle role of endothelium is a regulation of the vascular tone of the underlying vascular SMC by releasing various relaxing and contracting factors, we will focus on review of endothelium-derived relaxing and contracting substances.

a) Endothelium-derived relaxing substances

The most important endothelium-derived relaxing factors (EDRF) released by endothelium are nitric oxide (NO), prostacyclin (PGI_2), and an endothelium-derived hyperpolarizing factor (EDHF). The three mediators are not always equally important — NO may predominate in large arteries, whereas EDHF takes over in smaller blood vessels or in certain large arteries such as coronary arteries when the release of NO is curtailed.

Nitric oxide (NO)

Among endothelium-derived vasodilators, NO occupies a central position because changes in the release of endothelial NO play a crucial role in the perturbation of vascular homeostasis and in the development of endothelial dysfunction associated with various cardiovascular disorders (Annette Schmidt, 2012). Furchgott and Zawadzki in their landmark study discovered that the endothelium releases a factor, which is identified as endothelium-derived relaxing factor (EDRF), relaxing the underlying vascular smooth muscle and this was later shown to be nitric oxide (NO) (Furchgott and Zawadzki, 1980). In the endothelium, NO plays a pivotal role in controlling vascular tone and blood pressure (Moncada and Higgs 1993). In addition, NO is also considered as an antiatherogenic and antithrombotic molecule,

which can inhibit platelet aggregation, inflammatory cell adhesion, smooth muscle cell proliferation and migration (Lloyd-Jones and Bloch, 1996).

NO is synthesized by endothelial NO synthase (eNOS), which catalyzed the conversion reaction from L-arginine to L-citrulline and NO, a NADPH-dependent enzyme that requires calcium-calmodulin (Ca^{2+}/CaM), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydro-L-biopterin (BH_4) as cofactors (Forstermann, Closs et al. 1994). The production of NO depends on several factors, including eNOS mRNA and protein expression, its interaction with caveolin, camodulin and heat shock protein 90 (hsp90) (Garcia-Cardena, Fan et al. 1998) and its translational modifications such as phosphorylation (Rafikov, Fonseca et al. 2011) and S-nitrosylation at different sites (Ravi, Brennan et al. 2004). Besides, the production of NO also depends on the availability of L-arginine, which is influenced by arginase (which catalyzes arginine to ornithine and urea), therefore, this enzyme can compete with eNOS for arginine (Li, Meininger et al. 2001), and the abundance of asymmetric dimethylarginine (ADMA, an endogenous eNOS inhibitor that competes with L-arginine for binding to eNOS (Cooke 2004). In addition, NO production is also dependent on the availability of cofactors (including BH_4, NADPH and Ca^{2+}) (Katusic, 2001). Regarding BH_4, many studies have shown that the loss of BH_4 by oxidation to BH_2 leads to reduced BH_4 bioavailability for eNOS. Besides, the oxidation not only directly reduced BH_4 bioavailability, but also the oxidized BH_2, which has no cofactor activity, may compete with BH_4 for binding to eNOS (Bendall, Douglas et al. 2014).

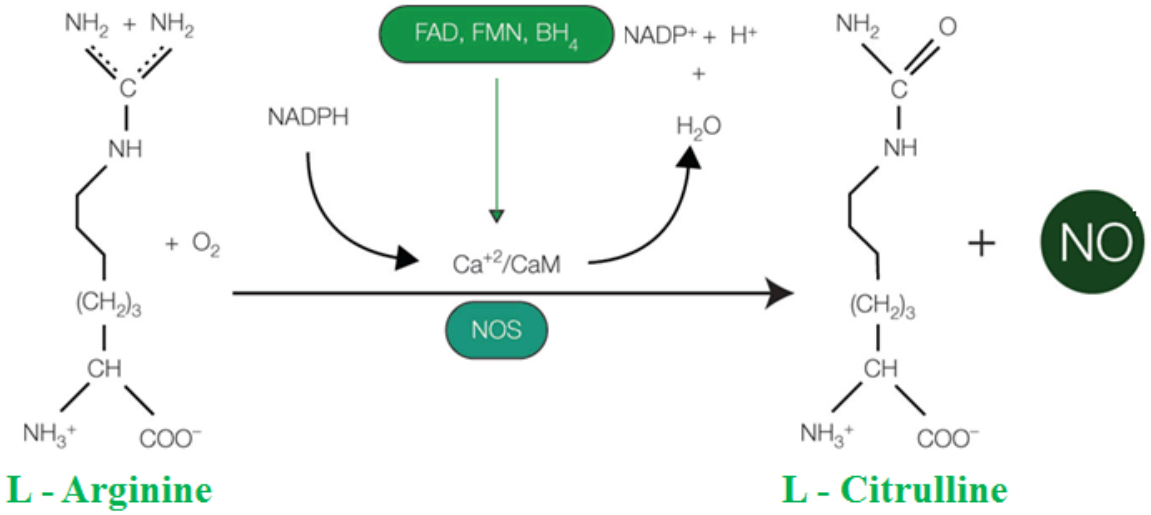

Figure 5. ***Nitric oxide synthesis***. Calcium (Ca^{2+}) binds to calmodulin (CaM) activating eNOS, which catalyses the oxidation of the terminal guanidinyl nitrogen of the amino acid L-arginine

to form L-citrulline and nitric oxide (NO) in the presence of oxygen, NADPH and co-factors such as flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), haem and tetrahydrobiopterin (BH_4). (Adapted from Forstermann, Closs et al. 1994).

After its synthesis NO diffuses towards the adjacent vascular smooth muscle cells (VSMC) and binds to the heme group of soluble guanylyl cyclase (sGC) promoting the conversion of guanosine triphosphate (GTP) to guanosine 3',5'-cyclic monophosphate (cGMP). Then, cGMP triggers a reduction in [Ca^{2+}] through the activation of cGMP-dependent protein kinase PKG. Activated PKG phosphorylates several key target proteins, including voltage-gated [Ca^{2+}] channels, promoting to the reduction of [Ca^{2+}]. In addition, the activations of Ca^{2+}/ATPase pump in the plasma membrane and the sarcoplasmic reticulum lead to the extrusion of [Ca^{2+}] across the plasma membrane and the uptake [Ca^{2+}] in sarcoplasmic reticulum, respectively, also mediate the reduced cytosolic level of Ca^{2+}. Taken together, the reduction of the cytosolic [Ca^{2+}] leads to smooth muscle relaxation (Lehen'kyi, Zelensky et al. 2005; Bruckdorfer 2005; Carvajal, Germain et al. 2000) (**Figure 6**).

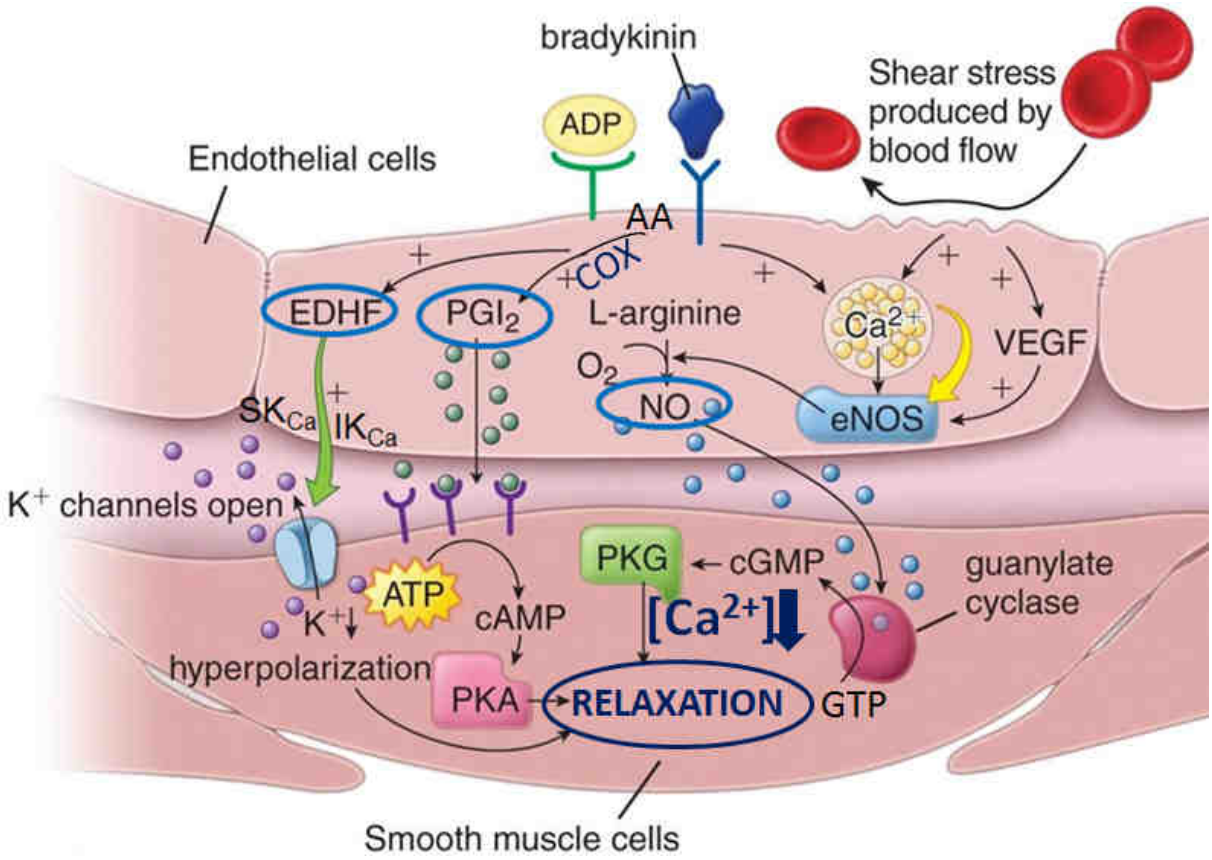

Figure 6. ***Endothelial-derived relaxing factors***. Relaxation of VSMC by diffusable vasodilator substances from endothelial cells. Nitric oxide (NO) activates soluble guanylyl

cyclase, yielding increased levels of cyclic guanosine 3',5'-cyclic monophosphate (cGMP). Prostacyclin (PGI_2) activates adenylyl cyclase, leading to increased production of cyclic adenosine 3',5'-cyclic monophosphate (cAMP). Endothelium-derived hyperpolarizing factor (EDHF), which causes Ca^{2+}-dependent K^+ channels activation leading to the hyperpolarization of VSMC. eNOS, NO synthase; COX: cyclooxygenase; GTP: guanosine triphosphate; ATP: adenosine triphosphate; PKA: cAMP-dependent protein kinase; PKG: cGMP-dependent protein kinase; SK_{Ca}: small conductance calcium-activated potassium channels; IK_{Ca}: intermediate conductance calcium-activated potassium channels; ADP: adenosine diphosphate; Blue sphere: NO molecules; Green sphere: PGI_2 molecules; Purple sphere: EDHF. Modified from https://s3.amazonaws.com.

Prostacyclin

Prostacyclin (PGI_2) is considered as the major endothelial cyclooxygenase (COX) metabolites (Bogatcheva, Sergeeva et al. 2005). PGI_2 is generated from arachidonic acid by COX in endothelial cells in response to neurohumoral mediators and physical forces, such as the shear stress exerted by the flowing blood (Furchgott and Vanhoutte, 1989; Okahara, Sun et al. 1998). After synthesis, PGI_2 diffuses to the smooth muscle, where it activates adenylyl cyclase through G-protein-coupled receptors, leading to increased production of 3',5'-cyclic adenosine monophosphate (cAMP), which, in turn, activates protein kinase A (Bogatcheva, Sergeeva et al. 2005). The activation of protein kinase A causes K^+ channels to open and produces smooth muscle hyperpolarization (Parkington, Coleman et al. 2004). In addition, voltage-sensitive Ca^{2+} channels will close, resulting in decreased intracellular free Ca^{2+} concentration, which, in turn, causes VSMC relaxation (**Figure 6**). Additionally, PGI_2 also inhibits platelet aggregation and prevents platelet-leukocytes adhesion to the vascular wall (Egan and FitzGerald, 2006).

Endothelium-derived hyperpolarization factor (EDHF)

In various blood vessels, endothelium-dependent relaxations are accompanied by endothelium-derived hyperpolarization (**Figure 6**). EDHF-mediated dilation occurs independently of a NOS or COX metabolite (Feletou 2011). Currently, it is well known that EDHF-mediated responses are initiated by an increase in the endothelial Ca^{2+} and the consequent activation of endothelial small conductance calcium-activated potassium channels (SK_{Ca}) and intermediate conductance calcium-activated potassium channels (IK_{Ca}) channels, which elicit the hyperpolarization of the endothelial cells. The endothelial hyperpolarization

could then spread to the adjacent SMC through myo-endothelial gap junctions or the efflux of K^+ through the endothelial SK_{Ca} and IK_{Ca} channels and elicit the hyperpolarization of the surrounding myocytes by activating inwardly rectifying K^+ (K_{IR}) channels and/or the Na^+–K^+-ATPase (Busse, Edwards et al. 2002). The relative importance of the EDHF mediated mechanisms to NO mediated mechanisms alters with vessel size. NO is an important endothelium-dependent mediator of vascular tone in relatively large arteries and larger arterioles. EDHFs are shown to be involved in vasodilation of smaller arteries (Feletou and Vanhoutte, 2009). However, the role of EDHF in ED in human CVDs remains elusive. This may be related to the difficulty that the function of EDHF can only be deciphered after impairment of NO and prostacyclin-mediated responses.

b) Endothelium-derived contracting substances

Major endothelium-derived vasoconstrictors are endothelin-1, thromboxane A_2, and prostaglandin H_2, angiotensin II and ROS. In addition, endogenous (for example, arachidonic acid, noradrenaline, thrombin) and pharmacological substances (for example, calcium, nicotine, high potassium), and physicochemical stimuli (shear stress, mechanical stress, hypoxia) can also stimulate endothelium-dependent vasoconstriction.

Endothelin-1

Endothelin 1 (ET-1), which is a 21-amino acid peptide, is known as one of the most potent vasoconstrictors (Yanagisawa, Kurihara et al. 1988). In human, ET-1 acts through specific receptors named ET_A and ET_B. ET_A receptors are represented only on SMC and have the function of promoting growth and mediating contractions. ET_A receptors have been shown to be coupled to the $G_{q/11}$ protein leading to activation of phospholipase C. The activation of $G_{q/11}$ stimulates cleavage phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) resulting in increased Ca^{2+} influx through Ca^{2+} channels (Neylon, 1999). Consequently, the increased intracellular [Ca^{2+}] stimulates phosphorylation of myosin light chain leading to smooth muscle contraction (Wynne, Chiao et al. 2009). In contrast, ET_B receptors are located on both endothelial and SMC, with different effects (**Figure 7**). Smooth muscle cell ET_B receptors evoke contractions, whereas endothelial ET_B receptors induce relaxation by the production of endothelium-derived relaxing factors, including nitric oxide (NO) and prostacyclin. Thus, this explains the mechanism why ET-1, which is a physiological substance, can shift from a physiological to a

pathological role in CVD (Haynes and Webb 1998). In blood vessels, the ETA receptor is dominant under normal conditions in terms of ET-1 effects on contraction.

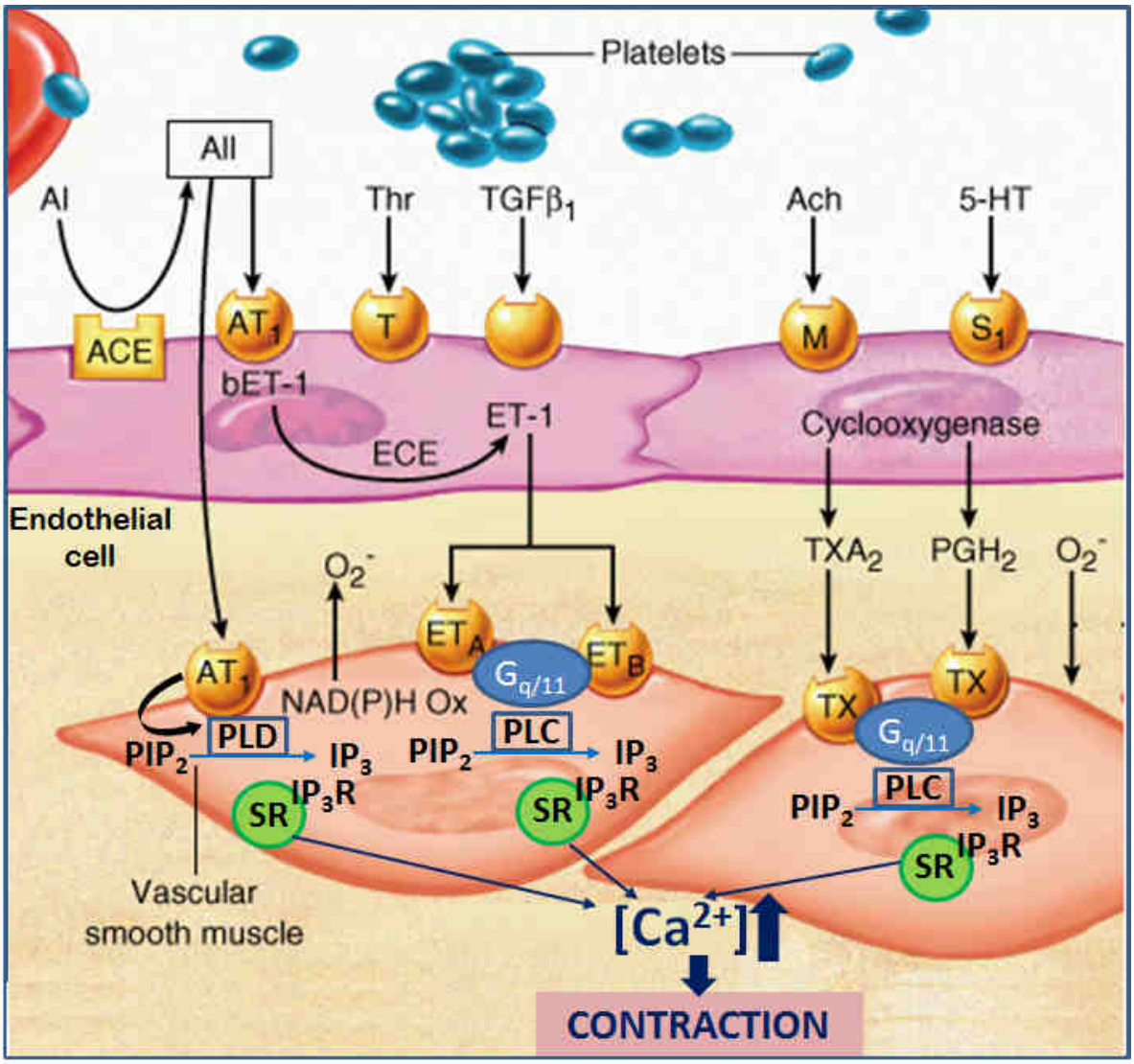

Figure 7. ***Release of endothelial-derived contracting factors***. AI: angiotensin I; AII: Angiotensin II; AT1: angiotensin II receptor 1; AT2: angiotensin II receptor 2; ACE: angiotensin-converting enzyme; ECE: endothelin converting enzyme; ET-1: endothelin-1; ET_A, ET_B: endothelin-1 receptors; NADPH Ox: nicotinamide adeninedinucleotide phosphate oxidase; NO: nitric oxide; PGH_2: prostaglandin; TXA_2: thromboxane; TX: thromboxane prostanoid receptor; ACh: acetylcholine; M: muscarinic receptor for acetylcholine; 5-HT: serotonin; S1: serotonin receptor; Thr: thrombin; T: Thrombin receptor; TGFβ1: Transforming growth factor beta 1; PLC: phospholipase C; PLD: phospholipase D; PIP_2: phosphatidylinositol 4,5-bisphosphate; IP_3: inositol 1,4,5-trisphosphate; IP_3R: inositol 1,4,5-trisphosphate receptor; SR: sarcoplasmic reticulum. (Modified from Ruschitzka et al. 1999)

Vasoconstrictor prostanoids

Vasoconstrictor prostanoids produced by endothelium are included prostaglandin H_2 (PGH_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane A_2 (TXA_2) (Feletou, Huang et al. 2010). Firstly, PGH_2 is formed from arachidonic acid by COX. Then, PGH_2 is metabolized to $PGF_{2\alpha}$ and TXA_2 by specific synthase (Smith 1992). The vasoconstrictor prostanoids are recognized by the thromboxane prostanoid receptors (TX) located in VSMC (**Figure 7**). Once activated, TX are coupled to either $G_{q/11}$ or $G_{12/13}$ proteins, which, in turn, evokes the biosynthesis of IP_3 by coupling to the phospholipase C and activates Rho kinase (Momotani, Artamonov et al. 2011). The generation of IP_3 stimulates intracellular Ca^{2+} release from the sarcoplasmic reticulum into the cytoplasma (Hisatsune, Nakamura et al. 2005; Berridge, Lipp et al. 2000). Consequently, the increase intracellular [Ca^{2+}] leads to open the Ca^{2+} channel on the cellular membrane, resulting in the elevation of [Ca^{2+}], which initiates contractile response by activation of myosin light chain kinase (Hill-Eubanks, Werner et al. 2011). Moreover, the activation of Rho kinase leads to inhibition myosin light chain phosphotase (Tsai and Jiang, 2006). Overall, the phosphorylated myosin light chain increases the activity of actin-activated Mg^{2+}-ATPase leading to actin-myosin interaction and smooth muscle contraction (Wakabayashi, 2015). It has been published that arachidonic acid induces endothelium-dependent contractions in arteries and veins, which can be inhibited by cyclooxygenase blockers. In addition, TXA_2 is also a potent activator of platelets, while activated platelets, in turn, release a large amount of TXA_2 to promote thrombosis (Taddei, Ghiadoni et al. 2003).

Angiotensin II

Angiotensin II (Ang II), which is the bioactive peptite in the renin-angiotensin system, plays the major role in regulation of blood pressure, water and sodium homoeostasis and neuronal function. Ang II is converted from Angiotensin I by enzyme angiotensin-converting enzyme (ACE). ACE is found mostly in the lung, but it also presents in endothelial cells (**Figure 7**) (Higuchi, Ohtsu et al. 2007). The effects of Ang II are mediated by two G-protein-coupled receptors (AT1 and AT2), which are expressed on vascular SMC (Ohtsu, Suzuki et al. 2006). In SMC, Ang II signaling cascade via AT1 receptor stimulates phospholipase C, which leads to cleavage IP3 and DAG, and phospholipase D, which also generates DAG. Like prostanoids, Ang II rapidly increases the intracellular [Ca^{2+}] by its release from the sacroplasmic reticulum. Then the sustained increase in [Ca^{2+}] facilates increased cytosolic [Ca^{2+}] from extracellular space resulting in the activation of myosin light chain kinase.

Besides, the binding of Ang II to AT1 receptor also activates RhoA/Rho kinase, which inhibits the dephosphorylation of myosin light chain by myosin light chain phosphotase. Consequently, both of these processes lead to the contraction of SMC (Touyz and Berry 2002; Kanaide, Ichiki et al. 2003). In contrast to AT1 receptor, angiotensin II *via* AT2 receptors stimulates vasorelaxation by increased production of NO and cGMP (Lemarie and Schiffrin 2010; Wiemer, Scholkens et al. 1993). Besides, AT1 receptor is also found in endothelial cells (Pueyo and Michel 1997). Although the endothelial AT1 signal transduction and function are much less known than those in VSMC, it has been suggested that the AT1 receptor signaling regulates the balance between NO and ROS in endothelial cells (Nickenig 2002; Watanabe, Barker et al. 2005).

Reactive oxygen species (ROS)

Reactive oxygen species are reactive molecules and free radicals derived from molecular oxygen. Many ROS are free radicals, which possess unpaired electrons, including molecules such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), nitric oxide ($NO^{\bullet}$), and lipid radicals. Other ROS are molecules derived from molecular oxygen, such as hydrogen peroxide (H_2O_2), peroxynitrite (OONO–), and hypochlorous acid (HOCl) have oxidizing effects that contribute to oxidant stress. The cellular production of one kind of ROS may lead to the production of several others via radical chain reactions. ROS are produced by various oxidase enzymes, including nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase, uncoupled endothelial NO synthase (eNOS), xanthine oxidase, cyclooxygenase, lipooxygenase, glucose oxidase, cytochrome P450, and mitochondrial electron transport (Cai and Harrison, 2000). Under physiological conditions, ROS are produced in low concentrations and act as a signaling molecule that regulate VSMC contraction (**Figure 7**) and participate in VSMC growth (Touyz and Schiffrin, 1999). A number of studies has been shown the ROS-induced contractile response in the basilar artery of the dog (Katusic, Shepherd et al. 1987); (Katusic, Schugel et al. 1993) and the renal artery of the rat (Gao and Lee 2005). In other blood vessels, they may act as a facilitator of endothelium-dependent contractions by reducing the bioavailability of the NO produced by the endothelial cells (Cosentino, Sill et al. 1994; Miyagawa, Ohashi et al. 2007). Moreover, several intracellular signal events stimulated by ROS have been defined, including the two members of mitogen-activated protein kinase family (ERK1/2 and big MAP kinase, BMK1), tyrosine kinases (Src and Syk) and different isoenzymes of PKC (Abe and Berk 1999; Sundaresan, Yu et al. 1995). ROS regulate of signal

transduction components including the modification in the activity of transcriptional factors such as NF-κB and others that result in changes in gene expression and modifications in cellular responses. Thus in the vascular system, ROS plays a physiological role not only in the regulation of differentiation and contractility of VSMC, but also in the control of vascular endothelial cell proliferation and migration, in the induction of platelet activation and haemostasis, and in the immune response (Vara and Pula, 2014).

1.2.3.2. Endothelial dysfunction

Endothelial dysfunction is characterized by a shift in the actions of the endothelium toward reduced vasodilation, a proinflammatory state, and prothrombic properties, by the increased vasocontraction, the enhaced expression of adhesion molecules and upregulation of smooth muscle cell proliferation and migration. It is associated not only with most forms of CVD, such as hypertension, coronary artery disease, chronic heart failure, peripheral vascular disease, but also with diabetes, chronic kidney failure, and severe viral infections. Initially, endothelial dysfunction is characterized by the diminished production or availability of NO and a disrupted balance in the relative contribution of endothelium-derived relaxing and contracting factors. This imbalance predisposes the vasculature to vasoconstriction, pro-oxidation, leukocyte adherence, platelet activation, thrombosis, impaired coagulation, vascular inflammation, mitogenesis, increased permeability and atherosclerosis (**Figure 8**).

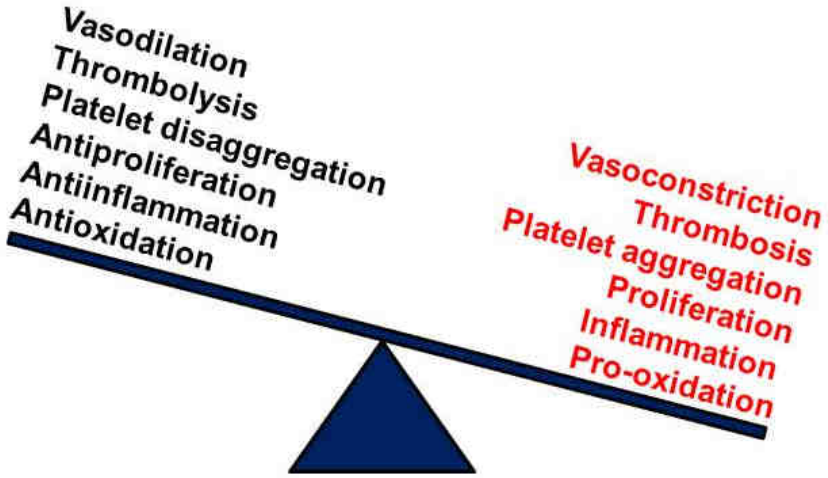

Figure 8. ***Endothelial dysfunction*** is characterized by the imbalance between vasodilation and vasoconstriction, thrombolysis and thrombosis, platelet aggregation and disaggregation, growth inhibition and growth promotion, anti-inflammation and proinflammation, and also antioxidation and pro-oxidation.

It is well accepted that endothelial dysfunction occurs in response to cardiovascular risk factors and precedes the development of atherosclerosis (Shimokawa 1999; Ross 1999). Endothelial dysfunction actively participates in the process of lesion formation by promoting the early and late mechanisms of atherosclerosis, including upregulation of adhesion molecules, increased chemokine secretion and leukocyte adherence, increased cell permeability, enhanced LDL oxidation, platelet activation, cytokine elaboration, and vascular smooth muscle cell proliferation and migration. Endothelial dysfunction has been implicated in the pathogenesis and clinical course of all known CVDs and is associated with future risk of adverse cardiovascular events (Schachinger, Britten et al. 2000; Verma and Anderson 2002). Molecular processes contributing to endothelial dysfunction include impaired endothelium-dependent vasorelaxation by decreased NO bioavailability, increased generation of ROS, increased secretion of others vasoconstrictors such as endothelin-1, angiotensin II, increased expression of adhesion molecules, synthesis of pro-inflammatory and pro-thrombotic factors, enhanced permeability, reduced number of circulating endothelial progenitor cells and increased molecular markers of damage (circulating endothelial microparticles derived from activated or apoptotic cells) (Reriani, Lerman et al. 2010). An impaired endothelium-dependent vasorelaxation is a hallmark of the development and progression of endothelial dysfunction.

Mechanism involved in the impairment of endothelium-dependent vasorelaxation

The impaired endothelium-dependent vasorelaxation may arise from several mechanisms, including decreased production of endothelium-derived relaxation factors (EDRF), enhanced generation of endothelium-derived constricting factors (EDCF), enhanced inactivation of EDRF, impaired diffusion of EDRF to the underlying SMC and decreased responsiveness of the smooth muscle to EDRF (**Figure 9**) (De Vriese, Verbeuren et al. 2000).

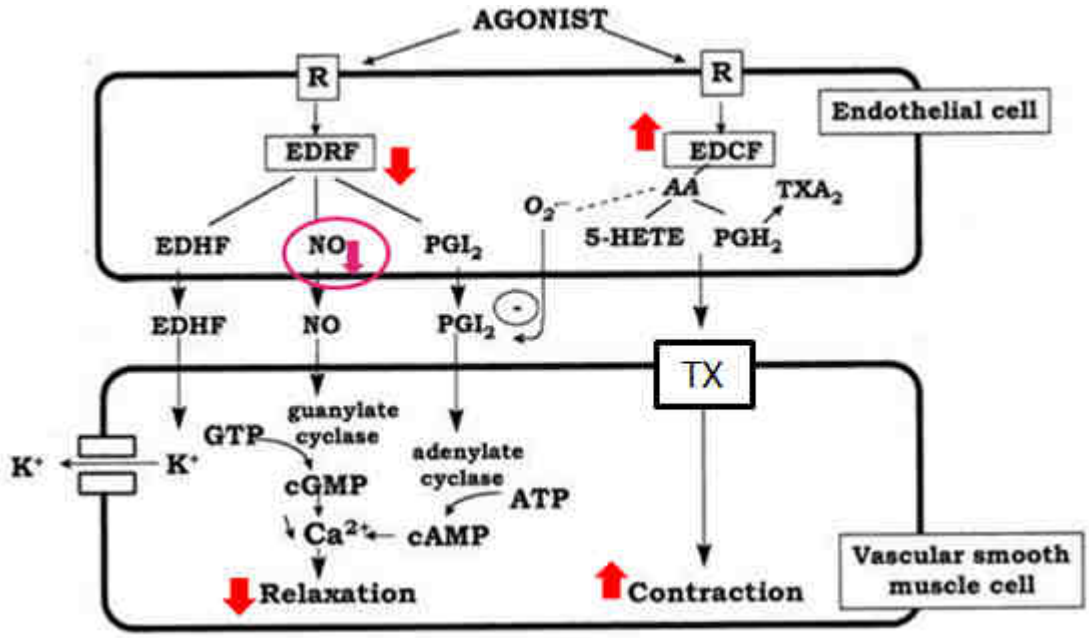

Figure 9. ***Mechanisms of impaired endothelium-dependent vasorelaxation***. Impaired endothelium-dependent vasodilatation arises from several mechanisms: decreased production of endothelium-derived relaxation factors (EDRFs), enhanced inactivation of EDRF, impaired difusion of EDRF to the underlying VSMC, decreased responsiveness of the smooth muscle to EDRF and enhanced generation of endothelium-derived constricting factors (EDCF). Abbreviation: R: receptor of agonist; EDRF: endothelium-derived relaxing factor; EDHF: endothelium-derived hyperpolarizing factor; PGI_2: prostacyclin; EDCF: endothelium-derived constricting factors; TXA_2: thromboxane A_2; PGH_2: prostaglandin H_2; 5-HETE: 5-hydroxyeicosatetraenoic acid; TX: tromboxane prostanoid receptor; $O_2^{\bullet-}$: superoxide anion; cGMP: cyclic guanosine 3',5'-cyclic monophosphate; cAMP: cyclic adenosine 3',5'-cyclic monophosphate; GTP: guanosine triphosphate; ATP: adenosine triphosphate. Modified from (De Vriese, Verbeuren et al. 2000).

Although mechanisms leading to the impaired endothelium-dependent vasorelaxation are multiple, a reduction in NO bioavailability plays the fundamental role (**Figure 10**). A lack of L-arginine, a substrate for eNOS, an abundance of asymmetric dimethylarginine (ADMA: an endogenous eNOS inhibitor that competes with L-arginine for binding to eNOS) (Cooke, 2004) and the availability of cofactors such as tetrahydrobiopterin (BH_4) and NADPH (Katusic, 2001) can contribute to reduce the bioavailability of NO. Specially, a decline in NO bioavailability could be due to decreased eNOS expression. However, several studies have shown that cardiovascular risk factors are associated with an increase rather than a decrease in eNOS expression (Li, Wallerath et al. 2002). The increased expression of eNOS in vascular

disease is likely to be a consequence of an excess production of H_2O_2. H_2O_2, the dismutation product of $O_2^{\bullet-}$, can increase eNOS expression through transcriptional and posttranscriptional mechanisms (Drummond, Cai et al. 2000). On the other hand, an accelerated degradation of $NO^{\cdot}$ (by its reaction with $O_2^{\bullet-}$) is likely to occur in vascular disease (Masha, Dinatale et al. 2011). It has been shown that the interaction between $NO^{\cdot}$ and $O_2^{\bullet-}$ occurs at an extremely rapid rate of 6.7×10^9 mol/L^{-1}/s. This is 3 times faster than the reaction rate for $O_2^{\bullet-}$ with SOD. Given this rapid reaction rate, there is always likely existing $O_2^{\bullet-}$ reacting with $NO^{\cdot}$ within cells and in the extracellular space (Cai and Harrison, 2000). Under physiological conditions, endogenous antioxidant defenses minimize this interaction and maintain what seems to be a tenuous balance between $O_2^{\bullet-}$ and $NO^{\cdot}$. This balance is altered in a CVD state. $NO\cdot$ and $O_2^{\bullet-}$ react avidly to form peroxynitrite ($ONOO^-$), which in turn leads to eNOS uncoupling and enzyme dysfunction. Since $ONOO^-$ is a highly cytotoxic reactive nitrogen species, it avidly reacts and damages proteins, lipids, and DNA. For instance, $ONOO^-$ reacts with tyrosine residues of proteins to produce nitrotyrosine, which can be detected in the plasma and various tissues. Alternatively, ROS can initially react with tyrosine to produce tyrosyl radicals that can, in turn, oxidize NO and generate nitrotyrosine. In addition, nitrotyrosine can be formed from an interaction of nitrogen dioxide with tyrosine residues of proteins. Consequently, nitrotyrosine abundance is generally considered as an indicator of NO oxidation by ROS (Forstermann 2010; Higashi, Noma et al. 2009). Therefore, enhanced NO inactivation caused by excess ROS production plays often an important role in the impairment of endothelium-dependent vasodilation rather than decreased NO production. Endothelial dysfunction has been shown to be associated with an increase in ROS in atherosclerotic animal models and human subjects with atherosclerosis (Cai and Harrison, 2000). In addition, NADH/NADPH oxidase, which is a major source of production of ROS in vessel walls, is activated in experimental models of atherosclerosis (Hegde, Srivastava et al. 1998). The concentrations of antioxidant scavengers, such as SOD, GPx catalase, and vitamins C and E, are decreased in patients with atherosclerosis (Irani, 2000).

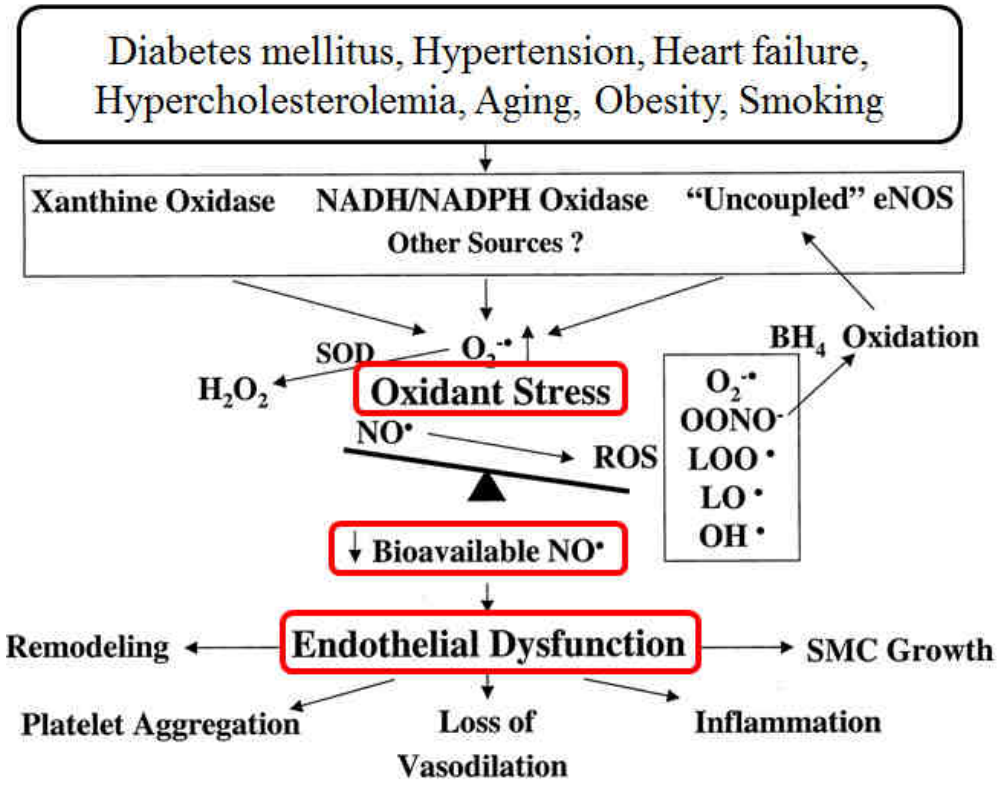

Figure 10. ***Mechanisms by which cardiovascular risk factors induce ED.*** ROS: reactive oxygen species; NO: nitric oxide; $O_2^{\bullet-}$: superoxide anion; $OONO^-$: peroxynitrite; $LOO^\cdot$: peroxyl radical; $LO^\cdot$: alkoxyl radical; $OH^\cdot$: hydroxyl radical; H_2O_2: hydrogen peroxide; NADPH: nicotinamide adenosine dinucleotide phosphate; SOD: superoxide dismutase; eNOS: endothelial NO synthase; SMC: smooth muscle cell. Reproduced from (Higashi, Noma et al. 2009).

1.2.4. Endothelial dysfunction in diabetes

In diabetic patients, endothelial dysfunction is also characterized by the reduced endothelial-dependent vasodilation response to an endothelium-dependent vasodilator (Nitenberg, Valensi et al. 1993; De Vriese, Verbeuren et al. 2000), the increased proinflammatory and prothrombotic state (Devaraj, Dasu et al. 2010; Isordia-Salas, Galvan-Plata et al. 2014); Sena, Pereira et al. 2013), the enhanced expression of adhesion molecules (Nozaki, Ogura et al. 2002; Rubio-Guerra, Vargas-Robles et al. 2009), and upregulation of smooth muscle cell proliferation and migration (Panchatcharam, Miriyala et al. 2010; He, Xue et al. 2012). The combination of all these phenomenons leads to the endothelial dysfunction and provides the foundation for the development of micro- and macro-vascular disease. Therefore, diabetes is also considered as a vascular disease because of its effect on macro and microcirculation of

many vascular beds. Endothelial dysfunction has been documented in patients with type 2 diabetes (Hogikyan, Galecki et al. 1998; Watts, O'Brien et al. 1996; Avogaro, Fadini et al. 2006) and also in individuals with type 1 diabetes (Clarkson, Celermajer et al. 1996; Johnstone, Creager et al. 1993; Funk, Yurdagul et al. 2012). The endothelial dysfunction phenomens, such as the reduced NO production (Lin, Ito et al. 2002; Tessari, Cecchet et al. 2010), the increased platelet aggregation (Angiolillo, Bernardo et al. 2006; Kajita, Ishizuka et al. 2001; Vinik, Erbas et al. 2001), the enhanced expression of adhesion molecules (Bai, Tang et al. 2003; Nozaki, Ogura et al. 2002), chemokines and cytokines (Alba, Planas et al. 2008; Goldberg, 2009), and the increased production of ROS from the endothelium have been observed in diabetic subjects (Kolluru, Bir et al. 2012).

Various risk factors converge on the artery to promote the alteration of its functions under diabetic conditions. These factors include insulin resistance, hyperglycemia, hyperinsulinemia, hypertension, oxidative stress, and genetic predisposition. In addition, increased advanced glycation end products (AGEs), and increased free fatty acids (FFAs) and enhanced circulatory proinflammatory and prothrombotic cytokines also contribute to induce endothelial dysfunction. Besides, multiple lifestyle behaviours such as physical inactivity, smoking, poor diet and alcohol consumption also contribute into the induction of endothelial dysfunction (Pilacinski and Zozulinska-Ziolkiewicz 2014; Patino-Alonso, Recio-Rodriguez et al. 2015). It has been demonstrated that hyperglycemia, insulin resistance, hyperinsulinemia and increased free fatty acids, which are the main mechanisms, can induce endothelial dysfunction (**Figure 11,** Beckman, Creager et al. 2002).

In diabetic patients with defiency of insulin or insulin resistance, skeletal muscles decrease the utilization of glucose and free fatty acids, causing **hyperglycemia** and increased levels of circulating free fatty acids (FFAs). Moreover, in diabetic patients with the insulin resistance, the pancreatic β-cells initially try to compensate by producing more insulin, resulting in **hyperinsulinemia** (Wilcox, 2005). However, this period of pancreatic β cell compensation is followed by the failure of pancreas, which secretes insufficient insulin and diabetes ensues (Kasuga, 2006). Consequenltly, it precedes the development of **hyperglycemia** and usually associated with elevated levels of FFAs (Libby and Plutzky, 2002). This **excess of FFAs** will lead to the overproduction of triglyceride-rich lipoprotein particles. **Hypertriglyceridemia** is accompanied by a concomitant decrease in HDL. Finally, the formation of advanced glycation end products (AGEs) from glycated macromolecules can damage vasculature through different mechanisms (discuss details in section 1.2.4.4) (Nesto

2004). Although the aetiology of vascular dysfunction in diabetes has been extensively investigated in humans and in animal models of diabetes, no one clear pathway emerges as the basic mechanisms underlying endothelial dysfunction in diabetes.

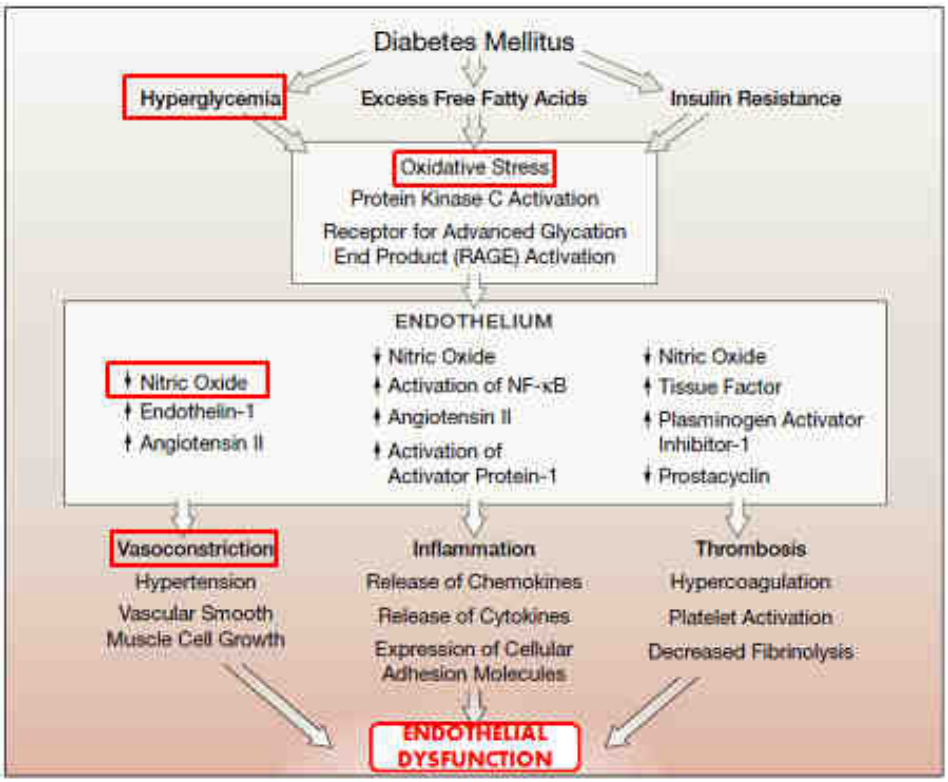

Figure 11. ***Endothelial dysfunction in Diabetes.*** Modified from (Beckman, Creager et al. 2002)

1.2.4.1. Role of insulin-resistance in endothelial dysfunction

Approximately 90-95% of patients with diabetes mellitus have T2D, which is characterized by insulin resistance or the inability of insulin to exert its metabolic actions. Numerous studies have demonstrated that the insulin resistance is commonly associated with endothelial dysfunction (Wiernsperger, 1994; Arcaro, Cretti et al. 2002). The actions of insulin are mediated through two major pathways. One pathway is through the activation of insulin-receptor substrate-1/2 (IRS-1/2), phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase 1 (PDK-1), and Akt. This pathway can lead to phosphorylation and activation of eNOS, which in turn regulates endothelial production of NO. The other insulin-signaling pathway leads to the activation of mitogen-activated protein kinases (MAPK), including mitogen-activated protein kinase kinase MEK-1, ERK-1, and ERK-2, which have major effects on growth, proliferation and differentiation but are also

involved in insulin-stimulated secretion of the vasoconstrictor ET-1 (**Figure 12**; (Cusi, Maezono et al. 2000).

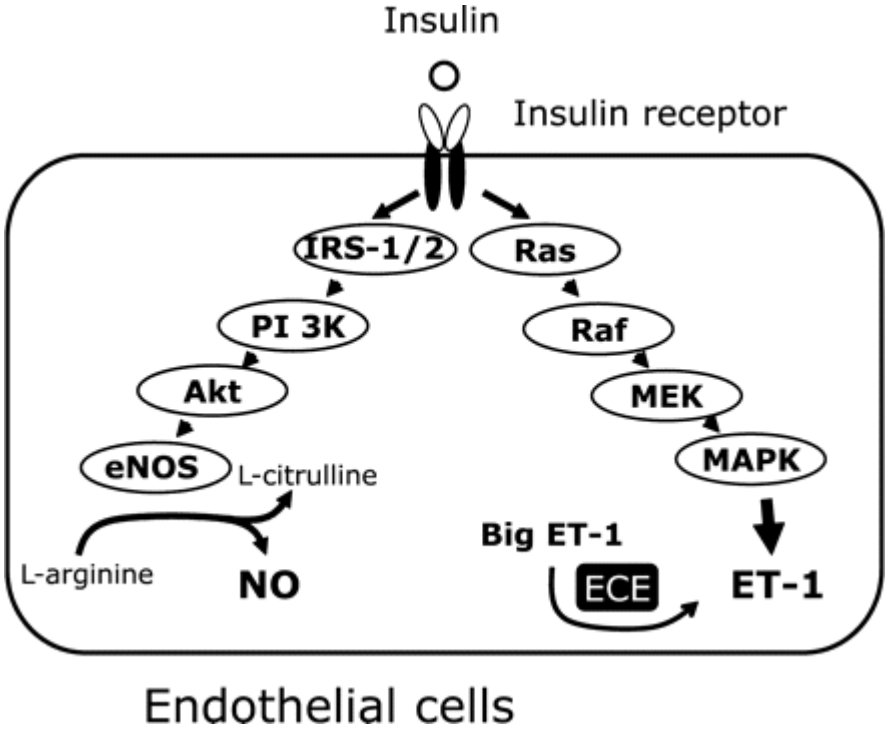

Figure 12. ***Insulin-signaling pathways in endothelium***. IRS-1/2: insulin-receptor substrate-1/2; PI 3K: phosphatidylinositol 3-kinase; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; MEK: mitogen-activated protein kinase kinase; MAPK: mitogen-activated protein kinases; ET-1: endothelin-1; ECE: endothelin-converting enzyme. Adapted from (Potenza, Addabbo et al. 2009)

In states of insulin resistance, only the PI-3K dependent signaling is impaired, whereas the MAPK pathway is unaffected (Cusi, Maezono et al. 2000). This explains how insulin resistance induces a relative loss in NO bioactivity with subsequent endothelial dysfunction (**Figure 12**). Experimental and clinical evidences support the concept that defects immediately upstream or downstream from PI3K-signaling pathways result in the insulin-mediated endothelial dysfunction. For example, transgenic mice with insulin resistance in endothelium show a significant reduction in NO bioavailability secondary to increased generation of ROS even in the absence of a metabolic phenotype (Duncan, Crossey et al. 2008). Similarly, it has been shown that Akt/eNOS phosphorylation is decreased in aorta of diabetic animals, as well as in type 2 diabetic patients (Cai, Khoo et al. 2005; McVeigh, Brennan et al. 1992).

1.2.4.2. Role of hyperinsulinemia in endothelial dysfunction

As suggested by many *in vitro* and *in vivo* studies, hyperinsulinemia overdrived the unaltered MAPK-dependent pathways leading to an imbalance between PI3K- and MAPK-dependent pathways of insulin. As a consequence, it promotes the secretion of ET-1 and decreases the production of NO resulting in endothelial dysfunction (Potenza, Gagliardi et al. 2009; Piatti, Monti et al. 1996). Besides, in endothelial cells *in vitro*, elevated insulin levels stimulate the increased expression of adhesion molecules VCAM-1 and E-selectin via MAPK-dependent signaling pathway, thus facilitating the interaction of activated monocytes and further contributing to the endothelial dysfunction and atherosclerosis (Hink, Li et al. 2001). Hence, the increased insulin signaling via MAPK-dependent pathway can accelerate endothelial dysfunction by mediating vasoconstriction, inflammation and thrombosis (Potenza, Marasciulo et al. 2005).

1.2.4.3. The excess of free fatty acids

As insulin inhibits lipolysis in adipose tissue and stimulates the uptake of free fatty acids (FFA) for skeletal muscle, diabetic patients are characterized by deficiency of insulin or insulin resistance leading to elevated circulating level of FFA (Boden, 1999; Kelley and Simoneau 1994). In response to the excess of FFA, the liver produces very-low-density lipoprotein production and increases cholesteryl ester synthesis resulting in hypertriglyceridemia, dyslipidemia and obesity. Elevated triglyceride concentrations lower HDL by promoting cholesterol transport from high density lipoprotein to very-low-density lipoprotein (Sniderman, Scantlebury et al. 2001). It has been shown that infusion of FFA reduces endothelium-dependent vasodilation in animal models and in humans *in vivo* (Steinberg, Tarshoby et al. 1997; de Kreutzenberg, Crepaldi et al. 2000; Kim, Tysseling et al. 2005; Sarabi, Vessby et al. 2001). Co-infusion of the antioxidant ascorbic acid improves endothelium-dependent vasodilation in humans treated with FFA, which indicates that oxidative stress contributes to FFA-mediated endothelial dysfunction (Pleiner, Schaller et al. 2002). In addition, the elevation of FFA concentrations activates PKC and decreases insulin receptor substrate-1–associated phosphatidylinosital-3 kinase activity (Inoguchi, Li et al. 2000). These effects on signal transduction may decrease NOS activity. Besides, it has been also demonstrated that both hypertriglyceridemia and low HDL have been associated with endothelial dysfunction (Kuhn, Mohler et al. 1991; de Man, Weverling-Rijnsburger et al. 2000). Based on the published studies, FFA may impair endothelial function through several

mechanisms, including the increased production of ROS, the activation of PKC, and the exacerbation of dyslipidemia leading to endothelial dysfunction (Dresner, Laurent et al. 1999).

1.2.4.4. Role of hyperglycemia in endothelial dysfunction

A number of studies in endothelial cells, animal models and clinical studies have consistently reported that hyperglycemia is strongly associated with endothelial dysfunction. It has been reported that the presence of hyperglycemia inhibits the eNOS and increases the production of ROS, leading to further inhibition of eNOS (De Vriese, Verbeuren et al. 2000; Du, Edelstein et al. 2001). The vascular endothelium also loses its ability to produce NO-activated tissue plasminogen activator, a fibrinolytic (anti-clotting) protein that inhibits the ability of inflammatory cells to adhere to the endothelial surface (Grant 2007). In addition, the high D-glucose-induced impairtment of endothelium-dependent relaxation also was observed in human brachial arteries (Bhargava, Hansa et al. 2003; Kim, Park et al. 2003). However, a complete biochemical understanding of the mechanisms by which hyperglycemia causes vascular functional and structural alterations associated with the diabetes still is not well understood.

The literature has reported that hyperglycemia is the major causal factor in the development of endothelial dysfunction in diabetes mellitus. The tissue-damaging effects of hyperglycemia occur only in the few cell types because most cells are able to reduce the transport of glucose inside the cell when they are exposed to hyperglycemia, so that their internal glucose concentration stays constant. Diabetes selectively damages cells, like endothelial cells in blood vessels, mesangial cells in the renal glomerulus, neurons and Schwann cells in peripheral nerves because they are unable to regulate uptake of glucose during hyperglycemia, allowing intracellular glucose to rise concomitantly with extracellular glucose concentrations (Kaiser, Sasson et al. 1993; Heilig, Concepcion et al. 1995; Mandarino, Finlayson et al. 1994). Since only the level of intracellular glucose was different amongs the cells, we focus on mechanisms induced by hyperglycemia going on inside these cells, particurlally endothelial cells (Brownlee, 2005).

In diabetic cells with high intracellular glucose concentration, there is more glucose-derived pyruvate being oxidized in the tricarbocylic acid cycle, increasing the flux of electron donors (such as NADH and dihydroflavine-adenine dinucleotide ($FADH_2$)) into the electron transport chain. As a result of this, the voltage gradient across the mitochondrial membrane

increases until a critical threshold is reached. At this point, electron transfer inside complex III is blocked, causing the electrons to back up to coenzyme Q, which donates the electrons one at a time to molecular oxygen, thereby generating superoxide anion, consequently, leading to an increased level of oxidative stress (**Figure 13;** Brownlee, 2005).

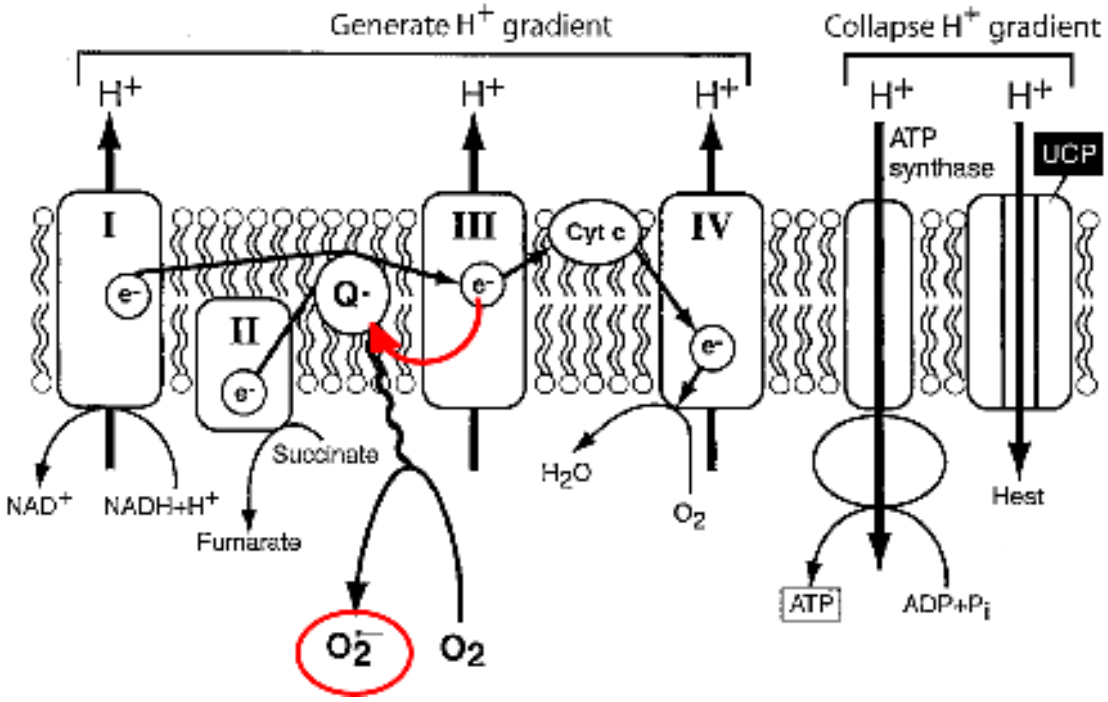

Figure 13. ***Hyperglycemia-induced production of superoxide anion by the mitochondrial electron transport chain.*** Modified from (Brwolee, 2005).

ROS, which are generated in mitochondria, cause strand breaks in nuclear DNA, which activate poly-adenosine diphosphate ribose (polyADP-ribose) polymerase (PARP). Once activated, PARP splits the NAD^+ molecule into its two component parts, nicotinic acid and ADP-ribose. PARP then proceeds to make polymers of ADP-ribose, which accumulate on glyceraldehyde- 3 phosphate dehydrogenase (GAPDH), a key glycolytic enzyme, thereby reducing its activity. Finally, decreased GAPDH activity activates different metabolic pathway, (1) increased flux of sorbitol through polyol pathway; (2) overactivity of hexoamine pathway; (3) activation of PKC and (4) increased formation of advanced glycation end-products (AGEs) and activation of their receptors (Brownlee 2005). Through these pathways, there are an increased synthesis of growth factors, cytokines, and angiotensin II and other EDCF; a decreased bioavailabity of NO and decreased activity of eNOS. The imbalance between NO bioavailability and accumulation of reactive oxygen species (ROS) and EDCF, in turn, induces a diffuse endothelial dysfunction and contributes to the progressive

development of micro- and macrovascular complications and multiorgan damage (**Figure 14;** (Giugliano, Ceriello et al. 1996).

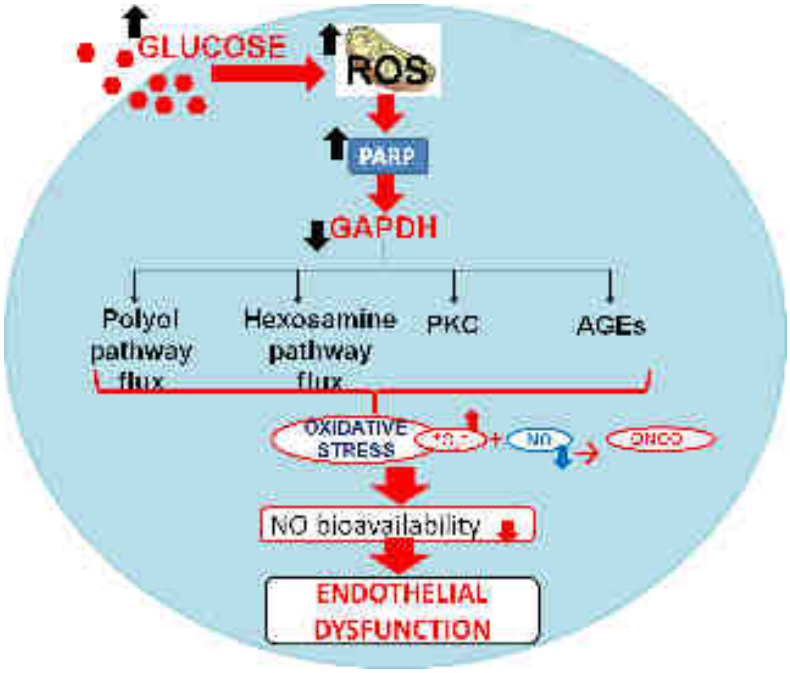

Figure 14. ***Mechanism of endothelial dysfunction induced by hyperglycemia.*** ROS, reactive oxygen species; PARP, poly-adenosine diphosphate ribose polymerase; GAPDH, glyceraldehyde- 3 phosphate dehydrogenase; PKC, protein kinase C; AGEs, advanced glycation end-products.

a) Activated the polyol pathway flux

The polyol pathway has been implicated in the pathogenesis of vascular complications, through the action of aldose reductase (AR), the first and rate-limiting enzyme in this pathway. AR reduces the aldehyde form of glucose to sorbitol. In physiological situations, sorbitol is then oxidized to fructose by sorbitol dehydrogenase and then enters again into glycolysis (**Figure 15**). In the presence of HG concentrations, the production of sorbitol overcomes the potential of its oxidation, with accumulation within the cells. This causes deregulation of the cellular osmotic status, a reduction of Na^+/K^+-ATPase activity, an increased cytosolic NADH/NAD^+, and a decreased cytosolic NADPH (Oyama, Miyasita et al. 2006). In addition, the reduction of glucose to sorbitol by AR consumes NADPH. As NADPH is required for regenerating reduced glutathione (GSH) and GSH is an important scavenger of reactive oxygen species (ROS), this could induce or exacerbate intracellular oxidative stress (Brownlee, 2001; **Figure 15**). Ramana and colleagues suggest that NO inhibits AR by reversible S-thiolation of the protein, indicating a new role of NO in regulating glucose

metabolism (Ramana, Chandra et al. 2003). It has been also demonstrated that restoring the NO levels in diabetic animals increases glutathiolation of cellular proteins, inhibits aldose reductase activity and prevents sorbitol accumulation (Giacco and Brownlee, 2010). Based on those findings, potential therapeutic strategies are focused on AR inhibitors (ARIs) like ranirestat, epalrestat. However, the majority of ARIs to date have been less effective in human patients than in experimental models (Funk, Yurdagul et al. 2012).

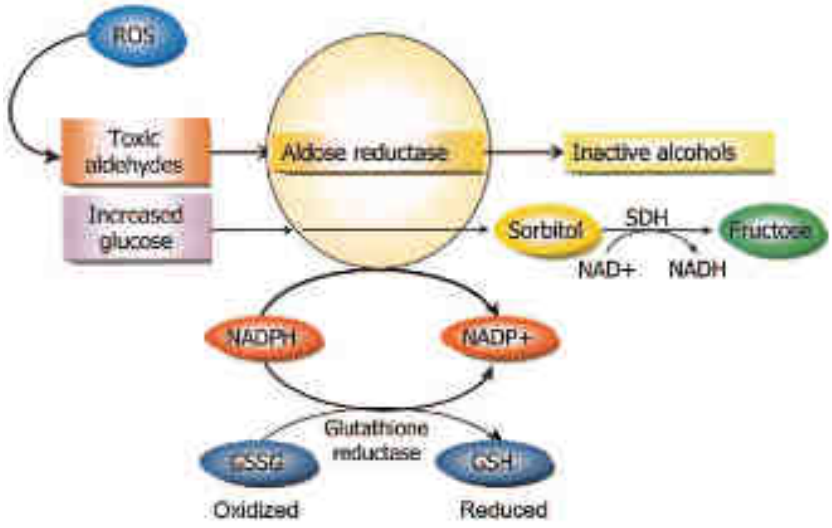

Figure 15. ***Hyperglycemia increases flux through the polyol pathway***. ROS, reactive oxygen species, SDH, sorbitol dehydrogenase; NAD+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP+, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; GSH, reduced glutathione; GSSG, glutathione disulfide. Adapted from (Brownlee, 2001).

b) Overactivity of hexoamine pathway

In the physiological setting, glucose is metabolized through glycolysis, going first to glucose-6 phosphate, then fructose-6 phosphate, and then on through the rest of the glycolytic pathway (**Figure 16**). However, in case of the excess intracellular glucose, there is an increased flux of fructose 6-phosphate into the hexosamine pathway. In this way, fructose 6-phosphate is converts to glucosamine-6 phosphate by an enzyme called glutamine, fructose-6 phosphate amidotransferase (GFAT) and finally to uridine diphosphate *N*-acetyl glucosamine (UDPGlcNAc). UDPGlcNAc is a nucleotide donor utilized in the endoplasmic reticulum and Golgi for glycosylation of various glycoproteins, lipids, and proteoglycans. For example, O-linked glycosylation by O-GlcNAc transferase (OGT), which involves the addition of N-acetylglucosamine (GlcNAc) to the intracellular protein's serine and threonine residues.

Many cytoplasmic and nuclear proteins and transcription factors are dynamically modified by O-linked GlcNAc, resulting in induction of perturbations in normal enzyme function (**Figure 16**). For example, hyperglycemia-induced O-GlcNAcylation of eNOS leads to decrease serine 1177 phosphorylation in eNOS (a regulator site of eNOS activation) and therefore reduced NO production (Du, Edelstein et al. 2001). In addition, Federici and colleagues found that endothelial O-GlcNAcylation is increased in carotid plaques of type II diabetic patients, and inhibition of the hexosamine pathway is sufficient to reverse O-GlcNAcylation-mediated eNOS inhibition in human coronary endothelial cells in hyperglycemic conditions (Federici, Menghini et al. 2002; Sayeski and Kudlow 1996). In addition, GlcNAc has also been implicated in the activation of the transcriptional factor Sp1, which is associated with increased synthesis of transforming growth factor-β1 and plasminogen activator inhibitor-1, which in turn are associated with the development of vascular complications (Brownlee, 2001). In contrast to the polyol pathway, until now no therapeutics have been developed targeting hexosamine stress. Although the glutamine analogs 6-diazo-5-oxo-L-norleucine and azaserine are frequently used to study the hexosamine pathway by inhibiting GFAT activity, the role of GFAT in glycosylation, and the ubiquitous nature of O-GlcNAcylation make GFAT an unattractive therapeutic target (Frunk, 2012).

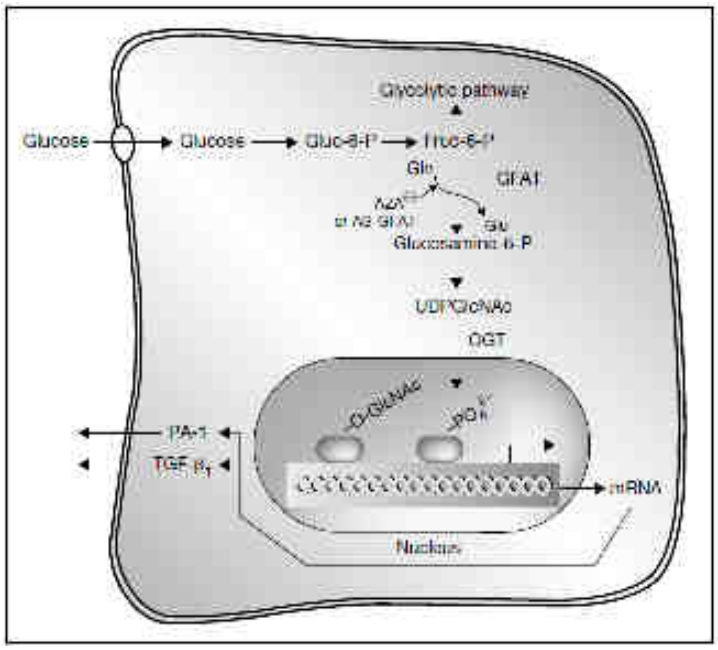

Figure 16. ***Hyperglycemia increases flux through the hexosamine pathway***. The glycolytic intermediate fructose-6-phosphate (Fruc-6-P) is converted to glucosamine-6-phosphate by the enzyme glutamine, fructose-6-phosphate amidotransferase (GFAT). Intracellular glycosylation by the addition of N-acetylglucosamine (GlcNAc) to serine and threonine is

catalyzed by the enzyme O-GlcNAc transferase (OGT). Increased donation of GlcNAc moieties to serine and threonine residues of transcription factors such as Sp1, often at phosphorylation sites, increases the production of factors as plasminogen activator inhibitor-1 (PAI-1) and transforming growth factor-β1 (TGF-β1). AZA, Azaserine; AS-GFAT, antisense to GFAT. Reproduced from Brownlee, 2001.

c) Protein kinase C (PKC) activation

Hyperglycemia can stimulate the synthesis of diacylglycerol (DAG), followed by the activation of protein kinase C (PKC). DAG, which is formed from glycolytic intermediates such as dihydroxy-acetone phosphate and glyceraldehyde-3-phosphate, is the major endogenous cellular co-factor for PKC activation (**Figure 17**). Once activated, PKC is responsible for different structural and functional changes in the vasculature including alterations in relaxation, cellular permeability, inflammation, angiogenesis, cell growth, extracellular matrix expansion, and apoptosis (Geraldes and King 2010). PKC family has at least 11 isoforms that are widely distributed in mammalian tissues. The specific PKC isoform activated by hyperglycemia varies across tissues and species (Kang, Alexander et al. 1999). Particularly, hyperglycemia primarily activates the β and δ isoforms of PKC in cultured vascular cells (Xia, Inoguchi et al. 1994; Koya, Jirousek et al. 1997).

In vascular endothelial cells, hyperglycemia-induced activation of PKC increases $O_2^{\bullet-}$ production via NADPH oxidase (Christ, Bauersachs et al. 2002). After generated, $O_2^{\bullet-}$ inactivates NO to form OONO–, which is a powerful oxidant, easily penetrates across phospholipid membranes and induces substrate nitration. Protein nitrosylation blunts activity of antioxidant enzymes and eNOS (Hink, Li et al. 2001). In addition, PKC affects NO availability not only via intracellular accumulation of ROS but also by decreasing eNOS activity through increasing eNOS phosphorylation at the inhibitory site (Thr 495) and reducing the phosphorylation at activator site Ser 1177 (Hirata, Kuroda et al. 1995; Du, Edelstein et al. 2001). Experimental evidences support the notion that hyperglycemia decreases NO bioavailability in diabetes. When normal rabbit and rat's aortic rings are incubated in a hyperglycemic milieu, endothelium-dependent relaxations are impaired (Tesfamariam, 1991; Zhang, Dellsperger et al. 2012). Similarly, endothelium-dependent vasodilation is reduced in healthy subjects during hyperglycemic clamping (Masha, Dinatale et al. 2011).

Endothelial dysfunction in diabetes is not only the result of the impaired NO availability but also of the increased synthesis of EDCF. Hyperglycemia increases PKC activation, leading to an increased production of prostanoid substances by the endothelium (Tesfamariam, Brown et al. 1991). Besides, PKC-mediated COX-2 up-regulation is associated with an increase of TXA_2 and a reduction of PGI_2 release (Cosentino, Eto et al. 2003). PKC also leads to an increased production of ET-1 promoting vasoconstriction and platelet aggregation (**Figure 17**). The enhanced ET-1 production caused by hyperglycemia is partly mediated via activation of PKC-β and δ isoforms (Park, Takahara et al. 2000). In addition, Cardillo and colleagues have demonstrated that the blockage of ET_A receptor results in vasodilation in patients with diabetes but not in controls suggesting that the activity of endogenous ET-1 on ET_A receptors is enhanced in resistance vessels of patients with diabetes (Cardillo, Campia et al. 2002). These findings suggest that PKC is the upstream signalling molecule affecting vascular homeostasis in the setting of hyperglycemia (**Figure 17**).

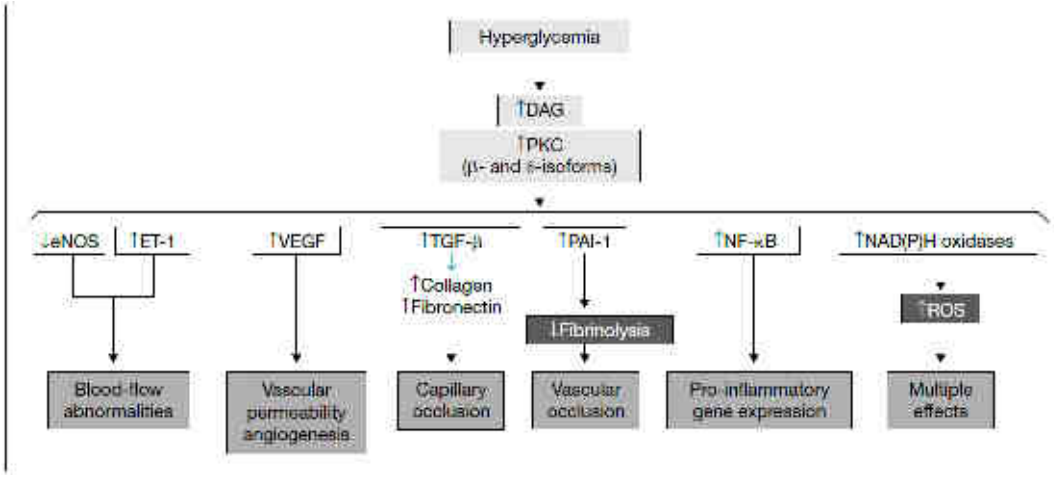

Figure 17. ***Consequences of hyperglycemia-induced activation of protein kinase C (PKC)***. DAG, diacylglycerol; eNOS, endothelial NO synthase; ET-1, endothelin-1; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor-β; PAI-1, plasminogen activator inhibitor-1; NF-κB, nuclear factor κB; NAD(P)H oxidase, nicotinamide adeninedinucleotide phosphate oxidase; ROS: reactive oxygen species. Adapted from (Brownlee, 2005).

In addition, in the vessel wall, hyperglycemia-induced ROS production triggers NF-κB-mediated vascular inflammation (**Figure 17**). Indeed, ROS lead to up-regulation of the nuclear transcription factor NF-κB subunit p65 and, hence, transcription of pro-inflammatory genes encoding for monocyte chemoattractant protein-1 (MCP-1), selectins, vascular cell

adhesion molecule-1 (VCAM-1), and intracellular cell adhesion molecule-1 (ICAM-1) are up regulated. These latter events lead to monocyte adhesion, rolling, and diapedesis with formation of foam cells in the sub-endothelial layer, consequently forming atherosclerosis plaques (Kouroedov, Eto et al. 2004). The PKC increases the production of growth factors by the endothelium, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and transforming growth factor (TGF-β). These reactions lead to the migration and proliferation of SMC.

To date, there are several studies suggesting that PKC inhibitors may regulate endothelial dysfunction and activation in animal models. The expression and activation of PKCα and PKCβII have been shown to be enhanced in the diabetic microvasculature (Das Evcimen and King 2007), and the general PKC inhibitor bisindolylmaleimide-I blunted hyperglycemia-induced leukocyte binding to mesenteric postcapillary venules *in vivo* (Booth, Stalker et al. 2002). It has been shown that the PKCβ inhibitor ruboxistaurin reduced retinal and renal complications in diabetic rats while enhancing acetylcholine-induced aortic vasodilation (Nangle, Cotter et al. 2003). In line with these studies, transient hyperglycemia blunted endothelium-dependent vasodilation in otherwise healthy human subjects, and PKCβ inhibition with ruboxistaurin restored normal endothelial function in these patients (Beckman, Goldfine et al. 2002).

d) Increased formation of advanced glycation end-products (AGEs)

Hyperglycemia-induced ROS formation also increases the intracellular levels of advanced glycation end products (AGEs) synthesis. The nonenzymatic reaction between glucose and proteins or lipoproteins forming advanced glycation end-products (AGEs) in arterial walls is one of the most important mechanisms responsible for the accelerated vascular complication in diabetes. It has been shown that receptor-independent effects of AGEs include extracellular matrix modification, NO scavenging, and glycation of both signaling proteins, low density lipoprotein (LDL) and nucleic acids (Funk, Yurdagul et al. 2012). The accumulation of AGEs on macromolecules can interfere with their normal functions by disrupting molecular conformation, alter enzymatic activity, reduce degradation capacity and interfere with receptor functions (Singh, Bali et al. 2014). Hyperglycemia causes excessive glycation of proteins found in serum (e.g., albumin, hemoglobin, and LDL) and in the vessel wall (e.g., collagen, fibronectin). It has been demonstrated that hyperglycemia stimulates considerable glycation of extracellular matrix proteins resulting in vessel stiffening

through cross linking type I collagen and elastin (Diez 2007). In addition, AGEs can also increase the transcription of adhesion molecules, growth factors, cytokines and chemokines promoting oxidative stress, inflammation, endothelial injury, vessel wall hypertrophy, thus, promoting to atherosclerosis (Singh, Bali et al. 2014).

It has been noted that AGE formation by intracellular glycolysis-derived dicarbonyl precursors occurs at a rate several orders of magnitude higher than non-enzymatic glycation, suggesting that these intermediates may be primarily responsible for both intracellular and extracellular AGE production (Degenhardt, Thorpe et al. 1998; Brownlee 2001). For example, methylglyoxal, one of AGE precursors, is produced from the glycolytic intermediates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Methylglyoxal is detoxified by conversion into pyruvate via the multienzyme glyoxalase system, of which glyoxalase-I is rate-limiting enzyme. Brouwers and colleagues found that transgenic overexpression of glyoxalase-I in a rat model reduced vascular AGE formation and improved vasoreactivity (Brouwers, Niessen et al. 2014).

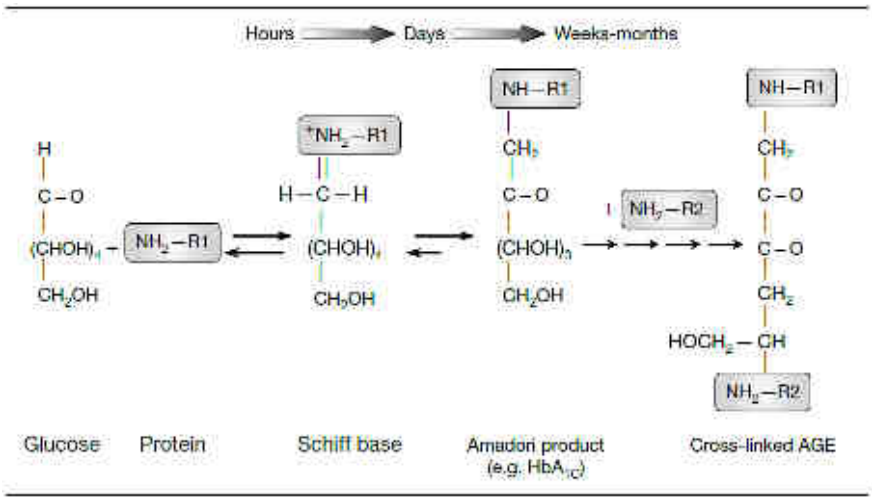

Figure 18. ***Formation of advanced glycosylation end-products (AGE)***. Formation of the Schiff base from carbonyl group of glucose and free amino group of protein is relatively fast and highly reversible and represents an equilibrium reaction in which the amount of Schiff base formed is dictated by glucose concentration. When glucose is removed or lowered, the unstable Schiff base reverses within minutes. Formation of Amadori product from the Schiff base is slower but much faster than the reverse reaction, and therefore tends to accumulate on proteins. Equilibrium levels of Amadori products are reached in weeks. Proteins bearing Amadori products are referred to as advanced glycosylation end-products, which are stable and virtually irreversible. (Adapted from Doron Aronson, 2002).

Moreover, AGEs interact with a specific receptor (RAGE) present on endothelail cells, SMC and monocyte-derived macrophages. The interaction of AGEs with their receptor leads to the induction of oxidative stress and proinflammatory responses resulting cellular dysfunction (Bierhaus, Humpert et al. 2005). It has been demonstrated that AGE-RAGE interaction stimulates endothelial ROS production (Yan, Schmidt et al. 1994), decreases eNOS expression and L-citrulline production (readout of NO production) in endothelial cells (Xu, Ji et al. 2005), and inhibits histamine-induced NO production in endothelial cells associated with reductions in eNOS serine 1177 phosphorylation (Xu, Chibber et al. 2003).

The AGEs concentration is directly related to glucose concentration. The formation and accumulation of AGEs has been observed in various tissues such as coronary atheroma, renal cortex, mesangial and glomerular basement membrane. Such histological findings suggest AGEs to be related with the pathogenesis of diabetic complications. Pharmacological inhibitors of AGE formation (alagebrium chloride (ALT-711), pyridoxamine dihydrochloride) significantly reduced atherosclerotic plaque formation in animal models of diabetes (Watson, Soro-Paavonen et al. 2011; Forbes, Yee et al. 2004; Usta, Kendirci et al. 2006). Moreover, early clinical trials using ALT-711 demonstrated enhanced arterial compliance (Kass, Shapiro et al. 2001) and improved flow-mediated vasodilation (Zieman 2007) in aged hypertensive patients. At the moment, no clinical data is available on the effect of ALT-711 in the prevention of diabetic macro- and microvascular complications. Despite these early successes, Alteon (the producer of ALT-711) encountered financial hardship and halted drug development (Funk, 2012). Besides, RAGE antagonists (TTP488) and humanized sRAGE are making their way through early clinical trials for diabetic nephropathy, Alzheimer's disease, and acute lung injury. However, no trials for CVD are currently underway (Calcutt, Cooper et al. 2009).

1.3. New therapeutic approaches for diabetic patients

1.3.1. Oral antidiabetic treatment of vascular protection

Recently, it has been demonstrated that a poor control of hyperglycemia appears to play a significant role in the development of CVDs in diabetes (Lehto, Ronnemaa et al. 1997). Diabetes control and complications trial/ epidemiology of diabetes interventions and complications study for 30 years (DCCT/EDIC) in 1,441 subjects with T1D has demonstrated the effectiveness of intensive glucose control (TNT) in reducing the long-term complications of T1D and improving the prospects for a healthy life span compared to conventional therapy

(CONV). TNT aimed at achieving levels of glycemia as close to the nondiabetic range as safely possible, otherwise, CNV aimed to maintain safe asymptomatic glucose control. DCCT/EDIC demonstrated a 35–76% reduction in the early stages of microvascular disease with INT, with a median HbA1c of 7%, compared with CONV, with a median HbA1c of 9%. The major adverse effect of INT was a threefold increased risk of hypoglycemia, which was not associated with a decline in cognitive function or quality of life (Nathan 2014). In addition, another group has selected five prospective randomised controlled trials of 33 040 patients with T2D to assess the effect of an intensive glucose-lowering regimen on death and cardiovascular outcomes compared with a standard regimen (Ray, Seshasai et al. 2009). They found that the mean HbA_{1c} was 0.9% lower for participants given intensive treatment than for those given standard treatment. Intensive glycaemic control resulted in a 17% reduction in events of non-fatal myocardial infarction (odds ratio 0·83, 95% CI 0.75–0.93), and a 15% reduction in events of coronary heart disease (0.85, 0.77–0.93). Intensive glycaemic control had no significant effect on events of stroke (0.93, 0.81–1.06) or all-cause mortality (1.02, 0.87–1.19). In addition, another meta-analysis has reported no benefit of intensive glycemic control on macrovascular and microvascular complications ($p > 0.1$) in the intensive control group when the target HbA1c level was < 7.0%, even worse that INT gave a higher rate of severe hypoglycemia ($p < 0.00001$). When the target HbA1c level was lowered to 7.0 – 7.9%, intensive glycaemic control showed benefits on the reduction of microvascular events ($p < 0.05$) without increasing the risk of severe hypoglycemia ($p = 0.74$), but no influence on macrovascular complications ($p > 0.1$). They have suggested that a target HbA1c level of 7.0-7.9% may be a better glycaemic control target than that of < 7.0% in patients with established T2D mellitus (Ma, Yang et al. 2009).

Treatment of hyperglycaemia in DM is necessary to relieve acute symptoms and to reduce the risk of chronic vascular complications. Lifestyle interventions, notably diet and exercise, are important but are generally insufficient to achieve or maintain glycaemic control. Therefore, pharmacologic therapy, using orally administered antidiabetic drugs (OADs), is necessary to achieve optimal glycemic control in the management of diabetes. The number of available OADs has increased significantly in the last decade. Most of the available OADs target the liver, pancreas, small intestine, skeletal muscle, or adipose tissue, in order to modulate pancreatic β-cells dysfunction and insulin resistance, consequently lower the blood glucose level in patients. For example, biguanides inhibit hepatic glucose production and possibly also improve insulin sensitivity. Sulfonylureas and meglitinides (glinides) are insulin

secretagogues, whereas thiazolidinediones (glitazones) are insulin sensitizers. Incretin-based therapies (glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors) stimulate glucose-dependent insulin release and decrease glucagon secretion. Alpha-glucosidase inhibitors slow the intestinal digestion/absorption of carbohydrates (Luna and Feinglos 2001).

However, the efficacy of the current anti-diabetic treatments vanishes over time, leading to a progression of the disease. This phenomenon is mostly explained by the reduction of the ß-cells' capacity to secrete insulin and by a desensitization of the insulin signalling pathways, while most of the treatments aim to induce insulin release or to improve insulin sensitivity. Likewise, in contrast to conventional anti-diabetics, a novel treatment option for diabetes – the sodium-glucose cotransporter 2 (SGLT2) inhibition is now available. Its action is independent of insulin secretion and signaling therefore it will not share these drawbacks. SGLT2 inhibitors reduce renal glucose reabsorption in proximal nephrons, leading to enhanced urinary glucose excretion (UGE) (Cheng and Fantus 2005). Interstingly, some studies have been published that this new class of drug has effect on reducing significantly cardiovascular events in diabetic patients (Zinman, Wanner et al. 2015; Vasilakou, Karagiannis et al. 2013; Dziuba, Alperin et al. 2014; Neal, Perkovic et al. 2013).

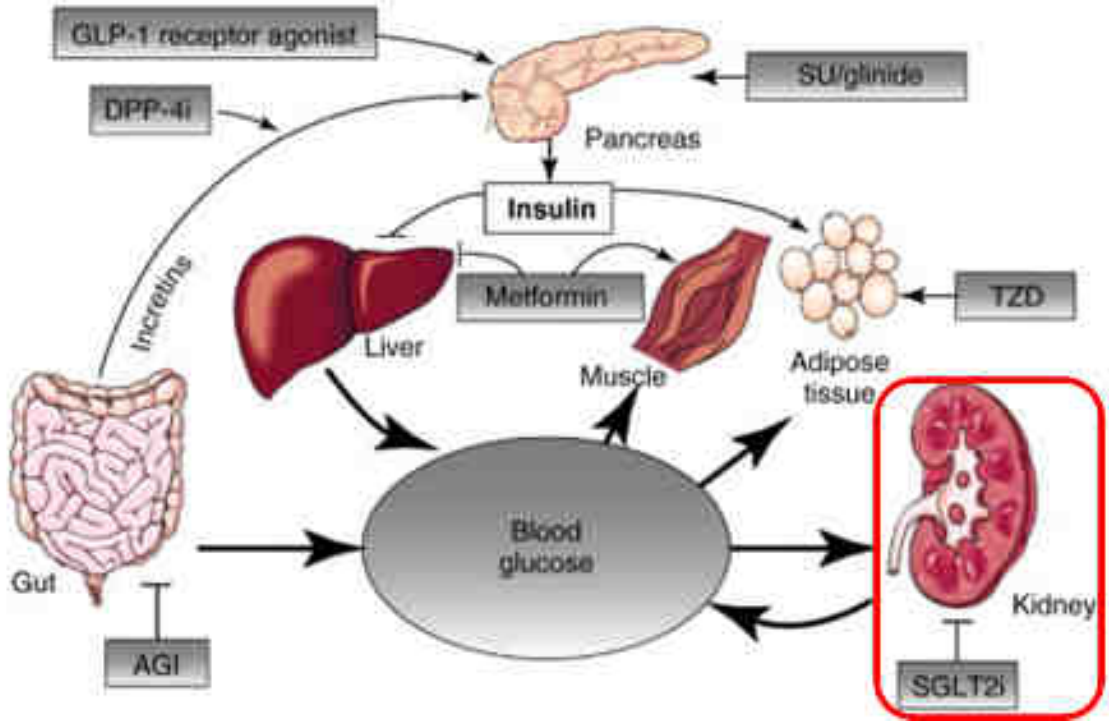

Figure 19. ***Sites of action of OADs.*** Metformin targets both the liver and skeletal muscle. The thiazolidinediones (TZDs) improve insulin sensitivity, reduce hepatic glucose production, and

improve glucose disposal indirectly by altering lipid metabolism in adipose tissue. Sulfonylureas, meglitinides (glinides) and GLP-1 receptor agonists target pancreatic β-cells and increase insulin secretion. GLP-1 agonists also reduce excess glucagon secretion by pancreatic α-cells and slow gastric emptying. DPP-4 inhibitors reduce the breakdown and increase the levels of endogenous incretin hormones GLP-1 and GIP. α-Glucosidase inhibitors (AGI) slow the rate of carbohydrate digestion by the small intestine. Selective SGLT2 inhibitors reduce glucose reabsorption by the kidney. Abbreviations, AGI, α-glucosidase inhibitor; DPP-4i, dipeptidyl peptidase-4 inhibitor; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; SGLT2i, sodium glucose cotransporter-2 inhibitor; SU, sulfonylurea. Modified from (Bailey, 2011).

1.3.2. New class of oral antidiabetic medication – Gliflozins

1.3.2.1. Physiological function of sodium - glucose cotransporters

a) Sodium - glucose cotransporters* (*SGLTs) family

The SGLTs are members of a sodium solute symporters family, which plays a role in transport both molecule Na^+ and glucose across the membrane with the same direction. The human SGLT protein family comprises 12 members, including Na-cotransporters for sugars, myo-inositol, iodide, short-chain fatty acids, and choline, all encoded by the *SLC5* genes (Wright, Loo et al. 2011). Table 3 summarised main SGLTs in human.

Table 3. ***Sodium-glucose cotransporters' family***. Adapted from (Wright, Hirayama et al. 2007).

SGLT member	Substrates	Distribution in human tissue
SGLT1	Glucose, galactose	Intestine, trachea, kidney, heart, brain, testis, prostate
SGLT2	Glucose	Kidney, brain, liver, thyroid, muscle, heart
SGLT3	Glucose	Intestine, testis, uterus, lung, brain, thyroid
SGLT4	Glucose, mannose	Intestine, kidney, liver, brain, lung, trachea, uterus, pancreas
SGLT5	Glucose, galactose	Kidney

SGLT6	D-chito-inositol	Brain, kidney, intestine

These 12 family members share an amino acid identity of 21-70%. There are characterized by 14 transmembrane domains, where the N-and C-termini face the extracellular (luminal) side of the cell (**Figure 20**; Augustin and Mayoux, 2014).

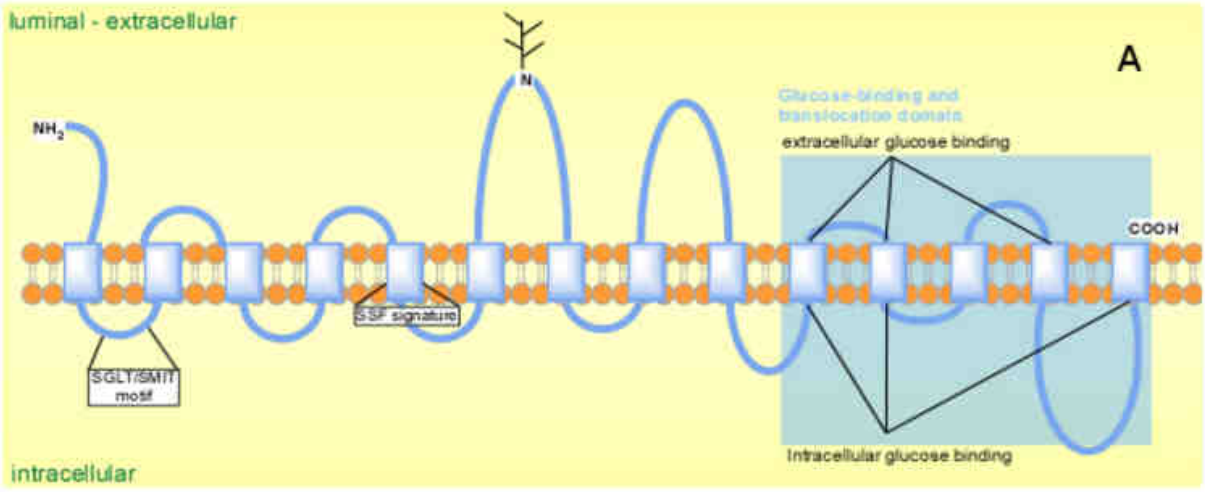

Figure 20. ***The secondary structure of human SGLT1.*** Human SGLT1 is an amino acid sequence arranged on a 14-transmembrane-helix model with both the N- and C- termini facing the extracellular side of the cell membrane. The glucose-binding and translocating domain is located towards the COOH terminal of the protein. Adapted from Augustin and Mayoux, 2014).

b) Role of SGLT1/2 in glucose homeostasis in kidney

Among this family, SGLT1 and SGLT2 are the most well characterized cotransporters. SGLT 1/2 play an important role in glucose homeostasis in kidney. Normal kidneys (with a glomerular filtration rate of ~125 mL/min) filter approximately 180 L of plasma each day. Thus, a healthy individual with an average plasma glucose concentration of 5–5.5 mmol/L (90–100 mg/dL) will filter approximately 160–180 g of glucose daily, all of which will be reabsorbed (Wright, Hirayama et al. 2007). The renal glucose handling occurs at two main sites. Firstly, SGLT2 is a high-capacity and low-affinity transporter, which is located in the S1 and S2 segments of proximal convoluted tubule, accounts for 90% of reabsorption through. Secondly, a high-affinity low-capacity transporter SGLT1 is located in the S3 segment (more distal and straight section) of the proximal tubule, which scavenges the remaining 10% filtered renal glucose (**Figure 21**). SGLT1/2 transports molecules $Na^+$and

glucose into the cells using sodium gradient created by Na^+/K^+ ATPase pumps. Glucose is then transported passively by GLUT1/2 along its concentration gradient into the interstitium (Bakris, Fonseca et al. 2009).

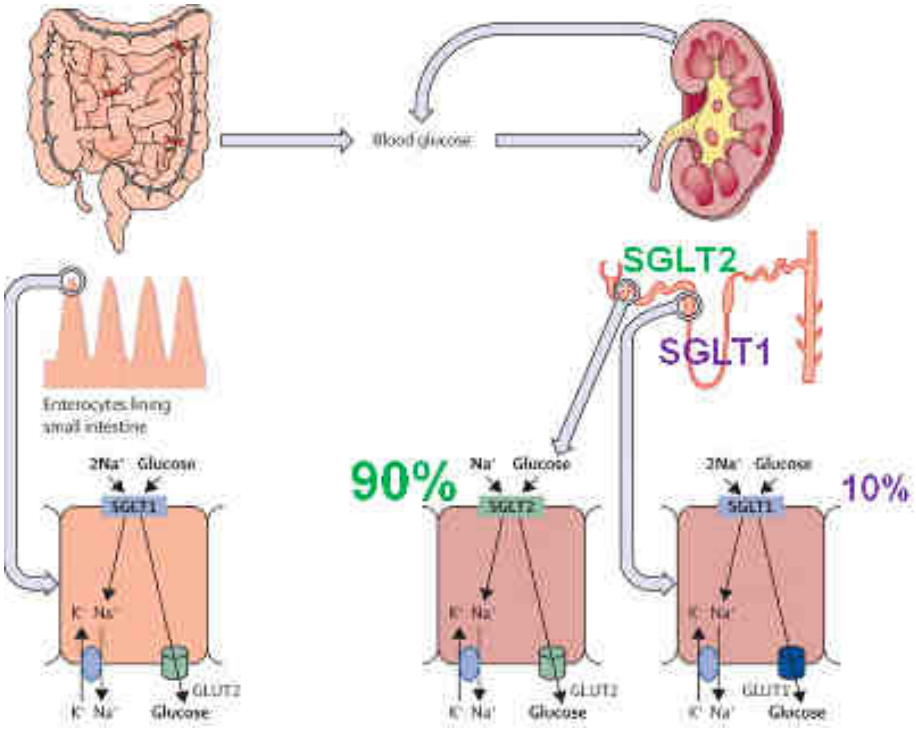

Figure 21. ***Role of sodium glucose cotransporters (SGLT1 and SGLT2) and facilitative glucose transporters (GLUT1 and GLUT2) in maintaining glucose homeostasis.*** SGLT1 and SGLT2 are secondary active cotransporters, driven by the Na^+ /K^+ -ATPase pump, which actively extrudes sodium across the basolateral membrane. Modified from (Tahrani, Barnett et al. 2013).

c) ***Renal glucose homeostasis in diabetic patients***

In patients with T2D, both renal and hepatic glucose release are increased in the post-absorptive state. It has been found that patients with T2D have increased renal glucose output in the post-absorptive state, causing increased release of glucose into the blood not only from the liver, but also with a significant contribution by the kidneys (Wilding, Woo et al. 2012). Specially, in another study, in patients with T1D, the maximum glucose transport capacity of glomerulus was reported to be up to 20% higher in patients with T1D than in healthy individuals (Jabbour, Hardy et al. 2014). In addition, the increase in glucose release over the 4.5-hour postprandial period has been shown to be roughly 30% higher in patients with T2D compared with healthy individuals, primarily due to increased endogenous glucose release. It

has been reported that glucose uptake by the kidneys is raised by more than twofold (21.0 ± 3.5 vs. 9.8 ± 2.3 g in diabetic vs. non-diabetic individuals) during a 4.5-h period following ingestion of 75 g of glucose (Meyer, Woerle et al. 2004). Specially, the increased glucose reabsorbtion from glomerular filtrate is well recognized in people with diabetes mellitus. One study has been reported that the transport maximum (Tm) increased from approximately 350 mg/min in normal individuals to approximately 420 mg/min in those with diabetes mellitus (Gerich, 2010). Due to these reasons, the kidneys of a diabetic person continue to reabsorb glucose. Consequently, hyperglycemia remains uncorrected and contributes to the ensuing problem of hyperglycemia.

1.3.2.2. Sodium - glucose cotransporter 2 inhibitors

This new class of drugs – the SGLT inhibitors – has a very long history. The first compound in this drug is phlorizin – O-glucoside phlorizin dihydrochalcones (a type of flavonoid), which was isolated in 1835 by French chemist from the apple tree bark. Later, in 1886, the famous diabetologist Joseph Vas Mering first described pharmacological role of phlorizin as ingestion caused glycosuria (Ehrenkranz, Lewis et al. 2005). Then phlorizin was found to improve glycemic control in diabetic animals (Rossetti, Smith et al. 1987; Rossetti, Shulman et al. 1987). However, the majority of phlorizin gets converted into an intermediary before it can be of any use, thereby requiring an increase in dose to achieve desired hypoglycemic effect. In addition, phlorizin is poorly absorbed in the small intestine and it has a short half-life, because the O-glucoside bond is highly susceptible to hydrolysis by intestinal glucosidases. Especially, the lack of selectivity towards SGLT2, which caused sufficient gastrointestinal side effects, is one main reason why phlorizin was precluded as an antidiabetic drug.

As the majority of glucose reabsorption occurs via the SGLT2 transporter, pharmaceutical companies have focused on the development of SGLT2 inhibitors. They aimed to find an ideal renal glucose reabsorption inhibitor for T2DM treatment, which would selectively inhibit SGLT2 and could exert some non-selective inhibition of SGLT1 to avoid the reduction of glucose absorption in the small intestine. In addition, it would require good bioavailability, safety and tolerability, and preferably a plasma half-life that would suit once-daily oral administration (Tahrani, Barnett et al. 2013). The search for such an ideal inhibitor led to the discovery of molecules such as canagliflozin, dapagliflozin, empagliflozin, ipragliflozin (**Table 4**). They are all SGLT2 selective inhibitors, have C-glucoside linkage so

they are not hydrolyzed by intestinal glucosidases. This class of antidiabetic medications effectively lowers blood glucose levels and offers additional benefits, including low propensity for causing hypoglycemia, weight loss, and reduction in blood pressure (Inzucchi, Zinman et al. 2015). It has been reported that all of these agents induce significant urinary glucose excretion, reduce blood glucose and reduce body weight in obese diabetic models (Bailey 2011).

Table 4. ***New SGLT2 inhibitors and their status and selectivity.*** Data is adapted from http://www.drugs.com/.

Name of drug	Brand name	Status	SGLT 2 selectivity over SGLT 1 (fold)	Dose (once daily)
Canagliflozin	Invokana	Available in USA	>250	100 mg; 300 mg
Dapagliflozin	Farxiga	Available in USA, EU, Australia	>1200	5 mg; 10 mg
Empagliflozin	Jardiance	Approved in Europe, USA	>2500	10 mg; 25 mg
Ipragliflozin	Suglat	Submitted in Japan	>550	25 mg; 50 mg or up to 100 mg
Tofogliflozin	Apleway	Approved in Japan	>1,875	20 mg
Sotagliflozin	LX 4211	Phase-II clinical trials	Dual SGLT1/2 inhibitor	200 mg; 400 mg

Mechanism action of SGLT2 inhibitors

SGLT2 inhibitors are a unique class of antidiabetic drugs, which works independently of insulin, and thereby its efficacy should be preserved without regard to the advancement of the pancreatic β-cell failure. SGLT2 is considered to mediate approximately 90% of renal glucose reabsorption. Therefore, SGLT2 inhibitors enhance urinary glucose excretion in kidney leading to remove the glucose from the body, consequently decreasing the blood glucose

level. It has been reported that SGLT2 inhibitors improve both fasting and postprandial hyperglycemia without causing weight gain and hypoglycemia (Dardi, Kouvatsos et al. 2015). In addition, SGLT2 inhibitors also contribute to the survival of pancreatic islet cells and preserve insulin production (Inzucchi, Zinman et al. 2015). SGLT2 inhibitors have been demonstrated to be effective in treatment of patients with T2D and are anticipated to be also effective in treatment of patients with T1D in number of clinical trials (Vasilakou, Karagiannis et al. 2013; Hattersley and Thorens 2015; Perkins, Cherney et al. 2014; Henry, Thakkar et al. 2015).

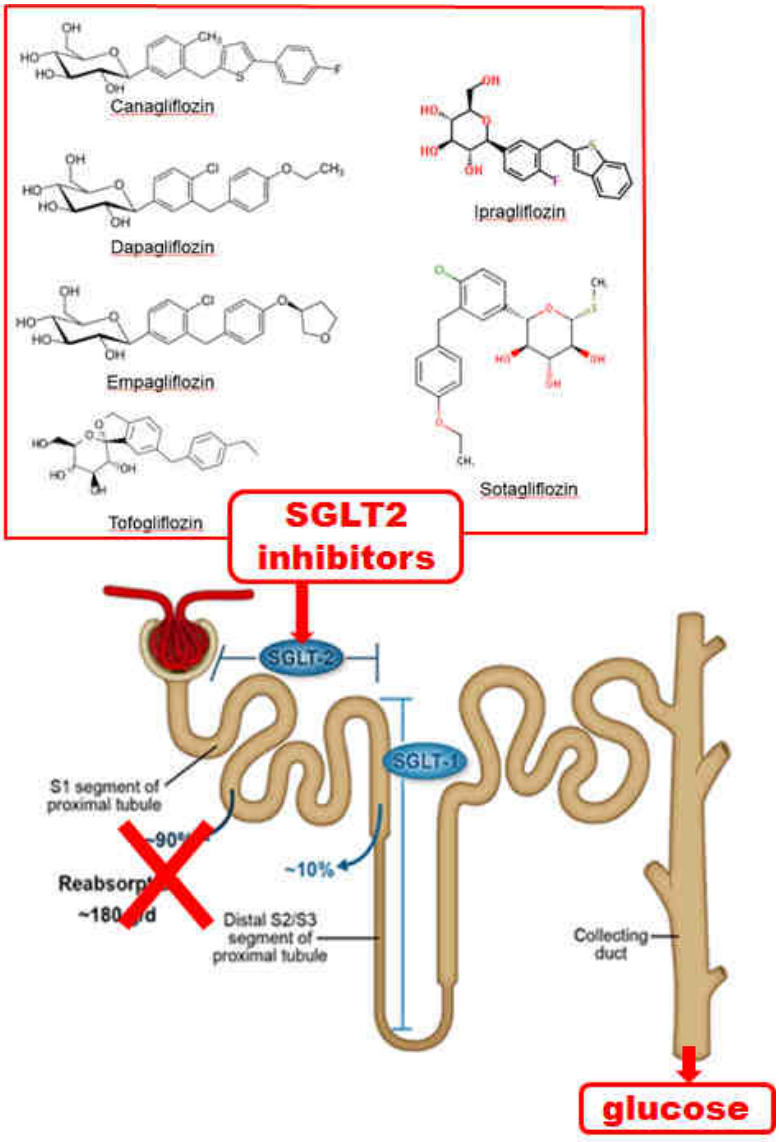

Figure 22. ***Mechanism action of SGLT2 inhibitors.*** Modified from (Dardi, Kouvatsos et al. 2015).

1.3.2.3. Pharmacological actions of sodium-glucose cotransporter 2 inhibitors

a) Effect on urinary glucose excretion

Since approximately 90% of renal glucose is reabsorbed by SGLT2, the inhibition of SGLT2 activity reduces the tubular glucose reabsorption and leads to the excretion of glucose in urine, hence reducing hyperglycemic state even after glucose loading. However, in clinical studies, SGLT2 inhibitors inhibit only 30–50% of the filtered glucose load in healthy individuals (Dardi, Kouvatsos et al. 2015). A number of explanations have been proposed to explain this paradox, the more satisfactory explanations are:

(i) high plasma protein binding reduces the filtered load of SGLT2 inhibitors and results in insufficient drug concentration in the tubular lumen to inhibit SGLT2 (Liu, Lee et al. 2012);

(ii) renal secrection and slow off rate of SGLT2i could also explain the limitation of SGLT2 inhibitors (Liu, Lee et al. 2012);

(iii) under conditions when SGLT2 is inhibited, SGLT1 is forced to reabsorb glucose at its maximum capacity resulting in the less amount of glucose excreted in the urine (Abdul-Ghani, DeFronzo et al. 2013).

b) Effects on plasma glucose levels (HbA1c, FPG and PPG)

It has been well known that the SGLT2 inhibitors significantly reduce the level of plasma glucose, which is shown by a reduction of the HbA_{1C} levels (Dardi, Kouvatsos et al. 2015). For example, at 24 weeks, dapagliflozin lowered HbA_{1C} by 0.44 % (placebo-adjusted) in patients with a baseline HbA_{1C} < 8.0 %, by 0.54 % in patients with baseline $HbA_{1C} \geq 8.0$ to < 9.0 %, and by 1.01% in patients with baseline $HbA_{1C} \geq 9$ % (Chao 2014). In addition, another study has shown placebo-adjusted reductions in HbA_{1C} from baseline to week 24 of 0.74 % ($P < 0.001$) and 0.85 % ($p < 0.001$) for the empagliflozin 10 mg and 25 mg doses, respectively. Corresponding, the reduction of HbA_{1C} values (~0.8%) was accompanied with a decrease in fasting plasma glucose (~2 mmol/L) (Hedrington and Davis 2015).

c) Effect on fasting hypoglycemia

Although hypoglycemia is the most potential side effect of several oral antidiabetic agents, SGLT2 inhibitors work independently from insulin, have minimal risk of producing hypoglycemia and do not overstimulate pancreatic beta cells. However, hypoglycemia may occur when SGLT2 inhibitors are used in combination with other antidiabetic drugs. For example, a low risk of hypoglycemia has been observed among patients treated with canagliflozin taken as monotherapy. In 26-week, randomized, double-blind, placebo-

controled, phase 3 trial, 584 subjects received canagliflozin 100 or 300 mg or placebo once daily. They reported that the percentages of subjects with hypoglycemia were similar with canagliflozin 100 or 300 mg and placebo (3.6, 3.0 and 2.6%, respectively) (Stenlof, Cefalu et al. 2013). Nevertherless, it has been documented that an increased incidence of hypoglycemia when canagliflozin was used in combination with insulin or sulfonylureas. The randomizes, double-blind, placebo-controlled evaluated canagliflozin as an add-on to metformin plus sulphonyluruea in T2D patients over 52-week treatment. They found that more patients treated with canagliflozin 100 and 300 mg than placebo had documented hypoglycemia (33.8%, 36.5% and 17.9%, respectively) (Wilding, Charpentier et al. 2013). Similar findings were observed with empagliflozin, a low rate of hypoglycemia (< 1%) has been shown for empagliflozin used as monotherapy (Rosenstock, Jelaska et al. 2014); (Roden, Weng et al. 2013). However, for empagliflozin added to metformin plus sulfonylureas in T2D, the frequency of hypoglycemia was greater for empagliflozin 10 mg (16,1%) and 25 mg (11,5%) versus placebo (8,4%) in 24-week, randomized, double-blind, placebo-controlled trial (Haring, Merker et al. 2013).

d) Effect on total body weight

In addition to improving the glycemic control, these agents also lead to other beneficial effects such as a reduction in weight. The glucosuria induced by SGLT2 inhibitors is typically associated with a net calorie loss of approximately 200 – 300 kilocalories per day leading to weight reductions about 2 – 3 kg over 24 – 52 weeks (List, Woo et al. 2009).

e) Effect on blood pressure

This group of drugs exerts a therapeutic control over the high blood pressure. All studies with SGLT2 inhibitors to date demonstrated significant reductions in blood pressure, with greater reductions seen in systolic (−1.66 mmHg to −6.9 mmHg) than diastolic (−0.88 mmHg to −3.5 mmHg), possibly by acting through net sodium loss without apparently changing the heart rate (Rosenwasser, Sultan et al. 2013). Importantly, this has been observed without a compensatory increase in heart rate (Bailey, Gross et al. 2010; Vasilakou, Karagiannis et al. 2013).

f) Unexpected effects

First of all, as sodium is cotransported with glucose by SGLT2, sodium is lost due to SGLT2-inhibited diuresis. This loss of sodium prevents hypernatremia, the trigger for thirst, which interferes with the ability to compensate for water loss, possibly leading to dehydration and

hypovolemia-related events. Second of all, due to increasing glucosuria, urinary tract infections and mycotic genital infections have been reported in patients treated with SGLT2 inhibitors, particularly in females. Third of all, 12 June 2015, the US Food and Drug Administration and the European Medicines Agency, respectively, have been reported that life-threatening cases of diabetic ketoacidosis have been found in patients with type 2 diabetes treated with SGLT2 inhibitors (Dardi, Kouvatsos et al. 2015).

g) Cardiovascular benificial effects

Since inhibition of SGLT2 leads to glucosuria with an accompanying diuresis, weight and blood pressure reductions, all of which are theoretically beneficial in patients with CVD. Besides, recent studies have also suggested that enhancing glucosuria with SGLT2 inhibitors improves insulin sensitivity as measured by peripheral glucose uptake (Merovci, Solis-Herrera et al. 2014). It has been reported that SGLT2 inhibitors are associated with a small increase in high-density lipoprotein as well as an increase in low-density lipoprotein with concomitant reductions in triglyceride levels (Monami, Nardini et al. 2014). Whether these small lipid changes could potentially offset any potential CV benefit with SGLT2 inhibitors will need further clarification. Moreover, SGLT2 inhibitors may also improve endothelial function by decline collagen, elastin, advanced glycation end-products and other components of connective tissue that participate in the process of arterial stiffening (Zimlichman, 2014; Cherney, Perkins et al. 2014). It is also conceivable that SGLT2 inhibitors have been shown to reduce leuokocytosis, inflammation and oxidative stress, which are processes involved in the pathophysiology of atherosclerosis in animal models (Tahara, Kurosaki et al. 2013; Osorio, Coronel et al. 2012).

1.3.3. Empagliflozin and cardiovascular benefits

Among SGLT2 inhibitors, empagliflozin was approved, in January 2014 in Europe and August 2014 in USA. Empagliflozin (Jardiance), which is commercialized at the doses of 10 mg and 25 mg once daily, is indicated for the treatment of T2DM with conditions as add-on to a background glucose-lowering therapy including metformin (Scheen 2015). Recent results from a randomized, placebo-controlled cardiovascular outcome trial of empagliflozin (EMPA-REG Outcomes) were highly impressed. It has been reported that empagliflozin led to significant reductions in HbA1c (by ~0.3–0.6%), weight (by ~2 kg) and systolic blood pressure (by ~3 mmHg) without any compensatory increase in heart rate. There were also significant rises in LDL-cholesterol and HDL-cholesterol levels as expected. Suprisedly,

empagliflozin significantly lowered death from cardiovascular causes (by 38%), heart failure hospitalisation (by 35%) and death from any cause (by 32%) (Zinman, Wanner et al. 2015). However, it also raises many questions, including the mechanisms behind these benefits, the cardiovascular benefits of empagliflozin is because of secondary effects of glycemic control, or a direct effect on the cardiovascular system.

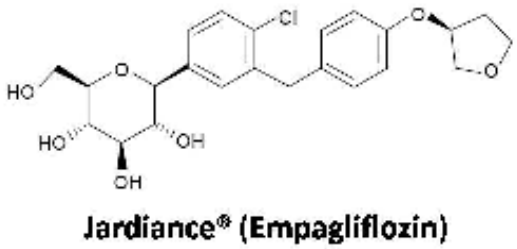

Figure 23. ***Chemical structure of empagliflozin*****.** Adapted from (Dardi, Kouvatsos et al. 2015).

Before the application of SGLT2 inhibitors as glucose-lowering drugs, metformin has been considered as the first choice in oral diabetic treatment thanks to its potential cardiovascular protective effect. Metformin exerts its antidiabetic activity by increasing peripheral glucose uptake and utilization, and decreasing hepatic gluconeogenesis. It has been reported that the metformin group experienced **a 36% relative risk** reduction in all-cause mortality (p = 0.01), a 39% relative risk reduction in myocardial infarction (p = 0.01) and a 30% relative risk reduction in all macrovascular end points (p = 0.02) compared with the conventional group in patients with T2D in a 10-year follow-upp study (UKPDS 34, 1998). Thus, metformin is the only OAD to demonstrate significant cardiovascular benefit over and above its glucose lowering effect in diabetes (1998). However, it has been mentioned some disadvantages of this class in treatment for patients with renal insufficiency. Because metformin is excreted by the kidney, therefore, metformin may accumulate in patients with kidney failure, leading to elevated blood lactate levels and ultimately inducing lactic acidosis (Inzucchi, Lipska et al. 2014). Consistently, metformin has also been found to associated with lactic acidosis (Fitzgerald, Mathieu et al. 2009). Therefore, the dose of metformin should be reviewed and reduced (e.g., by 50% or to half-maximal dose) in those with estimated glomerular filtration rate (eGFR) < 45 mL/min per 1.73 m^2, and renal function should be monitored closely (every 3 months). This drug should be stopped once eGFR falls to < 30 mL/min per 1.73 m^2 (Lipska, Bailey et al. 2011).

Regarding the insulin sensitizer, some studies have demonstrated that thiazolidinediones (TZD) reduced triglyceride levels and lower blood pressure. However, rosiglitazone, one of thiazolidinediones, was withdrawn of the trade because of its increased risk of heart problem including heart attacks and heart failure (Nissen and Wolski 2007; St. Onge, Motycka et al. 2009). The mechanism for the increased cardiovascular risk associated with rosiglitazone related to the fluid retention, the elevation of LDL (Palee, Chattipakorn et al. 2011). In regard to sulfonylureas, it is not yet clear whether sulfonylureas directly contributes to the increased risk of CVD (Shimoda and Kaku 2016). The UK Prospective Diabetes Study demonstrated that intensive glycemic control with either sulfonylureas or insulin resulted in significant reductions in microvascular complications, (1998) and a subsequent epidemiologic analysis demonstrated a reduction in macrovascular complications associated with improved glycemic control (Stratton, Adler et al. 2000). In contrast, other studies have shown no significant effect on the rates of major cardiovascular events with sulfonylureas (Gerstein, Miller et al. 2008; Duckworth, Abraira et al. 2009). Moreover, other disadvantages of sulfonylureas are the risk of weight gain and hypoglycemia.

In contrast with the traditional OADs, it has been revealed in recent studies that empagliflozin decrease the HbA1c, systolic blood pressure without significantly changing the heart rate and the body weight (Neumiller 2014). This drug also has a low intrinsic propensity to cause hypoglycemia. Although, the decrease in uric acid and increase in hematocrit is noticed as an additional beneficial effect, there is no impact on liver toxicity or elevation of liver enzymes (Malhotra, Kudyar et al. 2015). Regarding bone mineral density or bone fracture, the U.S Food and Drug Administration has strengthened the warning for canagliflozin related to the increased risk of bone fractures and decreased risk of bone mineral density (FDA Drug Safety Communication, 2015). These effects of empagliflozin have not yet been reported.

Empagliflozin has been used as a monotherapy in patients uncontrolled by diet and exercise; as an add-on therapy to metformin, or metformin plus sulfonylurea, or pioglitazone plus metformin; and lastly as an add-on to insulin in a 24-week trial. Empagliflozin monotherapy proved significantly beneficial in reducing the HbA1c levels by 0.74% (10 mg dose) and by 0.85% (25 mg dose) compared with placebo, and also reduced fasting blood glucose levels across all studies (25 mg preferred over 10 mg daily) (Roden, Weng et al. 2013). In addition, it has been also published that empagliflozin 10 and 25 mg for 24 weeks as add-on to metformin therapy significantly improved glycemic control. In this study, patients

with HbA1c levels of ≥7% to ≤ 10% while receiving metformin (≥1500 mg/day) were randomized and treated with once-daily treatment with empagliflozin 10 mg (*n* = 217), empagliflozin 25 mg (*n* = 213), or placebo (*n* = 207) for 24 weeks. At week 24, adjusted mean (SE, standard error) changes from baseline in HbA1c were −0.13% (0.05)% (−1.4 [0.5] mmol/mol) with placebo, −0.70% (0.05)% (−7.7 [0.5] mmol/mol) with empagliflozin 10 mg, and −0.77% (0.05)% (−8.4 [0.5] mmol/mol) with empagliflozin 25 mg (both $P < 0.001$) (Haring, Merker et al. 2014). These studies also showed significant reductions in fasting glucose, body weight and systolic blood pressure compared to placebo. The similar results were also found in evaluation studies of empagliflozin in combination with metformin plus sulfonylurea, and pioglitazone plus metformin (Haring, Merker et al. 2013; Lewin and Frias 2015; Kovacs, Seshiah et al. 2014). Moreover, it is recently shown that empagliflozin improved glycemic control and reduced weight without increasing the risk of hypoglycemia and with lower insulin requirements in obese inadequately controlled T2D patients (Rosenstock, Jelaska et al. 2014).

However, loss of glucose-lowering efficacy over time is a common limitation of existing treatments for diabetes and the long-term cardiovascular effects of SGLT2 inhibitors remain unknown. Several long-term vascular outcome trials are ongoing to assess specifically the cardiovascular safety of the various SGLT2 inhibitors (Table 6). The CANVAS study with canagliflozin as add-on to normal care was started preapproval. CANVAS is following about 4400 patients with T2D for more than 4 years, primarily to capture major adverse cardiovascular events (Retrieved from https,//clinicaltrials.gov/ct2/show/NCT01032629). The DECLARE study with dapagliflozin as add-on to normal care also will investigate major adverse cardiovascular events in about 17 000 patients and is planned to continue for about 6 years (Retrieved from https,//clinicaltrials.gov/ct2/show/NCT01730534). Regarding empagliflozin, the EMPA-REG OUTCOME, was recently published (Zinman, Wanner et al. 2015). In this trial, patients with T2D at high cardiovascular risk were randomized to receive empagliflozin 10 mg or 25 mg or placebo once daily. Patients treated with empagliflozin had lower rates of the primary outcome, which was death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke [10.5% vs. 12.1%, HR 0.86 (CI 0.74, 0.99)], with a *P*-value of 0.04 for superiority. This was primarily driven by lower rates of death from cardiovascular causes, 3.7 vs. 5.9%, 38% relative risk reduction, which is **2-fold higher** compared with metformin – the best treatment for diabetic patients to reduce cardiovascular events. These findings suggested that empagliflozin reveals more cardiovascular benefits than

others OADs. In addition, hospitalization for heart failure (2.7 vs 4.1%, 35% relative risk reduction) and all-cause mortality (5.7 vs. 8.3%, 32% relative risk reduction) were both significantly reduced with empagliflozin versus placebo. This is the first study of a glucose lowering agent to demonstrate CV risk reduction in a dedicated cardiovascular outcomes trial.

Table 5. ***Ongoing trials regarding cardiovascular outcomes***. Adapted from (Katz and Leiter 2015)

Clinical trials	Treatment	N	Population	Endpoints	Results expected
CANVAS-R	Canagliflozin (100 and 300 mg) vs placebo	3,627	CVD or high risk for CVD	Primary: Progression of albuminuria Secondary: CV death, nonfatal MI or non fatal CVA	2017
CANVAS	Canagliflozin (100 and 300 mg) vs placebo	4,363	CVD or high risk for CVD	CV death, nonfatal MI or non fatal CVA	June 2018
CREDENCE	Canagliflozin (100 mg) vs placebo	3,627	eGFR $\geq$ 30 and < 90 mL/min/1.73 m^2 on ACEi or ARB	Primary: ESRD, doubling of serum creatine, renal or CV death Secondary: CV death, nonfatal MI or non fatal CVA	2019
DECLARE	Dapagliflozin (10 mg) vs placebo	27,000	CVD or high risk for CVD	CV death, nonfatal MI or non fatal CVA	2019

ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; CV, cardiovascular; CVA, cardiovascular accident; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; ESRD, end stage renal disease; MI, myocardial infarction.

The underlying mechanism for the cardioprotective effect is unlikely to be solely due to the reduction of the glucose reabsorption in kidney. Since empagliflozin reduced by about 0.6% the level of HbA1c while metformin by about 1.5%, the protective effect of empagliflozin most likely involves besides improving glycemia also other effects such as possibly on the vascular system.

To our knowledge, there are only few reports that investigated the effect of SGLT inhibitors on vascular function in normal conditions and diabetes. Oelze and colleagues have demonstrated that chronic treatment of streptozotocin (STZ)-treated rats with the empagliflozin prevents the development of endothelial dysfunction, oxidative stress, AGE/RAGE signaling and inflammation in a well-characterized animal model of T1D. In comparison with control rats, STZ injection caused endothelial dysfunction in diabetic rats, the ACh-dependent relaxation was reduced from 90.3 ± 1.4% to 80.3 ± 2.3%. This impaired ACh-dependent relaxation was improved up to 91.5 ± 0.7% and 85.5 ± 2.4% after 7 weeks' treatment with empagliflozin 10 and 30 mg/kg/d, respectively. In addition, in the same study, treatment with empagliflozin reduced oxidative stress in aortic vessels and in blood of diabetic rats. It has been shown that oxidative stress was increased in the diabetic rats and reduced by both SGLT2 inhibitors doses, although, still higher than to the control. Moreover, the phosphorylation of eNOS at Ser1177, which increases eNOS activity, was impaired in STZ-treated rats, and also found to be improved by treatment with empagliflozin (Oelze, Kroller-Schon et al. 2014). Another study investigating the role of canagliflozin in the regulation of vascular relaxation in mouse pulmonary and coronary arteries has observed that the acute *ex-vivo* treatment (20 min) with canagliflozin exerts an adverse effect on pulmonary arterial function (inhibited pulmonary vasodilation), whereas chronic treatment (16 weeks) with canagliflozin has a beneficial effect on the coronary arteries (enhanced coronary vasodilation) in T2D mice (Han, Cho et al. 2015). These findings suggested that SGLT2 inhibitors differentially regulate vascular relaxation depending on the type of arteries, duration of the treatment, and the health condition such as diabetes.

The *in vitro* and *in vivo* studies regarding expression of SGLT1/2 and the effect of SGLT1/2 on blood vessels are still limited. Besides kidney, several investigators demonstrate that SGLT1/2 are also expressed in other organs and tissues including heart, lungs and pulmonary and coronary arterial endothelial cells (Chen, Williams et al. 2010; Taubert, Rosenkranz et al. 2004; Wright, Hirayama et al. 2007). Han and colleagues have examined the mRNA expression level of SGLT subtypes in mouse and human endothelial and SMC of pulmonary and coronary arteries (Han, Cho et al. 2015). They found that SGLT2 are not

detected in both endothelial and SMC of pulmonary and coronary arteries, whereas SGLT1 was detected but had much less expression level compared to that of the intestine and kidney. Another group has demonstrated that, delphinidin-3-glucoside (Dp) - a member of a family of bioactive compounds known as anthocyanins, is transported into endothelial cells in a temperature, concentration, and time-dependent manner via SGLT1 both *in vitro* and *in vivo* studies (Jin, Yi et al. 2013). SGLT1 was noticeably expressed in the human umbilical vein endothelial cells (HUVECs) and vascular endothelium of the isolated thoracic aorta. They also found that Dp uptake by HUVECs in Na^+-containing medium was dramatically higher than that in Na^+-free medium. Dp uptake by HUVECs in the presence of 0.50 mM phlorizin resulted in a reduction of approximately 96%, (0.77 ± 0.27 vs. 20.18 ± 0.66 nmol per 106 cells for phlorizin and control groups, respectively), whereas a reduction of > 95% occurred following treatment with 3 M of D-glucose (0.94 ± 0.28 nmol per 10^6 cells). Furthermore, there was a significant reduction in Dp uptake by SGLT1 mRNA suppression. Together, these results support the hypothesis that the carrier-mediated process of Dp uptake was SGLT1-dependent in HUVECs. In contrast, the information about the expression of SGLT2 in blood vessels is still questionable.

Overall, a novel therapeutic approach by SGLT2 inhibition plays a central role in the diabetes management nowaday. They have glucose-lowering abilities similar to others existing glucose – lowering medications. Moreover, they have the other advantages such as weight loss, blood pressure reduction and a low risk for hypoglycemia. They are generally well tolerated and have favourable safety profiles. Clinical trials have consistently confirmed the antihyperglycaemic efficacy of SGLT2 inhibition, as monotherapy or add-on to other glucose-lowering therapies. However, the information regarding the impact of SGLT2 inhibitors on cardiovascular outcomes is still limited. Initial results indicate not only cardiovascular safety, but also a significant reduction in major cardiovascular events, as well as cardiovascular and total mortality (Zinman, Wanner et al. 2015). The result of another additional ongoing long-term studies is awaited soon (Retrieved from http,//www.ndei.org/conference-coverage-empa-reg-outcome-trial-empagliflozin-reduces-cardiovascular-deaths-SGLT2-inhibitor-EASD-congress-2015.aspx).

AIM OF THE STUDY

To our knowledge, diabetes is one of the most challenging public health problem that is approaching epidemic proportions globally. Despite many treatments available for diabetic patients on the market, the mortality of diabetic patients is still increasing every year. Since cardiovascular complications are commonly seen in diabetic patients this is the major risk factor for mortality. Regardless of other contributing factors, hyperglycemia remains a primary focus linking diabetes to vascular disease. Clinical and experimental investigations have shown that oxidative stress induced by hyperglycemia plays an important role in the pathogenesis and development of cardiovascular complications in diabetes. However, the exact mechanism by which oxidative stress contributes to and accelerates the development of cardiovascular complications in diabetes remains to be clarified. A number of evidences have supported the view that hyperglycaemia through oxidative stress mediates the impairment of endothelium-dependent relaxation by decreasing the bioavailability of NO and increasing the release of endothelial constrictor factors, particularly ROS. Besides, a recent therapeutic approach for diabetic patients – SGLT2 inhibitors, has shown a significant effect not only on reducing blood glucose levels, but also on decreasing the cardiovascular risk in animal models of diabetes and patients with T2D. Although the mechanism by which SGLT2 inhibitors reduce the cardiovascular risk factors is still not well understood. There are increasing evidences showing the beneficial effect of SGLT2 inhibitors on control of blood glucose level. However, reduction of plasma glucose concentration often has little effect on CVD risk as shown with other antidiabetic drugs. We hypothesized that SGLT 1 or 2 might exist on endothelail cells, and contribute to the transport of glucose. Therefore, SGLTs could contribute to reduce the increased level of oxidative stress induced by high glucose (HG) in endothelial cells in blood vessels in diabetes. Such a concept would explain that SGLT inhibitors have a direct effect on blood vessels by decreasing endothelial dysfunction induced by HG leading ultimately to a reduced cardiovascualar risk.

For these reasons, initially, our first goal was to establish an *ex vivo* model of HG induced endothelial dysfunction in different types of isolated arteries from animals (male Wistar rat and porc). Then, our goal was to clarify the contribution of SGLTs in the transport of glucose in endothelial cells under HG conditions to evaluate the protective effect of SGLT inhibitors (for example, empagliflozin) on the endothelial function. Contratry to our expectation, HG did not show any alteration either on contractile nor on relaxation responses in all tested types of artery. The absence of effect raised the question regarding the

contribution of oxidative stress to the impairment of endothelial relaxation of blood vessels. Therefore, our next mission was to determine the induction of endothelial dysfunction via oxidative stress by examining the effect of pyrogallol, which is known to be a generator of superoxide anions, on endothelial function in the rat aorta. Since, we have found that pyragallol significantly enhanced the contractile and attenuated the endothelium-dependent relaxation responses in aortic rings; we conclude that oxidative stress is able to cause endothelial dysfunction in the rat aorta. However, in our *ex vivo* model, oxidative stress induced by HG was not sufficient to impair the endothelium-dependent relaxation responses in aortic rings. In other word, the effect of HG on endothelium-dependent relaxation responses might too small to alter significantly vascular reactivity of healthy rats. It might also be related to certain factors, such as a gender, age, strain, species of studied animals, and conditions of housing of animals. Therefore, we co-incubated aortic rings with HG and pyrogallol in order to evaluate the effect of HG on endothelium-dependent relaxation responses in aortic rings with an increased level of oxidative stress (a lower healthy status) induced by a low concentration of pyrogallol. In addition, the lack of effect can also be accounted for a too short incubation time to HG. Then, we also examined the effect of a long-term incubation with HG on the relaxant activity of both rat aorta and porcine coronary artery.

In addition, our aim was also to characterize blood vessels of diabetic patients, either alone or in association with main cardiovascular risk factors such as hypertension and age by evaluating the level of oxidative stress and the expression levels of eNOS, SGLTs and the components of angiotensin system. We have collected human internal mammary arteries (IMA) harvested operatively from 58 patients underwent coronary artery bypass grafting between July 2013 and January 2016 in the New Civil Hospital of Strasbourg. Since the alterations of the endothelial properties of each IMA grafts in the presence of single or combined risk factors may have some relevance in the clinical setting. Therefore, firstly, we have analyzed the clinical charecteristics of the patients by several categogies including the time of havesting artery, age, sex, smoking, creatinin clearance, body mass index, duration of hospitalization and preoperative medications used. As diabetes and hypertension were the most frequent risk factors in bypass patients, we divided the patients into three groups: normotensive and non-diabetic, hypertensive, and diabetic and hypertensive patients. Secondly, we have evaluated the level of oxidative stress in IMA from patients in different groups in order to indicate the contribution of diabetes and hypertension in the level of oxidative stress. We also have determined the different sources of ROS in IMA from each

group. The results from identifying sources of ROS have showed that eNOS is one of the potential sources of ROS; hence, thirdly, we have evaluated the expression level of eNOS in IMA from diabetic patients. It has been found that the increased ROS induced by local angiotensin II in endothelial cells; therefore, fourthly, we also evaluated the expression level of components of the angiotensin system in IMA from diabetic patients. Finally, the expression level of SGLTs in the IMA has also been evaluated in order to clarify the association between diabetes with/without hypertension and the transport of D-glucose in endothelial cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. Reagent Information

Details of all reagents studied in this thesis are provided in Table 6.

Table 6. ***Reagents Information.*** Abbreviation: dH_2O: distilled water

Reagents	Abbreviations	Purchased from	Solvent
3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-d]pyrimidine	VAS-2870	Sigmal-Aldrich	DMSO
Acetylcholine	ACh	Sigmal-Aldrich	Krebs' buffer
Acrylamide 30 %		Sigmal-Aldrich	dH_2O
Alexa 633-conjugated goat anti-mouse IgG	Alexa anti-mouse	Invitrogen	Blocking buffer
Alexa 633-conjugated goat anti-rabbit IgG	Alexa anti-rabbit	Invitrogen	Blocking buffer
Ammonium persulfate	APS	Sigmal-Aldrich	dH_2O
Bovine serum albumin	BSA	Sigmal-Aldrich	TBST buffer
Bradykinin	BK	Sigmal-Aldrich	dH_2O
Calcium chloride	$CaCl_2$	Sigmal-Aldrich	dH_2O
Catalase	CAT	Sigmal-Aldrich	dH_2O
Clarity Western ECL Substrate	ECL	Bio-rad	
Dako fluorescence mounting medium	DAKO	Dako France SAS, Les Ulis	
DC™ Protein Reagent Assay Kit		Bio-rad	
D-glucose	D-glucose	Sigmal-Aldrich	Krebs' buffer
Dihydroethidium	DHE	Sigmal-Aldrich	DMSO
DMEM media	DMEM	Gibco	
Fungizone		Cambrex	
Indomethacin	Indo	Sigmal-Aldrich	dH_2O
Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	Sigmal-Aldrich	dH_2O
Mouse anti-eNOS	Ab eNOS	BD Biosciences	Blocking buffer
Mouse anti-Glyceraldehyde 3-phosphate dehydrogenase	Ab GAPDH	Merck Millipore	Blocking buffer

Reagents	Abbreviations	Purchased from	Solvent
Mouse anti-nitrotyrosine	Ab OONO–	Merck Millipore	Blocking buffer
Mouse anti-tissue factor	Ab TF	Sekisui Diagnostics	
Myxothiazol	Myx	Sigmal-Aldrich	Ethanol
N,N,N',N'-Tetramethylethylenediamine	TEMED	Sigmal-Aldrich	dH_2O
N(ω)-nitro-L-arginine	L-NA	Sigmal-Aldrich	dH_2O
N-acetylcysteine	NAC	Sigmal-Aldrich	dH_2O
OCT compound	OCT	Tissue-Tek, Sakura Finetek	
Penicillin		Cambrex	
Peroxidase-labeled anti–mouse IgG	anti–mouse IgG	Invitrogen	Blocking buffer
Peroxidase-labeled anti–rabbit IgG	anti–rabbit IgG	Invitrogen	Blocking buffer
Phenylephrine	PE	Sigmal-Aldrich	Krebs' buffer
Phosphate-buffered saline solution 10x concentrated	PBS	Euromedex	dH_2O
Polyethyleneglycol - catalase	PEG-CAT	Sigmal-Aldrich	dH_2O
Polyethyleneglycol - superoxide dismutase	PEG-SOD	Sigmal-Aldrich	dH_2O
Polymyxin B sulfate	Pol-B	Sigmal-Aldrich	dH_2O
Potassium chloride	KCl	Sigmal-Aldrich	dH_2O
Potassium cyanide	KCN	Sigmal-Aldrich	dH_2O
Potassium dihydrogen phosphate	KH_2PO4	Sigmal-Aldrich	dH_2O
Prestained markers		Invitrogen	
Pyrogallol	Pyr	Sigmal-Aldrich	dH_2O
Rabbit anti-nox1 (gp91phox)	Ab gp91phox	Santa Cruz	Blocking buffer
Rabbit anti-angiotensin converting enzyme	Ab ACE	Abbiotec	Blocking buffer
Rabbit anti-angiotensin II	Ab Ang II	Peninsula Laboratories	Blocking buffer
Rabbit anti-AT1 receptor	Ab AT1	Santa Cruz	Blocking buffer
Rabbit anti-AT2 receptor	Ab AT2	Santa Cruz	Blocking buffer
Rabbit anti-cyclooxygenase -1	Ab COX-1	Abcam	Blocking buffer

Reagents	Abbreviations	Purchased from	Solvent
Rabbit anti-cyclooxygenase -2	Ab COX-2	Abcam	Blocking buffer
Rabbit anti-p22phox	Ab p22phox	Santa Cruz	Blocking buffer
Rabbit anti-p47phox	Ab p47phox	Santa Cruz	Blocking buffer
Rabbit anti-P-eNOS (Ser1177)	Ab P-eNOS	Cell Signaling	Blocking buffer
Rabbit anti-Sodium-glucose transport-1	Ab SGLT1	Santa Cruz	Blocking buffer
Rabbit anti-Sodium-glucose transport-2	Ab SGLT2	Santa Cruz	Blocking buffer
Rotenone	Rot	Sigmal-Aldrich	Ethanol
RPMI 1640 media	RPMI 1640	PAN-Biotech	
Sodium bicarbonate	$NaHCO_3$	Sigmal-Aldrich	dH_2O
Sodium chloride	NaCl	Sigmal-Aldrich	dH_2O
Sodium dodecyl sulfate	SDS	Sigmal-Aldrich	dH_2O
Sodium nitroprusside	SNP	Sigmal-Aldrich	dH_2O
Streptomycin		Cambrex	
Sulfaphenazol	Sulfa	Sigmal-Aldrich	dH_2O
Superoxide dismutase	SOD	Sigmal-Aldrich	dH_2O
Thromboxane A_2 analogue U46619	U46619	Cayman Chemical Company	dH_2O
Tris HCl 1.0 M pH = 6.8		Euromedex	dH_2O
Tris HCl 1.0 M pH = 8.8		Euromedex	dH_2O
Tris/Glycine Buffer 10x concentrated	TG 10x	Euromedex	dH_2O
Tris/Glycine/SDS Buffer 10x concentrated	TG-SDS 10x	Euromedex	dH_2O
Tris-buffered saline solution 10x concentrated	TBS	Euromedex	dH_2O
Tris-buffered saline solution 10x concentrated and 0.1% Tween 20	TBST		dH_2O
Tween 20		Euromedex	TBS buffer

2.2. Ethics statement

This study conforms to the Guide of Care and the Use of laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and the present protocol has been approved by the local Ethics Committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale, France, approval number AL/01/09/09/05).

2.3. Preparation of isolated blood vessels

2.3.1. Isolated arteries from rat

Male Wistar rats (10-15 weeks old and weighing on range 200-350 g) were housed at a constant temperature of 21-22°C, with a 12 h light/dark cycle, and fed on standard pellet chow and water ***ad libitum***. Before sacrifice, rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally). The interested artery (aorta, main mesenteric, renal, carotid, femoral arteries) was rapidly removed and immersed in chilled Krebs' solution, composed of (mM), NaCl, 119; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and D-glucose, 11. After the connective tissue was carefully removed, rings approximately 4 mm in length were cut. Each arterial ring was mounted on two stainless steel hooks/clips placed through the lumen. One clip was anchored to the bottom of the organ bath while the other was connected to a strain gauge for measurement of changes of isometric tension with the aid of the µVessel software in an organ bath (10 mL) filled with Krebs' solution, maintained at 37ºC and bubbled with 95% O_2 / 5% CO_2 (pH 7.4).

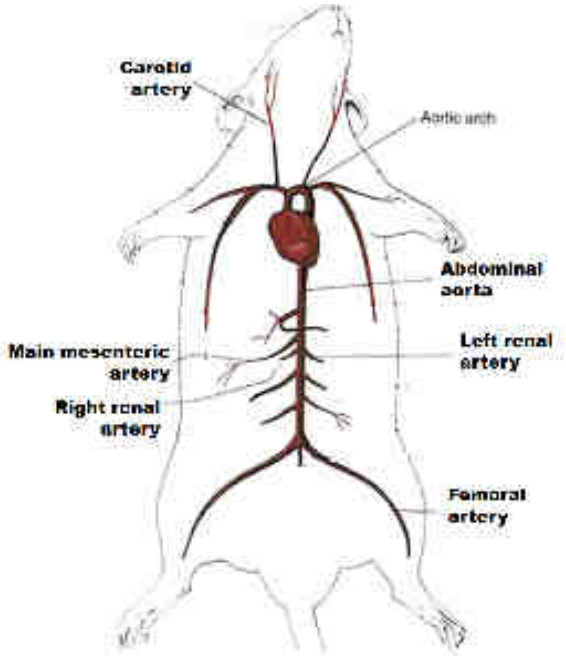

Figure 24. ***Schematic of the target rat arteries studied***. (Picture modified from http,//www.biologycorner.com/)

2.3.2. Porcine coronary artery

Pig hearts were collected from the local slaughterhouse (Copvial, Holtzheim). The left circumflex coronary arteries were excised, immersed in chilled Krebs' solution and cleaned of loose connective tissue. Then, rings of porcine coronary arteries approximately 5 mm in length were cut and suspended in organ baths. In some experiments, the endothelium was removed from the rings by gently rubbing the intimal surface of the blood vessel with a pair of watchmaker's forceps.

2.3.3. Human internal mammary artery

The study population comprised patients who would benefit from elective CABG. Permission was obtained from our local Ethics Committee, and all patients gave their written informed consent. Biological waste segments of internal mammary artery (IMA) from 58 bypass surgery patients were kindly provided by the vascular surgeons of the New Civil Hospital of Strasbourg between July 2013 and January 2016. Patients were divided into three groups, the control group included patients without cardiovascular risk factors, they are normotensive and non-diabetic; the second group incuded hypertensive patients and the third group included diabetic patients with hypertension. Details information regarding patients were described in **Table 7**. After removal, the IMA segment was incubated in Krebs' solution, transported at room temperature to the laboratory and processed within 3 h. Segments were cleaned of fat and connective tissues and cut into rings of 3-4 mm length. One ring of IMA was stored at -80°C for Western blot. Rings of IMA were frozen in a nitrogen bath, grinded using a mortar and pestle and then homogenized in extraction buffer (for details see section 2.5). One ring of IMA was embedded in OCT tissue Tek, stored at -80°C for evaluation of the formation *in situ* of reactive oxygen species (for details see section 2.6) and immunofluorescence staining (for details see section 2.7).

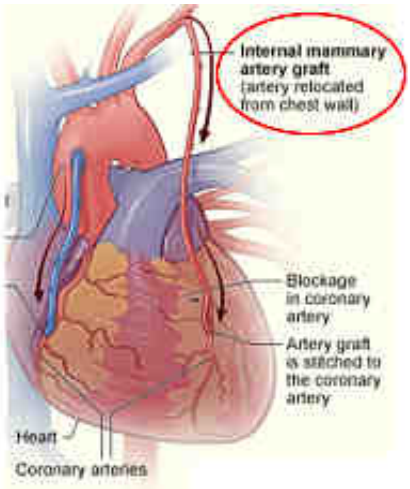

Figure 25. ***Internal mammary artery***. (Adapted from http,//drbenzur.com/).

Table 7. ***Clinical profile of the study patients***. Values are mean ± SD or percentage. Abbreviations, M, male; F, female; BMI, body mass index; ACE, angiotensin-converting enzyme; OAD, oral antidiabetic agents.

	Studied patients (n = 58) (Mean ± SD)
Risk factors	
Age, years	68.0 ± 9.3
Sex, male/female	51/7
BMI, kg/m^2	27.8 ± 4.5
Creatinin clearance (ml/min)	79.1 ± 35.1
Hypertension, n (%)	42 (72.4)
Diabetes, n (%)	20 (34.5)
Dyslipidemia, n (%)	42 (72.4)
Smokers, n (%)	6 (10.3)
Ex-smokers, n (%)	25 (43.1)
Preoperative medications used, n (%)	
Treatment with beta-blocker	47 (81.0)
Treatment with aspirin	46 (79.3)
Treatment with statin	44 (75.9)
Treatment with ACE Inhibitor	34 (58.6)
Treatment with diuretic	21 (36.2)
Treatment with OAD	17 (29.3)

2.4. Vascular reactivity studies

2.4.1. Arteries from rat

Vascular reactivity was performed to determine changes in isometric tension. The target rings were stretched step by step until an optimal resting tension (2 g for aortic rings and 1 g for all other arteries rings) was reached and then allowed to equilibrate for at least 45 min. During this period, tissues were washed every 10 min. After the equilibration period, the rings were exposed to Krebs bicarbonate solution containing a high concentration of potassium (KCl, 80 mM) until reproducible contractile responses were obtained in order to check the function of the vascular smooth muscle. After three times washing with Krebs bicarbonate solution, the rings were precontracted with the α_1 adrenoreceptor agonist phenylephrine (PE, 10^{-6} M) to

reach a stable plateau about 80% of the maximal contraction by high K^+ solution over approximately 15 min, before addition of acetylcholine (ACh, 10^{-6} M) to check the presence of a functional endothelium. Endothelium was considered to be functional when the ACh-induced relaxation was at least 80%. Endothelium was deemed absent when its response was not developed or was smaller than 20%. Then, each ring was washed and re-equilibrated for 30 min. After washout and equilibration period, rings were exposed to high D-glucose (Sigma Aldrich). The conditions of incubation including time, concentrations of D-glucose and additional pharmacological agents required for each test are described in the corresponding specific chapters. After incubation time, rings were again contracted with PE (10^{-6} M) before the application of increasing concentrations of either ACh (10^{-10} – 10^{-6} M) and/or sodium nitroprusside (SNP), (10^{-10} – 10^{-5} M, a NO donor) to construct concentration-relaxation curves. SNP, breaks down spontaneously to yield NO, thereby causing endothelium-independent relaxation by the same effector mechanism as NO released from endothelium (i.e. activation of sGC) (Murad 1986); (Bolotina, Najibi et al. 1994); (Perez-Vizcaino, Cogolludo et al. 1999). In some experiments, polymixin B (10 µg/ml) was added to avoid contamination (an inhibitor of lipopolysaccharides). In all cases, ACh or SNP was added to yield the next higher concentration only when the response to the earlier dose reached a steady state.

The experiment data were recorded then analysed using µVessels software. Tension values were collected for the baseline, for the contraction to KCl (plateau), and PE (plateau) and also for each concentration of the cumulative concentration-relaxation curve to ACh and SNP. Data were entered into Excel software and the KCl-, PE-induced increase in tone calculated (tension at KCl, PE plateau *minus* respective baseline tension). Each data point of the cumulative concentration-relaxation curve to ACh was then subtracted from the baseline tension and calculated as a percentage reversal of PE-induced tone (% relaxation). The concentration response data were fitted to a sigmoidal curve using nonlinear regression (GraphPad Software) to calculate the pEC_{50} (negative logarithm of the half maximal effective concentration). The maximum relaxation (E_{max}) to ACh or SNP was determined as a percentage of the PE precontraction (at 10^{-6} M). The cumulative concentration-contraction curve to PE was determined as the developed tension in grams (g) to PE. The data were then entered into Graphpad Prism Version 5.0 software for curve construction and statistical analysis (more details in section 2.8).

2.4.2. Porcine coronary artery

The porcine coronary artery (PCA) rings were stretched step by step until an optimal resting tension (5 g) was reached and then allowed to equilibrate for at least 60 min. During this period, tissues were washed every 15 min. After the equilibration period, the rings were exposed to Krebs bicarbonate solution containing a high concentration of potassium (80 mM) until reproducible contractile responses were obtained. After three times washing with Krebs bicarbonate solution, the rings were precontracted with the U46619, a thromboxane A_2 receptor agonist (U46199, 1–6.10^{-8} M) to reach a stable plateau about 80% of the maximal contraction to high K^+ solution over approximately 60 min, before addition of bradykinin (BK, 3.10^{-7} M) to check the integrity of the endothelium. Endothelium was considered to be present when the BK-induced relaxation was at least 80%. Endothelium was deemed absent when its response was not developed or was smaller than 20%. Then, each ring was washed and re-equilibrated for 30 min. After washout and equilibration period, rings were exposed to high D-glucose (Sigma Aldrich). The conditions of incubation including time, concentrations of D-glucose and additional pharmacological agents required for each test are described in the corresponding specific chapters. After incubation, rings were again contracted with U46199 before the application of increasing concentrations of either BK (10^{-10} – 10^{-6} M) or SNP, (10^{-10} – 10^{-5} M) to construct concentration-relaxation curves. Data were then analysed similarly as those for rat arteries (for details see in section 2.4.1).

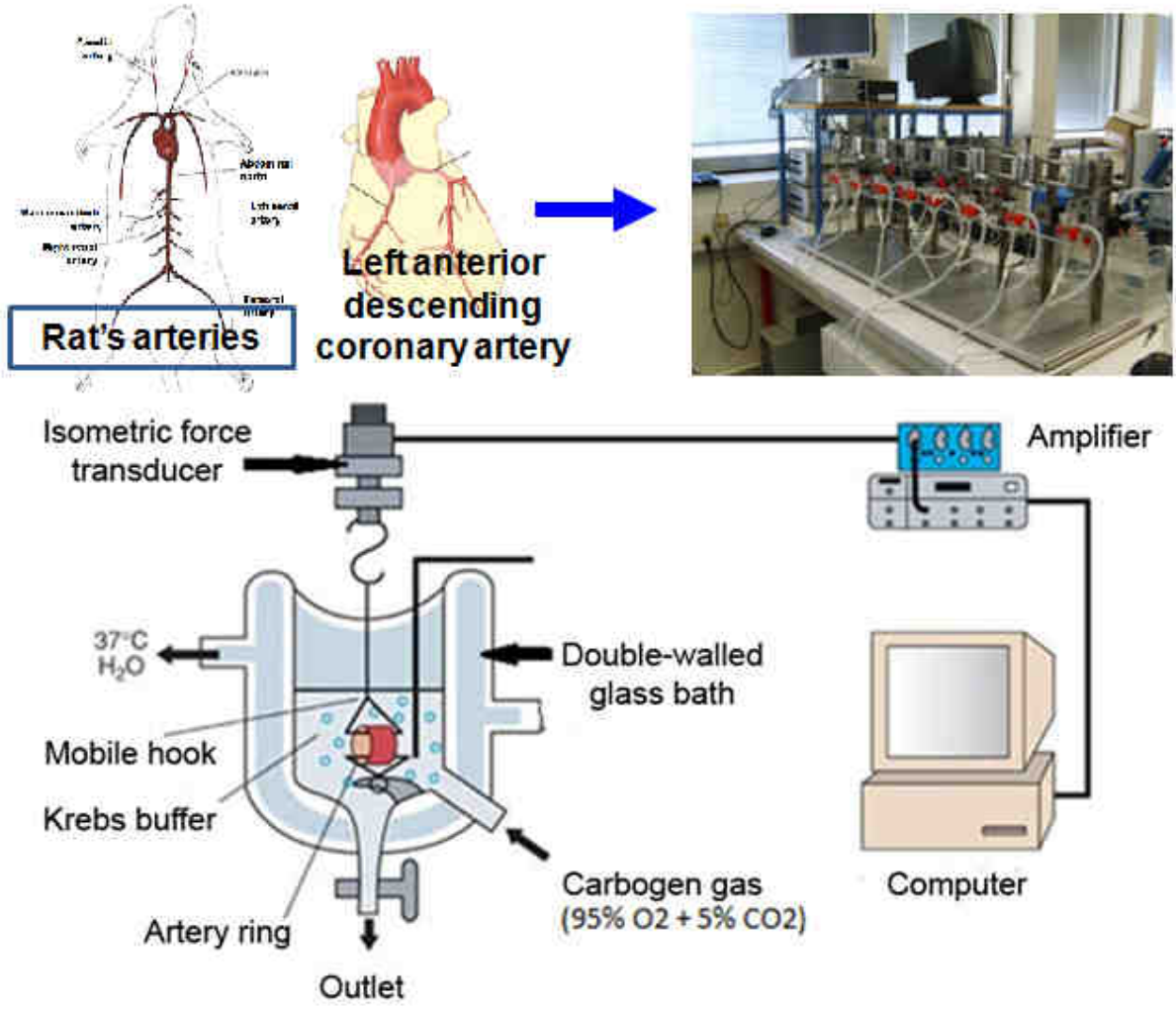

Figure 26. ***Schematic representation of vascular reactivity measurement system*****.** (Modified from the Department of Pharmacology, University of Montreal).

2.5. Western blot

2.5.1. Tissue lysate preparation

Human internal mammary artery rings (IMA) after carefully removing the connective tissue were frozen in a nitrogen bath and stored at -80°C. Then, frozen samples were grinded to a fine powder using a mortar and pestle prechilled with liquid nitrogen. Frozen sample powders were transformed to ice-cold RIPA extraction buffer (composition in mM, Tris/HCl 20 (pH 7.5; QBiogene), NaCl 150, Na_3VO_4 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogen). In general, 150 µl RIPA buffer was added for approximately 30 mg of tissue. The samples were homogenized thoroughly and kept on ice for 30 min, vortex occasionally. Then in order to break the cells or tissue up further and to shear DNA, the samples were sonicated twice at a power of about 40 Watts (in rounds of 10 seconds sonication/10 seconds rest for each cycle).

The samples were kept on ice during the sonication. Finally, by centrifugation at 16,000 g for 30 min at 4°C, the supernatants were collected as soluble proteins. The concentration of total proteins were determined by using Bio-rad *DC*™ Protein Reagent Assay Kit by ELISA at wavelength 650 nm. Samples were frozen at -80°C for long-term storage, or used immediately for Western blotting

2.5.2. Western blot

Total proteins (25 µg) from IMA were separated on 10% SDS-polyacrylamide (Sigma) gels at 70 V for 30 min to allow the proteins compact in the border of the gels, then 100 V for 60 min to start proteins seperation. Separated proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (Amersham) at 100 V for 120 min. Membranes were blocked with blocking buffer containing 5% bovine serum albumin Tris-buffered saline solution (Biorad) and 0.1% Tween 20 (Sigma) (TBS-T) for 1 h. For detection of interested proteins, membranes were incubated with an antibody directed against either eNOS (1/10000), P-eNOS Ser 1177 (1/1000), nitrotyrosine (1/2000), tissue factor (TF, 1/500) angiotensin-converting enzyme ACE (1/500), AT1 receptors (1/1000), AT2 receptors (1/1000), p47phox (1/1000), p22phox (1/1000), COX-1, (1/10000), COX-2, (1/10000) and SGLT1, (1/1000) and SGLT2, (1/1000) overnight at 4 °C. After five times washing by TBS-T, membranes were incubated with an appropriate secondary antibody (peroxidase-labeled anti–rabbit IgG, (3/10000) or anti–mouse IgG (1/10000); Cell Signaling Technology) at room temperature for 60 min. Prestained markers (Invitrogen) were used for molecular mass determinations. Ponceau staining was performed to verify the quality of the transfer and equal amounts of proteins in each lane. Immunoreactive bands were detected by enhanced chemiluminescence (Clarity Western ECL Substrate (Bio-rad). Then, by using densitometry, the quatification of protein bands from Western blot were assessed by Image J 1,4 2q software (National Institutes of Health, USA).

2.6. Dihydroethidium staining

The redox-sensitive fluorescent dye dihydroethidium (DHE, $C_{21}H_{21}N_3$) by virtue of its ability to freely permeate cell membranes and in the presence of superoxide anion ($O_2^{\bullet -}$) is oxidized to form a red fluorescent product (ethidium) with excitation/emission peaks (520/610 nm) (Munzel, Afanas'ev et al. 2002). Although, there are some limitations in $O_2^{\bullet -}$ quantification by using DHE, such as DHE might increase the $O_2^{\bullet -}$ dismutation rate to H_2O_2, which also oxidized DHE forming fluorescent oxidation products with excitation/emission peaks (490/600 nm) near the excitation/emission peaks of the ethidium, and this may cause

interference problems with the fluorescent detection of the $O_2^{\bullet -}$ by using DHE. In spite of these limitations, DHE is perhaps the most specific and least problematic dye, which is used extensively as ROS indicator (Owusu-Ansah, Yavari et al. 2008). DHE is used to determine the level of ROS in several animals and human tissues (Gomes, Fernandes et al. 2005). For example, recent study using DHE has shown increased ROS levels in human tissue in the setting of diabetes (Guzik, Mussa et al. 2002). Regarding our experiment, human internal mammary arteries (3–4 mm length) were embedded in OCT compound (Sakura Finetek, Villeneuve d'Ascq, France) and frozen in a nitrogen bath for cryostat sections. DHE (2.5 µM) was applied onto 25 µm unfixed cryosections of arteries for 30 min at 37°C in a light-protected humidified chamber to evaluate *in situ* formation of ROS.

To determine the nature and source of ROS, rings were incubated with several inhibitors including the antioxidant N-acetyl-cysteine (NAC, 10 mM), polyethylene glycol[1]-superoxide dismutase PEG-SOD (membrane-permeant superoxide dismutase, 500 UI/mL), polyethylene glycol-catalase PEG-CAT (membrane-permeant catalase, 500 UI/mL), superoxide dismutase (SOD, 500 U/mL), catalase (CAT, 500 U/mL), VAS-2870 (NADPH oxidase inhibitor and antioxidant, 300 µM), L-NA (NO synthase inhibitor, 300 µM), sulfaphenazol (cytochrome P450 inhibitor, 100 µM), indomethacin (cyclooxygenase inhibitor, 10 µM) or inhibitors of the mitochondrial respiration chain (myxothiazol, 0.5 µM + rotenone, 1 µM + KCN, 1 µM) for 30 min at 37°C before the addition of DHE. Sections were then washed, mounted in fluorescence mounting medium (Dako, Les Ulis, France) and cover-slipped. Images were obtained using a confocal laser-scanning microscope (Leica confocal microscope TSC SPE (× 63 HCX Pl Apo 1.32 NA objective, Wetzlar, Germany). Quantification of staining levels was performed using Image J 1,4 2q software (National Institutes of Health, USA).

2.7. Immunofluorescence determination of target proteins

Segments of the human IMA (3–4 mm length) were embedded in OCT compound (Tissue-Tek, Sakura Finetek) and snap-frozen in liquid nitrogen. Frozen arteries were cryosectionned at 14 µm. Sections were air-dried for 15 min and stored at −80°C until use. Sections were first fixed with paraformaldehyde at 4%, washed and treated with 10% milk in PBS containing 0.1% Triton ×100 for 1 h at room temperature to block non-specific binding. Sections were

[1] Poly(ethyleneglycol) (PEG) is the most commonly used candidate for drug delivery, which covalently conjugates to the molecule of interest like SOD and CAT. Therefore, PEG-SOD and PEG-CAT are membrane-permeant thank to the high aqueous solubility of PEG.

then incubated overnight at 4°C with an antibody directed against either eNOS (1/100), nitrotyrosine (1/200), angiotensin II (1/500), AT1 receptor (1/400), AT2 receptor (1/400), and ACE (1/200). Sections were then washed with PBS, incubated with the secondary antibody (Alexa 633-conjugated goat anti-rabbit IgG or Alexa 633-conjugated goat anti-mouse IgG) diluted (1/400) in the same buffer for 2 h at room temperature in the dark, and washed before being mounted in Dako fluorescence mounting medium (Dako France SAS, Les Ulis, France) and cover-slipped. For negative controls, primary antibodies were omitted. The samples were observed using a confocal laser-scanning microscope (Leica confocal microscope TSC SPE (× 63 HCX Pl Apo 1.32 NA objective, Wetzlar, Germany). Quantification of proteins levels was performed using Image J 1,4 2q software (National Institutes of Health, USA).

2.8. Statistical Analysis

Data are presented as mean ± SEM of n experiments. Mean values were compared by Student's *t* test (for paired data) or two-way ANOVA (for data from vascular reactivity experiments) and one-way ANOVA (for data from Western blot) followed by the Bonferroni post-hoc test to identify significant difference between treatments, using GraphPad Prism (Prism version 6.0, GraphPad Software, San Diego, CA, USA). In addition, the categorical data of patients are counted in percentage to the total number of patients in the group, then were compared by CHI-square test using GraphPad Prism. The difference was considered to be significant when the *p* value was less than 0.05.

CHAPTER 3

EFFECT OF HIGH D-GLUCOSE ON ENDOTHELIAL FUNCTION IN RAT ISOLATED ARTERIES

3.1. Introduction

As mentioned above, hyperglycemia-induced oxidative stress leads to DNA damage and activation of nuclear poly(ADP-ribose) polymerase, which can increase production of polymers of ADP-ribose reducing glyceraldehyde 3-phosphate dehydrogenase activity (Brownlee 2005). Consequently, the accumulation of glycolytic intermediates is increased, which in turn activate damaging mechanisms: polyol and hexosamine pathways, PKC pathway, and AGEs formation. Ultimately, all of these mechanisms lead to oxidative stress inducing a low-grade proinflammatory condition due to the activation of transcription factors such as nuclear factor-κB (NF-κB), to alter the functional property of matrix components, to enhance the vascular permeability, increase vascular tone and in particularly to induce endothelial dysfunction (Brownlee 2005).

Different methodological approaches have been used to evaluate the alterations of the endothelial function by high D-glucose (HG) in experimental studies. They include *in vivo* studies with diabetic animals (Lash and Bohlen 1991); (Fava, Azzopardi et al. 1997); (Tesfamariam, Jakubowski et al. 1989); (Kuusisto, Mykkanen et al. 1994), studies with isolated arteries of diabetic animals (Tesfamariam, Jakubowski et al. 1989); (Oyama, Kawasaki et al. 1986); (Taylor, Oon et al. 1994), with isolated arteries of non-diabetic animals exposed to high concentrations of D-glucose for a certain period of time (Tesfamariam, Brown et al. 1990); (Tesfamariam, Brown et al. 1991); (Tesfamariam and Cohen 1992); (Dorigo, Fraccarollo et al. 1997); (Wang, Xiong et al. 2005); (Qian, Wang et al. 2010); (Salheen, Panchapakesan et al. 2015), and with cultures of endothelial cells exposed to a medium with high concentrations of D-glucose (Kashiwagi, Asahina et al. 1996); (Asahina, Kashiwagi et al. 1995); (Kashiwagi, Asahina et al. 1994). Although an endothelial dysfunction has been observed in numerous animal models of diabetes and in diabetic patients, a clear mechanism explaining the direct effect of hyperglycemia on endothelial dysfunction has not been reported. It is due to the reason that the hyperglycemia is usually associated with insulin resistance, obesity and dyslipidemia in diabetic model. Therefore, it is hard to evaluate the effect of hyperglycemia without counting the contribution of the others factors. In our study, we have chosen an *ex vivo* model of HG (exposure isolated arteries to

physiological salt solution containing an elevated glucose) to evaluate the direct effect of HG on endothelial function in isolated arteries from the non-diabetic animals.

Using such of protocol, Tesfamariam reported that rings of rabbit aorta incubated for 6 h in 44 mM (790 mg/dL) D-glucose showed significantly decreased endothelium-dependent relaxations to ACh compared with rings incubated in D-glucose solutions of 5.5 mM (99 mg/dL) and 11 mM (198 mg/dL). Furthermore, relaxations in response to the endothelium-independent agent SNP was not different between rings exposed to control and elevated D-glucose, indicating that the hyperglycemia alters predominant the endothelium (Tesfamariam, Brown et al. 1990). Similarly, a Chinese group also found that a 6-h incubation of aortic rings of male Sprague-Dawley (SD) rats with HG (44 mmol/L) resulted in a significant inhibition of the endothelium-dependent relaxation to ACh, but had no effects on endothelium-independent relaxation to SNP. The E_{max} for ACh of rings exposed to 44 mM D-glucose was 43.7 ± 16.1% while the rings in control D-glucose (11 mM) reached 88.4 ± 12.3% (Wang, Xiong et al. 2005). In contrast, Qian and colleagues showed that after 0.5 or 1 h incubation of male Sprague-Dawley's aortic rings in HG (44 mM), ACh still evoked a normal relaxation. The E_{max} reached 80.5 ± 4.4% and 78.2 ± 3.6%; but after 2, 3 or 4 h exposure to HG, the E_{max} decreased to 70.1 ± 11%, 55 ± 5.6% and 51.2 ± 5.1%, respectively (Qian, Wang et al. 2006). Another study using SD rat aorta also observed that the endothelium-dependent relaxations to ACh in rat aortic rings exposed to HG (44 mM) for 4 h is markedly decreased to 44.8 ± 3.2% in comparison with rings exposed in normal D-glucose (11 mM) (E_{max} was 72.6 ± 6.0%) (Qian, Wang et al. 2010).

Besides, it has also been reported that the endothelium-dependent vasodilation to methacholine was attenuated by acute hyperglycemia in healthy subjects (Williams, Goldfine et al. 1998). The group of Williams has assessed the endothelium-dependent vasodilation through brachial artery infusion of methacholine chloride both before and during a 6 h period of local hyperglycemia (300 mg/dL) achieved by intra-arterial infusion of 50% dextrose. The forearm blood flow was determined by plethysmography. They observed that at the highest dose of methacholine (10 mg/min), forearm blood flow increased by only 13.3 ± 2.8 mL · min^{-1} · 100 mL^{-1} during hyperglycemia compared with 14.7 ± 1.5 mL · min^{-1} · 100 mL^{-1} during euglycemia.

Therefore, the aim of this chapter is to characterize the effect of HG on endothelium-dependent relaxation in male Wistar rat isolated arteries (including aorta, mesenteric, renal, carotid and femoral arteries) in order to determine the effect of gliflozin on HG-induced endothelial dysfunction.

3.2. Method

Male Wistar rats were sacrificed and arterial rings studied under isometric condition as described in 2.3 and 2.4. The rings were maintained at a resting tension of 2 g for aortic rings and 1 g for all other types of rings for at least a 45-min equilibration period. After checking the presence of a functional endothelium, rings were washed and prepared for another equilibration period before studing the effect of HG on the endothelium-dependent relaxation. Based on the studies by Tesfamariam et al and Qian et al, we exposed rat isolated arteries to HG for 4 h or 6 h. D-glucose was added at a concentration of 14 or 33 mM to give a total buffer concentration of 25 or 44 mM, respectively. In some experiments, polymixin B (10 µg/ml) was added to avoid the effect of contaminating lipopolysaccharides.

For long-term incubation studies, rings were incubated in Dulbecco's modified Eagle's medium (DMEM) media for 15 h and 22 h with additional D-glucose of 5 mM and 25 mM for normal and HG condition, respectively. In addition, DMEM media was supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 µg/ml) and polymixin B (10 µg/ml) in order to avoid contamination in long-term incubation period.

After the incubation time, rings were contracted with PE before the application of increasing concentrations of either ACh (10^{-10} – 10^{-6} M) to construct concentration-relaxation curves. Data were collected and analysed as described in 2.4.

3.3. Results

3.3.1. Effect of short term incubation in high D-glucose on the endothelial relaxation in the rat aorta

The effect of a short-term incubation with HG on PE-induced contraction and ACh-induced relaxation in aortic rings are shown in Table 8 and Figure 27. The incubation with 44 mM D-glucose for 4 and 6 h did not affect the relaxant response to ACh in aortic rings from male Wistar rat (n=6; Figure 27; Table 8). Neither the E_{max} for ACh nor PE-induced contraction level (Table 8) were affected by incubation with HG. Control data were obtained from rings incubated for a similar period in standard Krebs's buffer containing 11 mM D-glucose.

Table 8. ***Effect of HG on the contractile response to PE and endothelium-dependent relaxation to ACh in rat aortic rings following a short time incubation in HG***

Interventions	Time incubation	N	PE-induced contraction ± SEM (g)	E_{max} by ACh ± SEM (%)
Aorta				
Control	4 h	6	3.6 ± 0.5	93.4 ± 1.4
D-glucose (44 mM)			3.5 ± 0.4	84.5 ± 4.0
Control	6 h	6	3.5 ± 0.4	89.0 ± 2.4
D-glucose (44 mM)			2.8 ± 0.5	91.1 ± 4.4

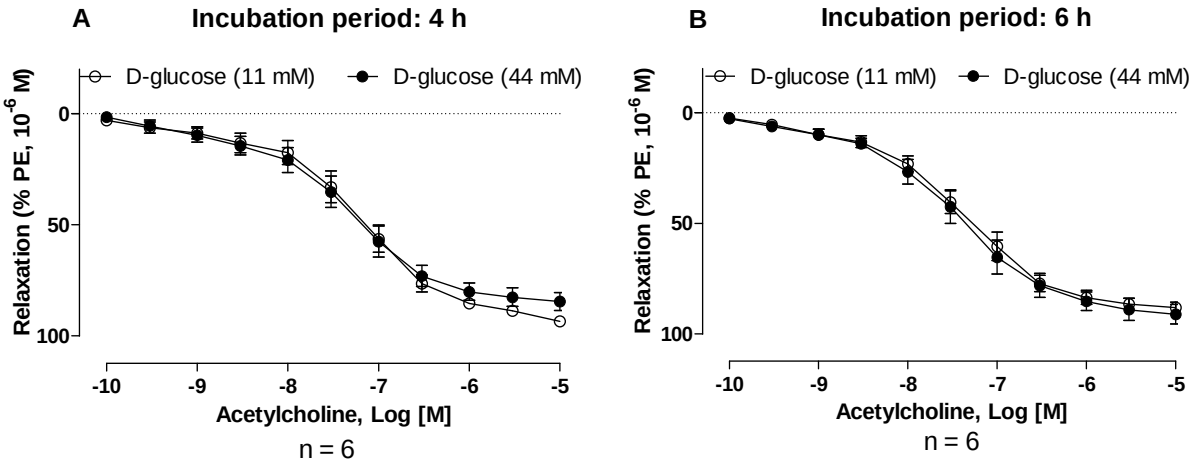

Figure 27. Effect of HG on the endothelium-dependent relaxation response to cumulative concentrations of ACh in aortic rings with additional pre-contracted with PE (10^{-6} M). Rings were exposed to 44 mM D-glucose for 4 h (A) and 6 h (B) in organ bath containing 10 ml Krebs' solution at 37°C bubbled with 95% O_2, 5% CO_2. The concentration-relaxation curves to ACh were unaffected by 44 mM D-glucose after a 4 or 6 h incubation period. Data are presented as the mean ± S.E.M of 6 different experiments.

Notably, although the incubation with 44 mM D-glucose for 6 h did not affect endothelium-dependent relaxation response to ACh, there was a non significant tendency of a reduced contraction induced by PE (10^{-6} M) in the HG group compared to the control group (PE-induced contraction of HG group: 2.83 ± 0.49 g *vs* control group 3.48 ± 0.44 g).

3.3.2. Effect of short term incubation in high D-glucose on the endothelial relaxation in other types of rat arteries

The effect of a short-term incubation with HG on PE-induced contraction level and ACh-induced relaxation in other types of rat arteries, including main mesenteric, renal, carotid and femoral arteries are shown in Table 9 and Figure 28. Incubation with 25 mM D-glucose for 3 h did not affect relaxation to ACh in all 4 types of arterial rings from male Wistar rast (n=5-9; Figure 28; Table 9). Neither the E_{max} for ACh nor the PE-induced contraction level (Table 9) were affected by incubation with HG. Time-matched control data were obtained with rings incubated for a similar period in standard Krebs's buffer containing 11 mM D-glucose.

Table 9. ***Effect of HG on the contractile to PE and endothelium-dependent relaxation to ACh in different types of arterial rings following a short-term incubation***

Interventions	Incubation time	N	PE-induced contraction ± SEM (g)	E_{max} by ACh ± SEM (%)
Mesenteric artery				
Control	3 h	5	1.2 ± 0.1	99.3 ± 0.5
D-glucose (25 mM)			1.1 ± 0.2	95.9 ± 2.5
Renal artery				
Control	3 h	9	1.9 ± 0.3	61.7 ± 9.1
D-glucose (25 mM)			1.70 ± 0.2	46.8 ± 8.8
Carotid artery				
Control	3 h	6	1.2 ± 0.1	87.5 ± 7.2
D-glucose (25 mM)			1.4 ± 0.2	83.3 ± 5.030
Femoral artery				
Control	3 h	9	1.1 ± 0.1	81.4 ± 4.5
D-glucose (25 mM)			1.2 ± 0.1	82.5 ± 4.3

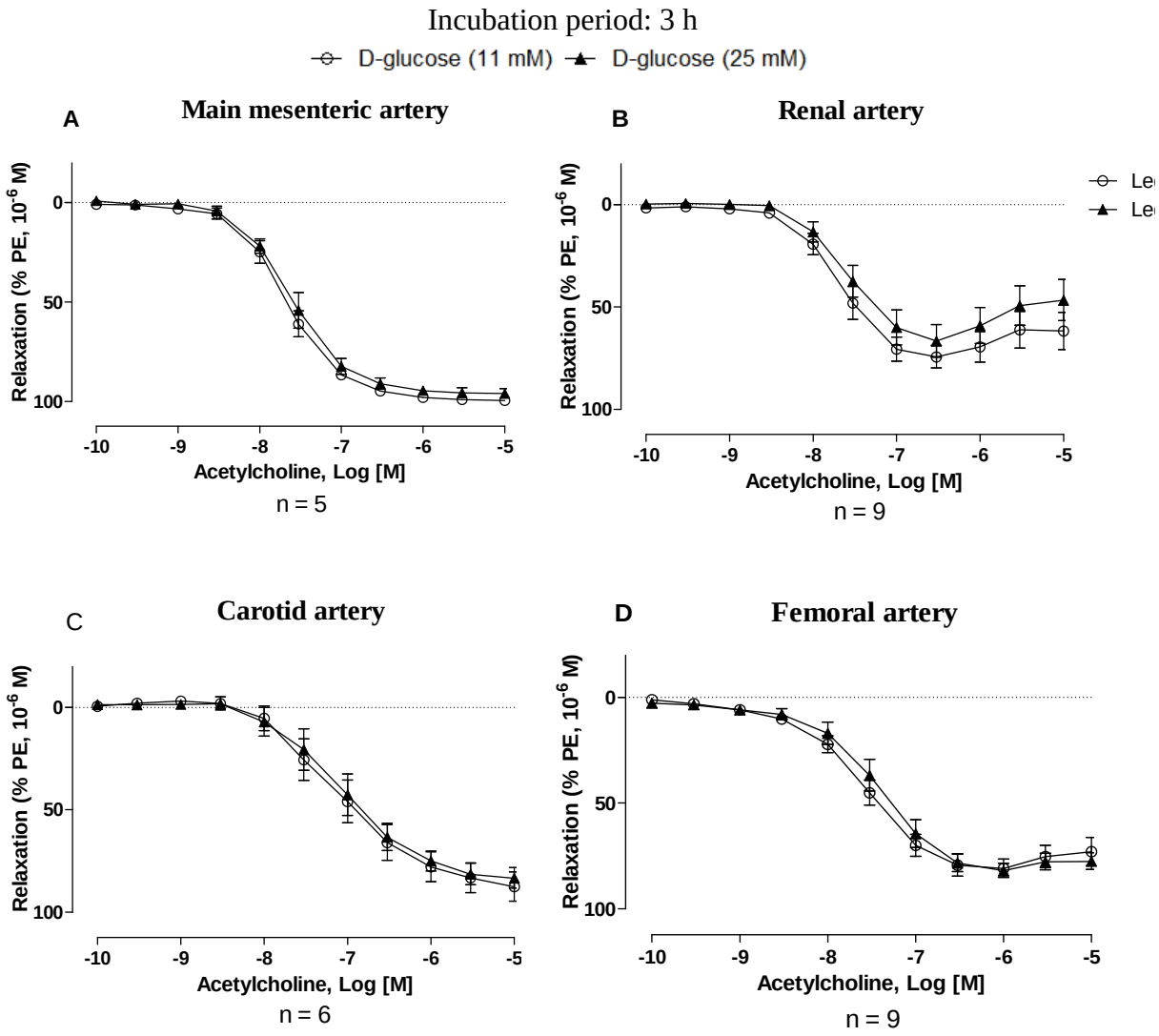

Figure 28. Effect of HG on the endothelium-dependent relaxation to cumulative concentrations of ACh in main mesenteric (A), renal (B), carotid (C) and femoral (D) arterial rings from male Wistar rat. Rings were exposed to 25 mM glucose for 3 h in organ bath containing 10 ml Krebs' solution at 37°C bubbled with 95% O_2, 5% CO_2. Concentration-relaxation curve to ACh was unaffected by 25 mM glucose after 3 h incubation period. Data are presented as the mean ± S.E.M of 5-9 different experiments.

The data in Table 9 and Figure 28 showed that the incubation with 25 mM D-glucose for 3 h did not affect both contraction level to PE and relaxation response to ACh in mesenteric, carotid and femoral artery rings. In addition, it should be noted that in renal arterial rings, there was a non significant trend to a decreased ACh-mediated endothelium-dependent relaxation. Although, the difference in relaxation response to ACh between renal arterial rings in control and HG groups was not significant, it has been observed that the

maximum relaxation to ACh in renal arterial rings exposed to HG fell from 61.68 ± 9.06 to 46.76 ± 8.83 *vs* control (n = 9, Table 9 and Figure 28, B).

3.3.3. Effect of long term incubation of rat aortic rings in high D-glucose on the endothelial relaxation

Since there was no induction of endothelial dysfunction in the previous experimental protocols using short-term incubation period of arterial rings in elevated glucose. The effect of a long-term incubation period (for either 15 h or 22 h) in DMEM media, which was supplemented by penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 µg/mL) and polymixin B (10 µg/ml) in order to avoid bacterial contamination was assessed.

The effect of HG for a long-term incubation on the endothelium-dependent relaxation response to ACh in aortic rings from male Wistar rats are shown in Table 10 and Figure 29. It should be noted that the contraction level to PE was depressed after long-term incubation period, possibly due to the induction of inducible NOS and COX expression by proinflammatory cytokines. In contrast, relaxations to ACh were not detected either in rings incubated with 25 mM D-glucose for 15 and 22 h and also not in the time-matched control rings.

Table 10. ***Effect of a long-term incubation with HG on the contractile to PE in rat aortic rings with endothelium***

Interventions	**Incubation period**	**PE-induced contraction (g)**		
		n = 1	**n = 2**	**n = 3**
Control	15 h	1.93	2.41	
D-glucose (25 mM)		0.66	2.06	
Control	22 h	0.84	0.98	0.88
D-glucose (25 mM)		0.59	0.75	0.63

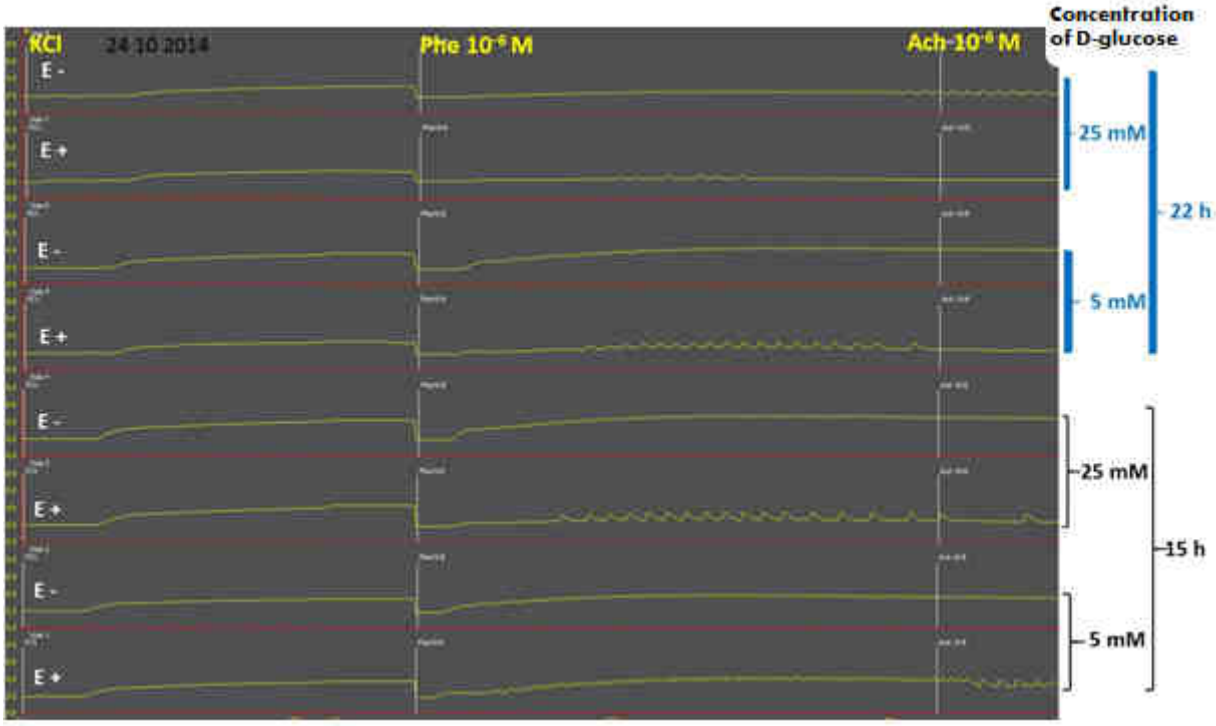

Figure 29. Effect of HG on the endothelium-dependent relaxation to ACh (10^{-6} M) in aortic rings pre-contracted with PE (10^{-6} M). Rings were exposed to 25 mM glucose for 22 and 15 h in DMEM media supplemented by penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 µg/mL) and polymixin B (10 µg/ml) in an incubator at 37°C supplied with 5% CO_2, before the organ chamber assay. Similar effects were observed in one (for 15 h incubation) and two (for 22 h incubation) additional experiments.

3.4. Discussion

The principal finding of this chapter was that the incubation with 44 mM D-glucose for 4 and 6 h did not affect the endothelium-dependent relaxation response to ACh in aortic rings with endothelium from male Wistar rat. Similar negative results were also observed in other types of artery (main mesenteric, renal, carotid and femoral arteries) following incubation with 25 mM D-glucose for 3 h. In addition, the long time incubation for 15 h and 22 h in 25 mM D-glucose reduced markedly the contractile response to PE, therefore it was difficult to assess the endothelium-dependent relaxation response to ACh induced by HG in aortic rings.

Although, the literature has shown that hyperglycemia appears to be a main cause of endothelial dysfunction, particularly, HG has been reported to attenuate the ACh-induced endothelium-dependent relaxation responses (Table 11).

Table 11. ***Experimental studies showing impaired endothelium-dependent relaxations in arterial rings exposed to HG.*** All the endothelium-dependent relaxations were significantly different between HG and control rings. Abbreviation: Wis: Wistar; SD: Sprague Dawley; NZW: New Zealand White rabbits; endothelium-dependent relaxation: EDR.

N°	Reference	Diabetes model	Vessel	D-glucose concentration time incubation	EDR response
1	***Tesfamariam et al. 1990***	Male NZW rabbits (2.2-2.5 kg)	Abdominal aorta	D-glucose 44 mM; 6 h	attenuated EDR to ACh, E_{max} for ACh reduced about 35% *vs* control (D-glucose 11 mM)
2	***Tesfamariam et al. 1991***	Male NZW rabbits (2.2-2.5 kg)	Abdominal aorta	D-glucose 22; 44 mM; 6 h	impaired EDR to ACh, E_{max} for ACh reduced about 15% and 35% in aortic rings in 22 and 44 mM D-glucose, respectively, *vs* control (D-glucose 11 mM)
3	***Tesfamariam & Cohen. 1992***	Male NZW rabbits (2.2-2.5 kg)	Abdominal aorta	D-glucose 44 mM; 6 h	impaired EDR to ACh, E_{max} for ACh reduced about 35% *vs* control (D-glucose 11 mM)
4	***Taylor et al. 1994***	Female Wis rats (220-250 g)	Third-order mesenteric arteries	D-glucose 45 mM; 2 h	impaired EDR to ACh, E_{max} for ACh reduced about 27% *vs* control (D-glucose 5.5 mM)
5	***Brouwers et al. 1994***	Male Wis rats (300–450 g)	Second-order mesenteric arteries	D-glucose 30 and 40 mM; 2 h	impaired EDR to ACh, E_{max} for ACh reduced about 17% *vs* control (D-glucose 5 mM)
6	***Zhang et al. 2004***	Male SD rats 9-month-old	Thoracic aorta;	D-glucose 44 mM; 5 h; D-glucose 20 mM in 7 h;	impaired EDR to ACh, E_{max} for ACh reduced about 20 % *vs* control (D-glucose 11 mM)

7	***Wang et al. 2005***	Male SD rats (180–200 g)	Thoracic aorta	D-glucose 44 mM; 6 h	impaired EDR to ACh, E_{max} for ACh reduced about 40% *vs* control (D-glucose 11 mM)
8	***Qian et al. 2006***	Male SD rats (average 250 g)	Thoracic aorta	D-glucose 44 mM; 0.5; 1; 2; 3; 4 h	impaired EDR to ACh, E_{max} for ACh reduced about 10, 15 and 20% for 2, 3 and 4 h incubation, respectively, *vs* control (D-glucose 11 mM)
9	***Goel et al. 2007***	SD Rat (225-250 g)	Thoracic aorta	D-glucose 46 mM; 3 h	impaired EDR to ACh, E_{max} for ACh reduced about 20% *vs* control (D-glucose 6 mM) in female rat; no significant difference in male rat
10	***Goel et al. 2008***	NZW rabbit (2.5-3 kg)	Thoracic aorta	D-glucose 46 mM; 3 h	impaired EDR to ACh, E_{max} for ACh reduced about 20% *vs* control (D-glucose 6 mM) in female rabbit; approximately 10% in male rabbit
11	***Ellis et al. 2008***	C57BL/6 mice; 8 weeks age;	Aorta; main mesenteric artery	D-glucose 30 mM; 20 h	impaired EDR to ACh (50 µM) about 20% *vs* control (D-glucose 11 mM) in aortic rings; impaired EDR to ACh about 10% *vs* control in mesenteric arterial rings.
12	***Qian et al. 2009***	Male SD rats (average 250 g)	Thoracic aorta	D-glucose 44 mM; 4 h	impaired EDR to ACh, E_{max} for ACh reduced about 28% *vs* control (D-glucose 11 mM)
13	***Dhar et al. 2010***	Male SD rats 11-week old	Thoracic aorta	D-glucose 15, 25 mM; 2 h	attenuated ACh-induced relaxation of rat aortic rings about 25 and 50% in 15 and 25 mM D-glucose, respectively *vs* control (D-glucose 5 mM)

14	***Sun et al. 2011***	SD rats of both genders (180–200 g)	Thoracic aorta	D-glucose 11, 25 mM; 24 h	impaired EDR to ACh, E_{max} for ACh reduced about 17% and 40% in aortic rings incubated in 11 and 25 mM D-glucose, respectively, *vs* control (D-glucose 5.5 mM)
15	***Rama Krishna Chaitanya and Chris 2013***	Male Wis rats of (average 300 ± 25 g)	Thoracic aorta	D-glucose 44 mM; 2 h	impaired EDR to ACh, E_{max} for ACh reduced about 21% *vs* control (D-glucose 5.5 mM)
16	***Salheen et al. 2015***	Male Wis rats (average 250 g)	Third order branch mesenteric artery	D-glucose 40 mM; 2 h	impaired EDR to ACh, the sensitivity to ACh pEC_{50} significantly reduced from 6.96 ± 0.10 to 6.36 ± 0.12 compared with control (D-glucose 11 mM)

Our results contrast with the previous results reported in the literature. Several experimental studies reported attenuated endothelium-dependent relaxation in both isolated arteries of diabetic models (Oyama, Kawasaki et al. 1986); (Pieper and Gross 1988); (Kamata, Miyata et al. 1989); (Abiru, Watanabe et al. 1990); (Cameron and Cotter 1992) and also in arteries of non-diabetic animals exposed to high concentrations of D-glucose for a certain period of time (Table 11). Even though these results differ to some extent between publications as shown in Table 11. Several possibilities exist to explain the discrepant results.

First of all, it has been documented that the gender difference can contribute differently to the impairment of endothelium-dependent vasodilation after acute exposure to HG both in aorta from rats and rabbits (Goel, Zhang et al. 2007); (Goel, Thor et al. 2008) (Table 11). Goel and colleagues have reported that the incubation of aortic rings taken from female rats for 3 h in Krebs containing 46 mM D-glucose attenuated ACh-induced relaxation compared to normal D-glucose (11 mM). The E_{max} to ACh was 77.8 ± 4.6% (before exposed to D-glucose) and 56.2 ± 4.3% after incubation with HG whereas that the sensitivity to ACh as assessed by $-\log EC_{50}$ (pEC_{50}) was similar for both cases. In contrast, ACh induced relaxation was not affected by HG in aortic rings taken from male rats (Goel, Zhang et al. 2007). These findings suggested that that a 3 h incubation with elevated level of D-glucose impairs ACh responses only in the female rat aortic rings. In another study by the same group, it has been observed that the extent of HG induced endothelium-dependent vasodilation impairment was significantly greater in female than male rabbit aorta: in male rabbit aorta E_{max} fell from 53.60 ± 3.42 to 45.84 ± 2.84% and in female rabbit aorta E_{max} fell from 57.8 ± 3.39 to 43.74 ± 3.74% in control and HG rings, respectively (Goel, Thor et al. 2008). It is consistent with the clinical data suggesting that a considerably higher diabetes-related relative risk for a major coronary heart disease event in diabetic women (hazard ratio 14.7) than in men (hazard ratio 3.8) (Juutilainen, Kortelainen et al. 2004). A meta-analysis of 37 studies of T2D and fatal coronary heart disease among a total of 447 064 patients has reported that the relative risk for fatal coronary heart disease associated with diabetes is 50% higher in women than in men (Huxley, Barzi et al. 2006). Although the exact mechanism of gender bias has not been elucidated. It has been suggested that in some cases this is due to the effects of sex hormones (Inada, Arai et al. 2007). Indeed, the effect of sex hormone can be contradictory in different animal models with, for example, male gonadectomy protecting against diabetes in some models such as non-insulin-dependent diabetes mellitus in a model rat (Otsuka-Long-Evans-Tokushima Fatty; (Shi, Mizuno et al. 1994)) while being ineffective or increasing

incidence in other models such as in transgenic mice expressing an insulin-ras hybrid gene (Efrat 1991).

Second of all, strain and species differences should also be carefully considered when choosing a model as background strains and different species have different susceptibilities to diabetes and treatments. Studies in the Table 11 have shown the different effect of HG on the endothelium-dependent relaxation response to ACh in SD and Wistar rats. The group of Dhar has found that the incubation with 25 mM D-glucose for 2 h significantly reduced about 50 % the maximum relaxation response to ACh in aortic rings from male SD rats (Dhar, Dhar et al. 2010). In contrast, in another study with the matched-age male Wistar rats: the same exposure time (2 h) to higher concentration of D-glucose (44 mM) significantly decreased ACh-induced relaxation, but with a smaller effect approximately 20% (E_{max} values of control: 85.01 ± 2.90 and HG: 64.68 ± 2.71%) (Rama Krishna Chaitanya and Chris 2013). The different degree of impairment of endothelium-dependent relaxation to ACh was shown in these two studies suggesting that the different types of animal strains contribute differently to the effect of HG on endothelial dysfunction. In addition, Goel and colleagues have reported that the incubation of in aortic rings from male SD rats 46 mM D-glucose for 3 h did not affect the endothelium-dependent relaxation to ACh, but did significantly reduced by about 10 % those in aortic rings from male New Zealand white rabbits (Goel, Zhang et al. 2007); (Goel, Thor et al. 2008). Therefore, the conflicting results may be due to the different strain and species of the animal models studied. Ideally, more than one species or strain should be investigated.

Third of all, the literature has also reported the fluctuation in the degree of impairment of endothelial relaxation induced by HG in different types of artery (Table 11). Data from Table 11 have shown the variation in the degree of impairment of endothelium-dependent relaxation to ACh in aortic rings exposed to elevated D-glucose. Some groups have found that the incubation in 44 mM D-glucose for 6 h induced about 20% impaired endothelial relaxation in male SD rat aortic rings (Qian, Wang et al. 2006); (Zhang, Yang et al. 2004), while others groups observed about a 40 % those (Wang, Xiong et al. 2005). With regard to the mesenteric artery, a similar variation was also observed in the literature. Taylor and colleagues have reported a significant impairment (about 30%) of the endothelium-dependent relaxation to ACh observed in third-order mesenteric arteries incubated in 45 mM D-glucose for 2 h in female Wistar rats (Taylor and Poston 1994). In male Wistar rats, it was observed only about 10% and 20% impairment of endothelial relaxation in third- and second-order mesenteric arteries, respectively, with the same exposure time (2 h) to 40 mM D-glucose (Salheen, Panchapakesan et al. 2015); (Brouwers, Niessen et al. 2010) (Table 11). This

discrepancy may be related to differences in the type of vessels studied and possibly also to differences in gender.

Regarding the other types of artery, unlike the aorta and mesenteric artery, the literature about endothelial relaxation response in renal, carotid and femoral arteries are limited. In contrast with our result, Tadros and collegueas have studied the effect of HG concentration and oxidative stress on transient receptor potential vanilloid 4 ion channel-mediated relaxation in rat carotid artery rings from adult male Wistar rats. They found that 2 h incubation in 40 mM D-glucose impaired the endothelium-dependent relaxation to ACh in carotid artery rings. The sensitivity pEC_{50} and E_{max} to ACh were both reduced significantly (pEC_{50}mannitol = 7.17 ± 0.14, pEC_{50}HG = 6.65 ± 0.15; E_{max} mannitol = 97 ± 4%, E_{max} HG = 78 ± 7%) (Tadros, 2015). It should be noted that the concentration of D-glucose (40 mM) used in this study is much higher than the one (25 mM) used in our protocol. It is possible that due to this reason we did not observe the same result as Tadros's group. Regarding the femoral artery, no information has been published. However, one group has studied the contractile responses to potassium chloride (KCl) of rat coronary and femoral arteries in solutions containing varying concentrations of D-glucose (2.75, 5.5 and 11 mM). When femoral arteries were superfused with 2.75 and 11 mM D-glucose and stimulated with KCl, the tension was significantly reduced when compared with those in normal glucose (5.5 mM): from 100% to 71.08% ± 8.51% in 2.75 mM and 79.30% ± 3.63% in 11 mM D-glucose. In contrast, when coronary arteries were superfused with 11 mM glucose the tension was significantly increased from 100% to 165.09% ± 5.97% *vs* control (5.5 mM) (n = 6) (Nava, Carbo et al. 2002). These findings indicate that the different type of artery could contribute differently to the PE-induced contraction level of arterial rings incubated in elevated D-glucose. Thus, the individual vascular beds exhibit differences in vascular reactivity therefore their relaxation and contraction responses may be affected differentially by hyperglycemia. Particularly, our results were not consistent with the previous findings. It might also be related to the different baseline of their health status of studied animals due to the diversity in animal age, food and water supplied, conditions of caging or housing system such as the temperature, humidity and cleanliness of stored room or the behavior of animals induced differently due to stress caused during the performance of experiments.

Forth of all, the duration of hyperglycemia might also affect the impairment of endothelial relaxation. It has been shown the triphasic response of endothelial function at different stages of diabetes induced in animal models by streptozotocin. In aortic rings contracted with noradrenaline, endothelium-dependent relaxation to acetycholine was

increased at 24 h following injection with streptozotocin compared with controls, kept stable after 1 and 2 weeks of disease, and then impaired from the 8th weeks of disease (Pieper 1999). Since *in vivo* studies have shown that the ACh-mediated endothelium-dependent relaxation is impaired in long-term period rather than in short-term period, similar tendency might be also found in *ex vivo* conditions. It may explain why we did not observe the HG-induced the impairment of endothelium-dependent relaxation response to ACh after 3 or 6 h incubation. Therefore, we changed the protocol with 15 and 22 h incubation aortic rings in 25 mM D-glucose. However, we were unable to establish the long-term incubation in HG model to induce endothelial dysfunction because of a depressed level of PE-induced contraction in aortic rings after long-term incubation. It is possible that this might be due to the induction of inducible NOS and COX expression by proinflammatory cytokines. In contrast, the group of Sun and Su has shown that the incubation of aortic rings with HG for 24 h resulted in a significant inhibition of endothelium-dependent relaxation, but had no effect on endothelium-independent relaxation. They reported that the exposure of aortic rings to elevated D-glucose (11 and 25 mM) for 24 h significantly attenuated relaxation responses to ACh in a dose-dependent manner compared with the control group: E_{max} to ACh was 73.18 ± 1.51% and 49.21 ± 1.65% in 11 mM and 25 mM groups *vs* 90.87 ± 1.97% in control group (5.5 mM), respectively (Su, Liu et al. 2008); (Sun, Su et al. 2011).

Regarding the results in human tissues, it has been demonstrated that the endothelium-dependent vasodilation is impaired by acute hyperglycemia not only in diabetic patients (Ceriello, Taboga et al. 2002); (Kawano, Motoyama et al. 1999); (Arora, Lidor et al. 2006) but also in healthy humans (Williams, Goldfine et al. 1998); (Beckman, Goldfine et al. 2001); (Grassi, Desideri et al. 2012). Although, the literature also showed the different effect of HG on endothelial relaxation in human blood vessels. The study of Williams et al has reported that after 6 h of local hyperglycemia (300 mg/dL) in brachial artery achieved by intra-arterial infusion of 50% dextrose, the forearm blood flow in response to a maximal concentration of methacholine chloride (10 mg/min) was significantly decreased from 14.7 ± 1.5 mL/min/100 mL to 13.3 ± 2.8 mL/min/100 mL (Williams, Goldfine et al. 1998). In contrast, the study of Houben and colleagues failed to demonstrate impaired NO–mediated vasodilation to ACh in the human forearm during a 24 h hyperglycemia (15 mM ≈ 270 mg/dL) induced by infusion of 5% glucose into the brachial artery (Houben, Schaper et al. 1996). In addition, another study also indicated that 3 h exposure to hyperglycemia (14 mM ≈ 252 mg/dL) induced by infusion of 33% glucose did not affect the vasodilatory response of coronary microcirculation in healthy subjects (Capaldo, Galderisi et al. 2005).

Overall, although *in vivo* studies in animals and human showed different effects of HG on the vasodilatory response, the *ex vivo* experiments in animals consistently showed that HG induced the impairment of ACh-mediated endothelium-dependent relaxation. Unexpectedly, our results did not confirm these previous findings. It might be connected to the following factors:

- The different species, strains, age and genders of animal used;
- The differences in health status of animal used, which might be affected by the conditions of housing system or the performance of experiments;
- The diversity in the type of the vessel used;
- The different incubation period of vessels in elevated D-glucose;
- The different material and facilities used.

CHAPTER 4

EFFECT OF PYROGALLOL ON ENDOTHELIAL FUNCTION IN RAT AORTA

4.1. Introduction

Although the literature has shown that the HG impaired the endothelium-dependent relaxation in isolated arterial rings of nondiabetic animals such as in rabbit aorta (Tesfamariam, Brown et al. 1990); (Tesfamariam, Brown et al. 1991); (Tesfamariam and Cohen 1992)), in guinea-pig aorta (Dorigo, Fraccarollo et al. 1997), in rat aorta (Wang, Xiong et al. 2005); (Qian, Wang et al. 2010) and in rat mesenteric artery (Salheen, Panchapakesan et al. 2015), our results in the previous chapter did not confirm this phenomenon. Since oxidative stress is regarded as the major contributor to the HG-induced impairment of endothelium-dependent relaxation response to ACh, we considered that is oxidative stress could lead to impaired endothelium-dependent relaxation response to ACh in isolated blood vessels. Oxidative stress is the overproduction of ROS including a major component - superoxide anion $O_2^{\bullet-}$. Superoxide anion, in turn, can elicit direct vasoconstriction and scavenge NO, the most potent endogenous vasodilator, to produce peroxynitrite ($OONO^{\bullet-}$) which oxidizes proteins, breaks DNA strands and reduces intracellular antioxidants such as glutathione and cysteine (Gryglewski, Palmer et al. 1986) resulting in the ED. Therefore, the direct inactivation of NO by $O_2^{\bullet-}$ is a key event in the decrease of the endothelium-derived NO bioactivity (Beckman and Koppenol 1996); (Thomas, Chen et al. 2003); (Tomasian, Keaney et al. 2000). In this chapter, we aimed to determine the oxidative stress induced the impairment of endothelium-dependent relaxation by examining the effect of pyrogallol - a generator of superoxide anion – on the endothelium-dependent relaxation in rat aorta.

Pyrogallol [$C_6H_6O_3$; 1,2,3-trihydroxybenzene or 1,2,3-benzenetriol] is known as a generator of $O_2^{\bullet-}$. This compound is often used to investigate the role of $O_2^{\bullet-}$ in biological systems. For example, pyrogallol has been shown to markedly increase the intracellular levels of $O_2^{\bullet-}$ in human glioma cells U87MG (Sawada, Nakashima et al. 2001), human brain microvascular endothelial cells (Li, Yang et al. 2014), human umbilical vein endothelial cells (Han, Moon et al. 2010) and in hypercholesterolemic rabbit aorta (Ohara, Peterson et al. 1993). It has been demonstrated that pyrogallol -induced oxidative stress leads to impaired endothelium-dependent relaxation response to ACh (Demirci, McKeown et al. 2008); (Yeh-Siang, Subramaniam et al. 2011). The group of Demirci has studied the effect of pyrogallol on vascular reactivity in aortic rings obtained from 12 - 14 weeks old male SD rats. Surprisingly,

they showed that the 20 min incubation with pyrogallol at low concentrations (10 nM - 1 μM) improved ACh-mediated relaxation (E_{max} to ACh was increased about 10% in rings exposed to pyrogallol *vs* control). Otherwise, during the same time exposure, pyrogallol at high concentrations (10 and 100 μM) attenuated endothelium-dependent relaxation to ACh (E_{max} to ACh was decreased by about 20 to 50% in rings exposed to pyrogallol *vs* control, respectively, (Demirci, McKeown et al. 2008). Demirci and his colleagues also showed that pretreatment with a $O_2^{\bullet-}$-scavenger, namely Tiron (4, 5-dihydroxy-1, 3-benzenedisulfonic acid, 1 μM) or mercapto-propionylglycine (MPG, 1 μM) for 20 min prevented the pyrogallol (10 μM)-induced impairment of endothelium-dependent relaxation response to ACh in aortic rings from SD rats. In addition, Yeh-Siang and colleagues also studied the ACh-mediated endothelium-dependent relaxation in the presence of pyrogallol in isolated aortic rings from 12-weeks old male SD rats. This group reported that the incubation with pyrogallol (10 μM) for 30 min reduced the maximal relaxation response to ACh (10^{-6} M) to about 30.3% compared to 93.4% in the control (Yeh-Siang, Subramaniam et al. 2011). However, the pEC_{50} of ACh-induced relaxation in the presence of pyrogallol did not significantly change compared to the control (6.30 ± 0.68 *vs* 7.18 ± 0.13, respectively). In contrast, another study showed that 20 min incubation with 30 μM pyrogallol significantly reduced the sensitivity to ACh (pEC_{50} fell from 7.66 ± 0.06 to 7.07 ± 0.06 in the control and pyrogallol group, respectively) in the third-order branch of the mesenteric artery from male Wistar rats (~ 8-weeks of age; (Salheen, Panchapakesan et al. 2015). However, the maximum relaxation to ACh was not affected: E_{max} (control) = 96 ± 1% and E_{max} (pyrogallol) = 95 ± 3%. Interestingly, these studies found that the addition of superoxide dismutase (SOD, 50 U/ml) as a scavenger of superoxide anion, improved pyrogallol -mediated impairment of endothelium-dependent relaxation both in the aorta and mesenteric arterial rings (Salheen, Panchapakesan et al. 2015); (Yeh-Siang, Subramaniam et al. 2011). Overall, these evidences have shown that pyrogallol induced the overproduction of $O_2^{\bullet-}$, which in turn leads to the impaired endothelium-dependent relaxation response to Ach.

For this reason, our goal in this chapter was to determine the effect of pyrogallol on the ACh-mediated endothelium-dependent relaxation in rat aorta to clarify whether oxidative stress can induce an impaired endothelium-dependent relaxation to ACh in our experimental model of isolated blood vessels. Then, as discussed above, the different baseline in health status of the studied animals due to certain factors, including gender, age, strain, species of studied animals, and conditions of caging or housing, might contribute to the lack of the effect of HG on endothelium-dependent relaxation to ACh in our *ex vivo* model. Probably, the effect

of HG on the ACh-mediated endothelium-dependent relaxation might be too small in our studied healthy rats. Therefore, it could not be detected. Thus, we have incubated aortic rings with HG in the presence of pyrogallol in order to evaluate the effect of HG on endothelium-dependent relaxation response in aortic rings with a partially impaired endothelial function by a low concentration of a $O_2^{\bullet -}$ generator – pyrogallol. Then we intended to evaluate the protective effect of a gliflozin on the impairment of endothelial relaxation induced by HG in the presence of a low concentration of pyrogallol.

4.2. Method

Male Wistar rats were sacrificed and aortic rings studied isometrically as described in sections 2.3 and 2.4. The aortic rings were maintained at a resting tension of 2 g for at least 45 min equilibration period. After checking the presence of a functional endothelium, rings were washed and prepared for another equilibration period before studying the effect of pyrogallol on the endothelium-dependent relaxation response. Rings were exposed to pyrogallol 10 and 30 µM for 30 min. After incubation with pyrogallol, cumulative concentration-contraction curves to PE were determined. Then, rings were again precontracted with PE (10^{-6} M) before application of increasing concentrations of ACh to construct a concentration-relaxation curve. In addition, the endothelium-independent relaxation was also studied by constructing cumulative concentration relaxation curve to SNP.

In addition, in order to evaluate the effect of HG on endothelium-dependent relaxation response to ACh in aortic rings with a lower healthy status induced by pyrogallol, we have co-incubated aortic rings for 30 min in 3 µM pyrogallol, then the 25 mM D-glucose was for 6 h in Krebs' solution in organ baths. After incubation, rings were again precontracted with PE (10^{-6} M) to evaluate the endothelium-dependent relaxation response by constructing cumulative concentration relaxation curve for ACh. We chose the low concentration of pyrogallol (3 µM) in co-incubation with HG to evaluate the effect of HG on the endothelial relaxation of aortic rings already slightly stimulated by pyrogallol. It is related to the reason that HG might induce oxidative stress in 6 h incubation, but the level of oxidative stress during this period is not enough to attenuate the endothelial relaxation in aortic rings of our highly healthy control rats. We hypothesized that the aortic rings with a lower healthy status induced by 3 µM pyrogallol might be more sensitively affected by HG. In addition, the low concentration of pyrogallol in co-incubation with HG can raise the level of oxidative stress, but not dominate the effect of HG. Moreover, in order to evaluate the protective effect of gliflozin, this inhibitor was added 30 min before incubation in HG. During incubation, polymixin B (10 µg/ml) was added to avoid contamination (an inhibitor of lipopolysaccharides).

4.3. Results

4.3.1. Effect of pyrogallol on contractile and endothelium-dependent relaxation responses

The results of the studies of evaluating the effect of pyrogallol on PE-induced contractile responses and relaxation responses to Ach and SNP are shown in Table 12 and Figure 30 and 31, respectively. The data showed that 30 min incubation with pyrogallol enhanced the contractile response to PE and attenuated the endothelium-dependent relaxation to ACh, but did not affect the endothelium-independent relaxation response to SNP in aortic rings from male Wistar rats.

Table 12. ***Effect of pyrogallol on PE-induced contractile responses and ACh-mediated endothelium-dependent relaxations in rat aortic rings (n = 5)***

Interventions	**Incubation time**	**PE-induced tone ± SEM (g)**	**E_{max} by ACh ± SEM (%)**	**pEC_{50} ± SEM**	**E_{max} by SNP ± SEM (%)**
Control	30 min	4.3 ± 0.6	91.5 ± 3.6	7.3 ± 0.1	101.8 ± 1.4
Pyr (10 µM)		5.1 ± 0.3 *	67.6 ± 3.9 *	7.0 ± 0.1	102.6 ± 1.3
Pyr (30 µM)		5.8 ± 0.5 *	34.3 ± 5.1 *	7.0 ± 0.2	100.5 ± 2.2
Gfz (10^{-7} M)	30 min	4.3 ± 0.2	92.9 ± 3.2	7.3 ± 0.1	104.1 ± 2.7
Pyr (10 µM) + Gfz (10^{-7} M)	30 min 30 min	5.1 ± 0.4 *	57.8 ± 12.2 *	7.2 ± 0.2	102 ± 1.9
Pyr (30 µM) + Gfz (10^{-7} M)	30 min 30 min	6.1 ± 0.5 *	39.8 ± 8.8 *	7.0 ± 0.2	100.6 ± 1.2
Pyr: pyrogallol; Gfz: gliflozin – SGLT2 inhibitor					

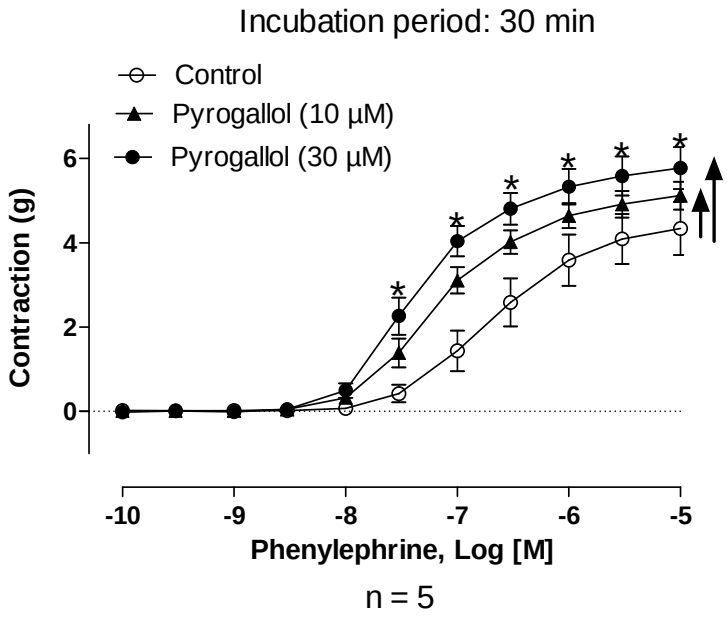

Figure 30. Effect of pyrogallol on concentration–dependent contractile curves for PE in rat aortic rings. Rings were incubated in 10 and 30 µM pyrogallol for 30 min before the addition of PE in organ baths containing 10 ml Krebs' solution at 37°C bubbled with 95% O_2, 5% CO_2. Contractile responses are expressed as the developed tension in grams (g) to PE. Values are the mean ± SEM of 5 different experiments. *$p < 0.05$ indicates a significant difference *vs* control.

As is shown in Figure 30, aortic rings exposed to pyrogallol (10 and 30 µM) for 30 min showed a significant higher contractile response from 3.10^{-8} to 10^{-5} M to phenylephrine compared with control rings (Figure 30, Table 12). Moreover, incubation with both 10 and 30 µM pyrogallol for 30 min significantly attenuated ACh-induced endothelium-dependent relaxation as compared with the control group (Figure 31 A; Table 12). Although, as the data is showing in the table 12, the pEC_{50} of ACh-induced relaxation in the presence of 10 and 30 µM pyrogallol were not significantly different in comparison with the control. In constrast, the E_{max} to ACh were significantly decreased in the presence of pyrogallol (E_{max} reduced about one and a half and three times in the rings exposed to 10 and 30 µM Pyr, respectively, compared to control rings (Table 12)). Conversely, pyrogallol completely did not affect the endothelium-independent relaxant responses to SNP (E_{max} by SNP were unaltered in the presence of pyrogallol *vs* control, data are shown in Figure 31 B, Table 12).

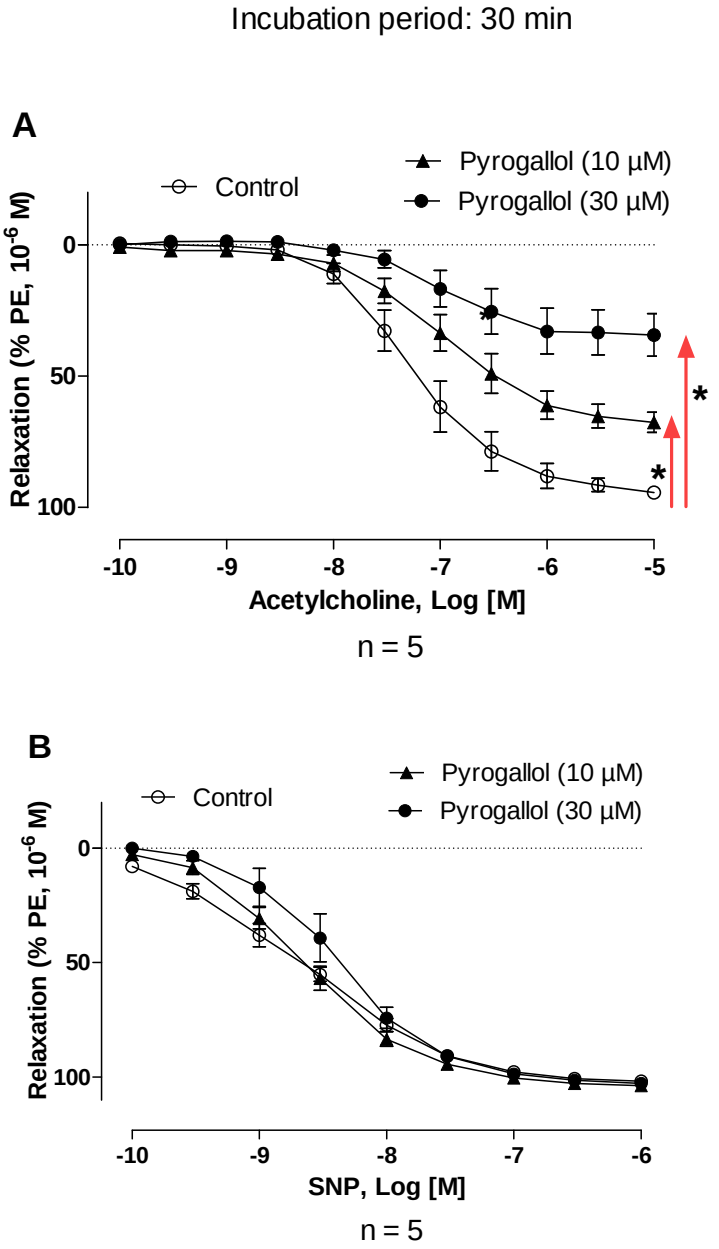

Figure 31. Effect of pyrogallol on endothelium-dependent relaxation response to cumulative concentrations of ACh (A) and SNP (B) in aortic rings contracted with PE (10^{-6} M). Rings were exposed to 10 and 30 µM pyrogallol for 30 min in organ bath containing 10 ml Krebs' solution at 37°C bubbled with 95% O_2, 5% CO_2. Data are presented as the mean ± S.E.M of 5 different experiments. *$p < 0.05$ indicates a significant difference versus control.

4.3.2. Effect of high D-glucose on endothelial function in the presence of low concentration of pyrogallol in the aorta

In another series of experiments, rat aortic rings were incubated separately with elevated glucose (25 mM) and low concentration of pyrogallol (3 μM), and their combination. Data obtained in these experiments are summarized in Table 13, and the results of studying the effect of HG on the endothelial relaxation response to ACh in the presence of pyrogallol are showed in Figure 32.

Table 13. ***Effect of D-glucose on contractile responses and endothelium-dependent relaxation to ACh in rat aortic rings (n = 6)***

Interventions	**Incubation time**	**PE-induced tone ± SEM (g)**	**E_{max} (%) by ACh ± SEM**	**pEC_{50} ± SEM**
Control	6 h	3.3 ± 0.4	85.7 ± 3.6	7.6 ± 0.1
D-Glucose (25 mM)		3.3 ± 0.4	87.3 ± 1.9	7.7 ± 0.1
Pyr (3 μM)	30 min	5.1 ± 0.6 *	75.0 ± 7.2 *	6.6 ± 0.1
Pyr (3 μM) + D-glucose (25 mM)	30 min + 6 h	5.3 ± 0.4 *	72.1 ± 6.4 *	6.6 ± 0.1
Pyr: Pyrogallol.				

The data presented in Table 13 and Figure 32 show that either the incubation with pyrogallol (3 μM) alone or in the combination with 25 mM D-glucose for 6 h significantly enhanced the contractile response to PE and attenuated the endothelium-dependent relaxation response to ACh in aortic rings. The PE-induced contractile response was significantly increased from 3.31 ± 0.41 g in the control rings to 5.07 ± 0.55 and 5.31 ± 0.36 g in rings incubated in 3 μM pyrogallol with and without 25 mM D-glucose, respectively (Figure 32; Table 13). Moreover, the endothelium-dependent relaxation response to ACh was also significantly decreased after 30 min incubation either in 3 μM pyrogallol alone or in combination with 25 mM D-glucose: the E_{max} to ACh was 74.96 ± 7.18 % and 72.07 ± 6.39, respectively, compared to 85.65 ± 3.58 % in the control.

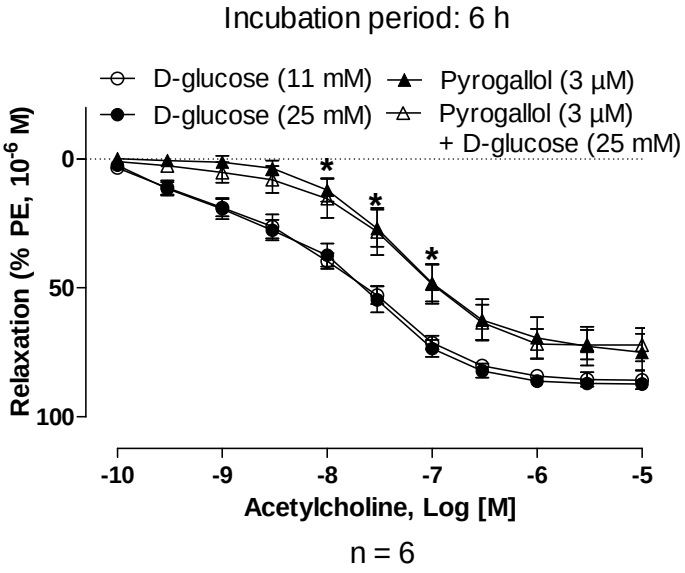

Figure 32. Effect of HG on the endothelium-dependent relaxation responses in aortic rings to cumulative concentrations of ACh in the absence and presence of pyrogallol (3 µM). Rings were pre-incubated in 3 µM pyrogallol for 30 min before exposed to 25 mM D-glucose for 6 h in organ bath in the presence of polymixin B (10 µg/ml). Relaxation responses are expressed as the percentage of the 10^{-5} M PE-induced contraction. Values are the mean ± SEM of 5 different experiments. *$p < 0.05$ indicates a significant difference versus control.

However, incubation with 25 mM D-glucose for 6 h in the presence of pyrogallol (3 µM) did not affected the endothelium-dependent relaxations evoked by ACh, nor the PE-induced tone in aortic rings in comparison with the rings exposed to pyrogallol alone (n = 6; Figure 32; Table 13). In other word, HG did not induce any alteration of the contractile response to PE and ACh-mediated endothelium-dependent relaxation response even in aortic rings with a lower healthy status induced by a low concentration of pyrogallol.

4.4. Discussion

The principal finding of this chapter was that preincubation with pyrogallol (10 and 30 µM) for 30 min markedly decreased endothelium-dependent relaxation response to ACh accompanying the increase of contractile response to PE in the isolated rat thoracic aorta. It also should be noted that pyrogallol did not alter the endothelium-independent relaxation response to SNP. Our results are consistent with previous ones in the literature, which indicate that $O_2^{\bullet-}$ from the auto-oxidation of pyrogallol elevates the level of oxidative stress resulting in the impared endothelium-dependent relaxation responses to ACh in rat thoracic aorta (Jin, Qian et al. 2009); (Demirci, McKeown et al. 2008); (Qian, Fu et al. 2012); (Yeh-Siang, Subramaniam et al. 2011) and rat mesenteric artery (Salheen, Panchapakesan et al. 2015). Returning to the question posed in the Introduction, the present data confirm the important

role of oxidative stress in the induction of the impaired endothelium-dependent relaxation in isolated aortic rings.

Besides, in another series of experiments, aortic rings were exposed for 6 h in combination with elevated glucose and pyrogallol to evaluate the effect of HG on endothelial function of aortic rings with a lower healthy status induced by pyrogallol. Inspite of the fact that 3 μM pyrogallol did slightly impair the endothelium-dependent relaxation response to ACh, aortic rings co-incubated with 25 mM D-glucose and 3 μM pyrogallol for 6 h did not show any difference in both PE-induced contractile and ACh-mediated endothelium-dependent relaxation responses compared to the rings exposed to pyrogallol alone. Thus, similarly in chapter 3, our result indicate that HG did affect neither contractile nor relaxation responses even in aortic rings with a lower healthy status induced by pyrogallol.

The lack of effect of HG in endothelium-dependent relaxation response in aortic rings might be related to the fact that the combination of HG and pyrogallol induced the enhanced formation of H_2O_2, which acts as an EDHF, and hence would compensate for the loss of NO. As HG and pyrogallol are associated with the increased formation of ROS in endothelial cells, a number of studies have shown the enhanced production of $O_2^{\bullet-}$ induced by HG (Peng, Ma et al. 2013); (Zhou, Su et al. 2015); (Kageyama, Yokoo et al. 2011) and pyrogallol (He, Ru et al. 2012); (Qian, Fu et al. 2012). Besides, several *in vitro* studies have also shown the increased formation of another type or ROS – hydrogen peroxide (H_2O_2) induced by HG (Peiro, Lafuente et al. 2001); (Ho, Liu et al. 2000; Qian, Fu et al. 2012). It has been reported that the HG–induced intracellular H_2O_2 formation was significantly increased in HUVECs exposed to elevated D-glucose (19 or 33 mM) for 24 to 48 h compared to the control (5.5 mM D-glucose) (Ho, Liu et al. 2000; Qian, Fu et al. 2012). Similarly, the formation H_2O_2 induced by HG was also found markedly increased after 24 h of incubation with 22 mM D-glucose medium compared to medium with low glucose (5.5 mM D-glucose) in human aortic smooth muscle cells (Peiro, Lafuente et al. 2001). Although, to our knowledge, there was no evidence about the formation of H_2O_2 induced by hyperglycemia in short-term period in *ex vivo* models. We postulated that the exposure with HG and pyrogallol might similarly potentiate the H_2O_2 production in endothelial cells of rat aortic rings for short time incubation as in long time incubation. Since, H_2O_2 has been reported to act like EDHF, i.e. as a freely transferable hyperpolarizing factor of vascular SMC (Shimokawa and Matoba 2004); (Graier and Hecker 2008), (Edwards, Li et al. 2008), it has been found H_2O_2-induced the endothelium-dependent relaxation in rat aorta (Yang, Zhang et al. 1999), (Mian and Martin), in rabbit aorta (Zembowicz, Hatchett et al. 1993) and rabbit mesenteric artery (Itoh, Kajikuri et al. 2003).

Similarly, the relaxant activity of H_2O_2 also has been found in diabetic animal models, such as in aorta from 10–12 weeks-STZ diabetic rats (Pieper and Gross 1988) and in coronary arterioles from db/db (Park, Capobianco et al. 2008). Based on these results, we suggest that H_2O_2 may act like an EDHF to compensate with the effect of HG to attenuate the ACh-mediated relaxation. Therefore, there was no impaired endothelium-dependent relaxation response to ACh observed in our results. Consistently, Matoba's group has shown that catalase (CAT), which dismutates H_2O_2 to form water and oxygen, inhibited ACh-induced endothelium-dependent relaxation and hyperpolarization attributed to EDHF in mouse small mesenteric artery (Matoba, Shimokawa et al. 2000). These findings suggested that the reduced NO bioavailability in diabetes may unmask the effect of EDHF as a back-up mechanism because under control conditions NO suppresss the action of EDHF (Cai 2005); (Shi, Ku et al. 2006). Moreover, CAT, when inactivated at its peroxide-binding site by aminotriazole, lost its inhibitory effect on the EDHF-mediated relaxation. These results indicate that the inhibitory effect of CAT on the EDHF-induced relaxation and hyperpolarization were indeed mediated by its enzymatic effect on H_2O_2 (Matoba, Shimokawa et al. 2000). It also has been suggested that H_2O_2 may fully compensate for the loss of NO in arterioles isolated from patients with coronary artery disease via an EDHF-type mechanism (Phillips, Hatoum et al. 2007). Taken together, the enhanced formation of H_2O_2, which may compensate for NO via its ability to act as an EDHF, might possibly be the key explanation for the lack of a relationship between HG and the impairment of ACh-mediated endothelium-dependent relaxation in our model. Thus, further studies are required to use CAT and PEG-CAT to block the effect of the extra- and intracellular of H_2O_2 on relaxation responses in order to evaluate the effect of HG on NO-mediated endothelium-dependent relaxation in rat isolated vessels.

In addition, the short incubation time of the artery in elevated D-glucose may also be a possibility explanation of the lack of the effect. As it has been revealed that HG incubation time-dependently stimulated ROS production in endothelial cells (Peng, Ma et al. 2013), the short time 6 h exposure in 25 mM D-glucose might not raise a sufficient hight level of oxidative stress to decline the NO bioavailability in aortic rings, consequently no alteration of NO-mediated endothelium-dependent relaxation are observed. Unfortunately, we did not have the opportunity to perform the experiment to measure the formation of ROS or to examine the expression level and activity of eNOS in the HG conditions as a function of time in isolated blood vessels. However, several *in vitro* studies have reported the time-dependent effect of HG on the formation of ROS, activity and expresson level of eNOS. It has been reported that

an upregulation of ROS was detectable after 6 h incubation in 25 mM D-glucose condition, however, the ROS accumulation was significantly increased after incubation for 12 and 24 h in cardiac microvascular endothelial cells (Peng, Ma et al. 2013). In addition, the group of Busik has shown no increase in endogenous ROS in human retinal endothelial cells were exposed to intermediate (15 mM) and high (25 mM) D-glucose for up to 96 h (Busik, Mohr et al. 2008). Besides, in another study, with the use of in vitro cultures of human umbilical vein endothelial cells, the interaction between eNOS and Hsp-90 has been shown to be impaired by long-term HG (33 mM) incubation (for a period over 24 h) (Lin, Lin et al. 2005). However, during early hours of HG incubation (<24 h), eNOS expression was found to be increased along with an increased interaction with heat shock protein 90, which is known as protein regulates eNOS phosphorylation at Serine 1177. Consequently, the NO generated during this early phase has been found increased. Similarly, the group of Ho has shown that eNOS protein expression was up-regulated by high D-glucose (33 mM) exposure for 2-6 h and gradually reduced after longer exposure times in cultured human umbilical vein endothelial cells (Ho, Liu et al. 1999). Besed on these observations, it is possible that 6 h incubation with HG might not be sufficient to induce the impairment of NO-mediated relaxation response in aortic rings. Consequently, we suggest that a longer period incubation (for example, 24 h) with HG may allow us to elucidate the NO-mediated relaxation response in HG conditions in isolated blood vessels. However, as reported in Chapter 3, the incubation with 25 mM D-glucose for 24 h showed a significant depression of the contractile response induced by PE in aortic rings most likely due to the induction of inducible NOS and COX expression by proinflammatory cytokines. Therefore, the experimental condition need to be changed to allow the study of both contractile and relaxation responses after a long time incubation in HG conditions.

Overall, this chapter has demonstrated the oxidative stress induced by pyrogallol significantly attenuated the endothelium-dependent relaxation responses to ACh and markedly enhanced the PE-contractile responses in the aortic rings. Besides, it has been also shown that HG did affect neither contractile nor relaxation responses even in aortic rings with a lower healthy status induced by pyrogallol. The contribution of other relaxant factors induced during the incubation time such as H_2O_2 and the short incubation time of the artery in elevated D-glucose may be the possible explanation of the lack of the effect of HG.

CHAPTER 5

EFFECT OF HIGH D-GLUCOSE ON ENDOTHELIAL FUNCTION IN THE PORCINE CORONARY ARTERY

5.1. Introduction

In the previous chapters, our results have shown that HG did not alter the relaxation response to ACh in several types of rat vascular beds (aorta, main mesenteric, renal, carotid and femoral arteries) with both normal and low healthy status. As mentioned in the Chapter 4, the short incubation time (6 h) of the different arteries in elevated D-glucose may be the possible explanation the lack of the effect. Moreover, we failed to examine the endothelium-dependent relaxation response in rat aorta incubated in HG for 24 h due to the depression of the PE-induced contractile response (more details in section 3.3.6). We now have focused on studying the effect of HG on endothelium-dependent relaxation response in porcine coronary artery (PCA). PCAs are characterized by a stiffer intima-media layer with elongated SMC and collagen fibers between the cells compared to rat aorta (Zoumi, Lu et al. 2004). Thus, PCAs may avoid the rapid deterioration of endothelial and smooth muscle cells during long period incubation. Consequently, PCA might exhibit different behavior after long time incubation with HG in comparison with rat arteries. In fact, isolated PCAs have been frequently used for 24 h incubation and shown a normal contractile and relaxation responses in a number of *ex vivo* studies (Ramaswami, Chai et al. 2004); (Bauersachs, Christ et al. 2002); (Shibano and Vanhoutte 1993); (Paladugu, Fu et al. 2003).

In addition, diabetes mellitus predisposes patients to premature atherosclerotic coronary artery disease (CAD), the leading cause of mortality among diabetic patients (Gu, Cowie et al. 1999). Therefore, studying the effect of HG on endothelial function of the coronary arteries plays an important role in contributing to explain the development of CAD in diabetic patients. It has been reported that endothelial cells from different vascular beds exhibit structural differences and may be affected differentially by hyperglycemia (Sobrevia and Mann 1997). Since the porcine heart closely resembles the human heart from the point of view of size, physiology and anatomy (Hearse and Sutherland 2000), PCAs are widely used to mimic human coronary artery in cardiovascular research (Zaragoza, Gomez-Guerrero et al. 2011); (Suzuki, Yeung et al. 2011). For these reasons, in this chapter, we choose PCA as study tissue to elucidate the effect of long time incubation with HG on relaxation response in PCA rings.

5.2. Method

The porcine coronary artery (PCA) rings were dissected and studied isometrically as described in sections 2.3 and 2.4. The rings were maintained at a resting tension of 5 g to equilibrate for at least 60 min. After checking the presence of a functional endothelium, each ring was washed and re-equilibrated for 45 min. PCA rings were exposed to elevated D-glucose concentrations (44 mM) for 6 h at 37°C in RPMI 1640 media supplemented by penicillin (100 U/ml), streptomycin (100 U/ml) and fungizone (250 µg/ml). In another series of experiment, PCA rings were exposed to HG concentrations (25, 30 and 44 mM) for 24 h also in RPMI 1640 media at 37°C. RPMI 1640, a less nutrient-rich medium, contains less calcium and more phosphate in comparison to DMEM media. Therefore, RPMI 1640 media can decrease release of pro-inflammatory factors, which might be the reasons for the depression of PE-induced contractile response in rat aorta after long period incubation (as reported in Chapter 3). In addition, polymixin B (10 µg/ml) - an inhibitor of lipopolysaccharides, was also added to the media in order to avoid the contamination in long time incubation. In comparison, we exposed PCA rings to HG (44 mM) for 6h in organ bath containing 10 ml Krebs' solution at 37°C bubbled with 95% O_2, 5% CO_2.

In addition, in order to assess the involvement of SGLT1/2 in glucose transport in endothelial cells under HG condition, gliflozin was added to the media 30 min before adding D-glucose. After incubation, rings were again contracted with U46199 before the application of increasing concentrations of BK to construct concentration-response curves. Then rings were again contracted with U46199 before the induction of endothelium-independent relaxations to SNP.

5.3. Results

5.3.1. Effect of short term incubation in high D-glucose

The results of studying the effect of short time incubation (6 h) in 44 mM D-glucose on the endothelial relaxation response in PCA rings are shown in Table 14 and demonstrated in Figure 33. Incubation with 44 mM D-glucose for 6 h did not affect relaxant responses to bradykinin (BK) in PCA rings (n = 2). Control data were obtained from preparations incubated for similar periods in RPMI 1640 media containing 11 mM D-glucose. Neither the E_{max} for BK nor U46199-induced contractile responses (Table 14) were affected by elevated D-glucose.

Table 14. ***Effect of D-glucose on contractile and endothelial relaxation responses in porcine coronary artery***

Interventions	PE-induced tone ± SEM (g)	E_{max} (%) by BK ± SEM	pEC_{50} ± SEM	E_{max} (%) by SNP ± SEM
Short time incubation (6 h), n = 2				
Control	15.7 ± 1.1	108.1 ± 1.3		
Glucose (44 mM)	20.9 ± 0.4	104.4 ± 4.6		
Long time incubation (24 h), n = 4				
Control	20.8 ± 3.4	95.8 ± 3.0	8.7 ± 0.05	99.5 ± 3.6
Glucose (25 mM)	19.2 ± 2.8	95.0 ± 3.3	8.6 ± 0.04	
Glucose (30 mM)	18.6 ± 2.7	92.5 ± 5.2	8.6 ± 0.04	
Glucose (44 mM)	16.8 ± 3.0	95.5 ± 3.0	8.3 ± 0.04	103.1 ± 7.1
Gfz (10^{-7} M)	15.4 ± 1.8	97.4 ± 2.4	8.8 ± 0.1	102.9 ± 4.4
Glucose (44 mM) + Gfz (10^{-7} M)	14.5 ± 2.1	97.9 ± 1.4	8.5 ± 0.1	100.6 ± 3.3

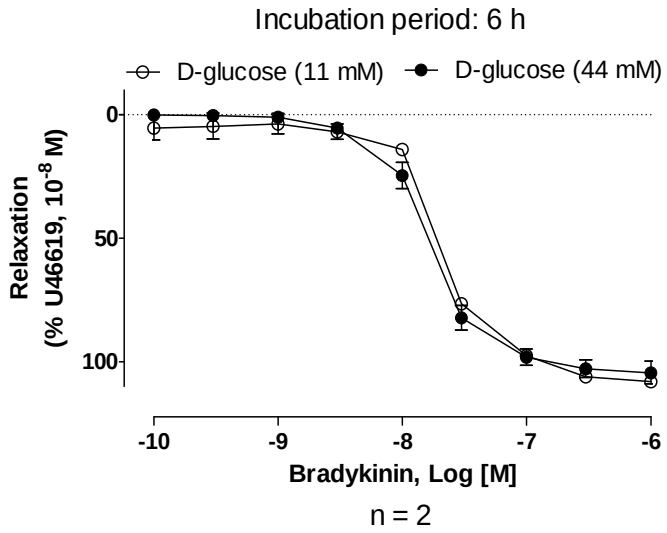

Figure 33. The short-term effect of HG on the endothelium-dependent relaxation response to cumulative concentrations of BK in PCA rings pre-contracted with U46619 (10^{-8} M). Rings were exposed to 44 mM D-glucose for 6 h in RPMI 1640 media supplemented by penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 µg/mL). Data are presented as the mean ± S.E.M of 2 different experiments.

5.3.2. Effect of long term incubation in high D-glucose

The dose-dependent effect of the incubation with HG for 24 h on the endothelium-dependent relaxation response to BK in PCA rings are shown in Table 14, Figure 34. It was observed that only 24 h incubation with 44 mM D-glucose attenuated slightly endothelium-dependent relaxation to BK, while PCA rings exposed to the lower concentrations of D-glucose (25 mM and 30 mM) did not show this effect. The 24 h incubation with 44 mM D-glucose shifted the concentration-relaxation curve to BK to the right compared to the curve of the control group (pEC_{50} from 8.7 ± 0.05 to 8.3 ± 0.04; n = 4) in PCA rings. It should be noted that the endothelium-dependent relaxation responses to BK in control and HG condition are significantly different only at BK's concentration of 3.10^{-9} M: the control rings reached about 72.6 ± 7.1% of relaxation while rings incubated in elevated D-glucose (44 mM) relaxed only approximately 36.9 ± 8.6%. However, the maximum relaxation to BK was not affected by HG (E_{max} = 95.8 ± 3.0% and 95.5 ± 3.0% in rings exposed in normal and 44 mM D-glucose, respectively).

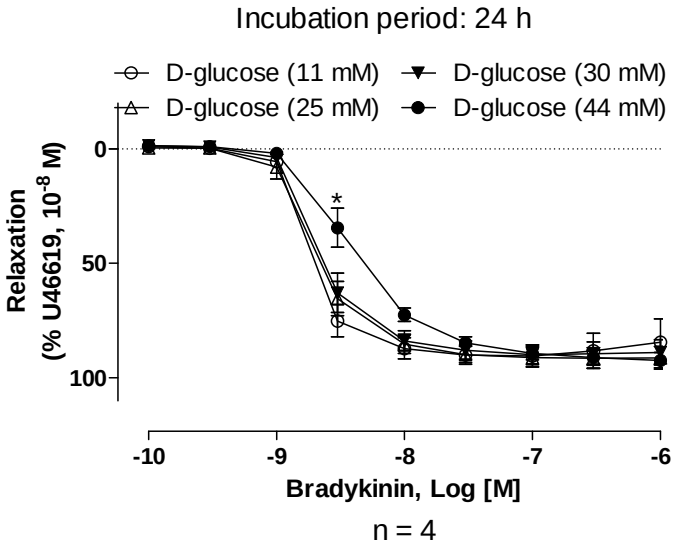

Figure 34. The long-term effect of HG on the endothelium-dependent relaxation response to cumulative concentrations of BK in PCA rings pre-contracted with U46619 (10^{-8} M). Rings were exposed to 25, 30, and 44 mM D-glucose for 24 h in RPMI 1640 media supplemented by penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 µg/mL) and polymixin B (10 µg/ml). Data are presented as the mean ± S.E.M of 4 different experiments. *$p < 0.05$ indicates a significant difference versus control.

It was also noted that although 24 h incubation in 44 mM D-glucose shifted the endothelial relaxation response to BK to the right to some extend, the endothelium-independent relaxant responses to SNP was not affected by long time incubation in HG (Figure 35).

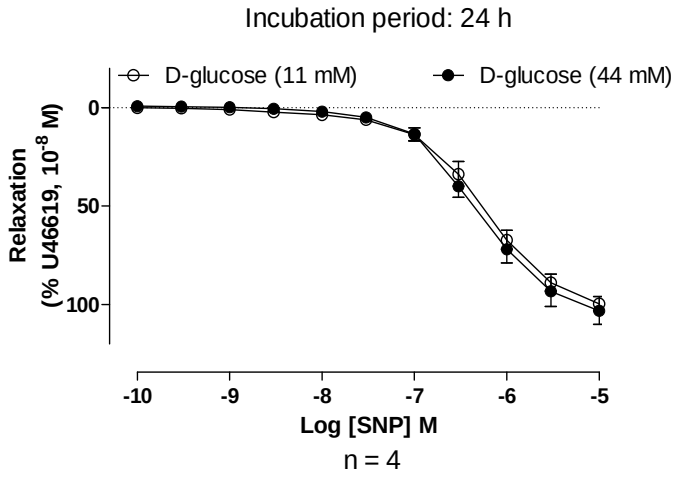

Figure 35. The long-term effect of HG on the endothelium-independent relaxation response to SNP in PCA rings with endothelium pre-contracted with U46619 (10^{-8} M). Rings were exposed to 25, 30, and 44 mM D-glucose for 24 h in RPMI 1640 media supplemented by penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 µg/mL) and polymixin B (10 µg/ml). Data are presented as the mean ± S.E.M of 4 different experiments.

The small HG-induced impairment in relaxation response to BK in PCA rings was not prevented by 30 min incubation with 10^{-7} M gliflozin (Figure 36). It might be because the long-term effect of HG on relaxation response in PCA rings was too small; therefore, the protective effect of gliflozin could not be detected.

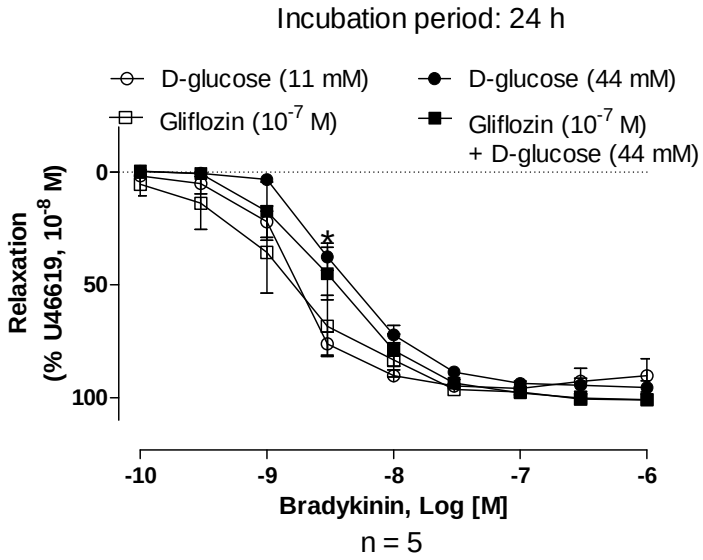

Figure 36. Effect of a gliflozin on the impaired endothelium-dependent relaxation induced by HG in PCA rings. The cumulative concentrations relaxation curves to BK in PCA rings pre-contracted with U46619 (10^{-8} M). Rings were exposed to 44 mM D-glucose for 24 h in RPMI 1640 media supplemented by penicillin (100 U/mL), streptomycin (100 U/mL), fungizone

(250 µg/mL) and polymixin B (10 µg/ml). Gliflozin was added to the media 30 min before adding D-glucose**.** Data are presented as the mean ± S.E.M of 5 different experiments.

5.4. Discussion

The principal finding of this chapter was that the 6 h incubation in 44 mM D-glucose concentrations did not cause endothelial dysfunction in isolated PCA rings, however, the 24 h incubation with 44 mM D-glucose shifted to some extent the concentration-relaxation curve of BK to the right compared to the control rings. It is worthwhile noting that the similar tendency was not observed in 24 h incubation with the lower concentrations of glucose (25 and 30 mM). In addition, endothelium-independent relaxation response to SNP was not affected by the long time incubation with HG in PCA rings. Our findings suggested that the shift in the relaxation response to BK was endothelium-dependent, which occurred only at the very high concentration of glucose (44 mM).

The result of effect of 6 h incubation with 44 mM D-glucose in PCA rings presented in Figure 33 was similar to the results in other vascular beds from male Wistar rats as reported in Chapter 3. As the previous chapters, the short time incubation (6 h) may be a possible explanation of the lack of effect in PCA as well as in rat arteries. In contrast, 24 h incubation with HG (44 mM) slightly shifted to some extent the endothelial relaxation response to BK to the right in comparison with the control. Probably, the level of oxidative stress in PCA rings incubated in HG for 24 h was higher than the one incubated in elevated D-glucose for only 6 h, therefore, a small shift of the relaxation curve to BK was observed only after a long incubation period. To date, the effect of 24 h incubation in HG on the relaxation response to BK has not been reported in PCA rings. However, *in vitro* studies have documented the dominant effect of long time incubation with HG in comparison with short time incubation on vascular superoxide anion formation in endothelial cells (Christ, Bauersachs et al. 2002); (Taubert, Rosenkranz et al. 2004). The group of Taubert has reported that the increased formation of superoxide anion was observed after 24 h, but not 2 h, incubation with high D-glucose (20 mM) in human umbilical vein endothelial cell (HUVECs, EA.hy 926) (Taubert, Rosenkranz et al. 2004). Similarly, in another study, the vascular superoxide anion formation in PCA rings incubated with 20 mM D-glucose for 48 h was significantly increased, while no such effect was observed in rings exposed to HG (20 mM D-glucose) for only 10 min, 4 h, and 8 h compared with respective controls (Christ, Bauersachs et al. 2002). Therefore, in our experiments, we suggest that the small impairment of the endothelium-dependent relaxation induced by 24 h incubation with 44 mM D-glucose in PCA rings can possibly be explained by

the HG-enhanced superoxide anion generation, which did not occur in 6 h incubation. The HG-enhanced formation of superoxide anion, in turn, can reduce the NO bioavailability, leading to impaired endothelium-dependent relaxation response. It has been also demonstrated that the 24 h incubation with D-glucose (25 mM) impaired Akt/eNOS signal transduction in HUVECs, promoting endothelial dysfunction in diabetes (De Nigris, Pujadas et al. 2015); (Varma, Lal et al. 2005). However, further studies will need to be performed to measure the level formation of $O_2^{\bullet -}$ and NO before and after 24 h incubation in HG to clarify the mechanism of the impaired endothelium-dependent relaxation response induced by HG in PCA rings.

In addition, the HG-induced impairment in relaxation response to BK in PCA rings was not prevented by 30 min incubation with 10^{-7} M gliflozin. It might be related to the reason that the HG-induced alteration of BK-mediated endothelium-dependent relaxation in PCA rings was too small: the significant difference of endothelium-dependent relaxation response to BK between normal and HG conditions was observed at only BK's concentration of 3.10^{-9} M (Figure 34). Therefore, we could not examine the protective effect of gliflozin. However, there are evidences in the literature that support the presence of SGLTs in endothelium of blood vessels. The group of Taubert has demonstrated that the elevation of the extracellular concentration of D-glucose would increase D-glucose entry not only via GLUT1, but also via SGLT, leading to a rise in intracellular Na^+, which, in turn, stimulates Ca^{2+} uptake through a Na^+/Ca^{2+}exchanger (NCX) involved in the regulation of Ca^{2+}-dependent eNOS activity (Taubert, Rosenkranz et al. 2004). In addition, they demonstrated that D-glucose-induced increase in intracellular Ca^{2+} and subsequent NO formation were completely blocked by the selective SGLT inhibitor phlorizin. Specially, they also found the expression of SGLT1 mRNA in aortic and coronary endothelium. Besides, another group has demonstrated that, delphinidin-3-glucoside - a member of a family of bioactive compounds, anthocyanins known to ameliorate oxidative stress, is transported into the human umbilical vein endothelial cells in a temperature, concentration, and time-dependent manner via SGLT1 both *in vitro* and *in vivo* studies (Jin, Yi et al. 2013). They also found that SGLT1 was noticeably expressed in the human umbilical vein endothelial cells and vascular endothelium of the isolated thoracic aorta. Moreover, Han and colleagues have examined the mRNA expression level of SGLTs in mouse and human endothelial cells and SMC of pulmonary and coronary arteries (Han, Cho et al. 2015). They found that SGLT1 was detected in both mouse and human endothelial cells and SMC of pulmonary and coronary arteries, but had much less expression level compared to that of the intestine and kidney. In contrast, the mRNA expression level of SGLT2 is not

detected in both endothelial cells and SMC of pulmonary and coronary arteries. Hence, the information about the expression of SGLT2 in blood vessels is still questionable.

Overall, it is worthwhile noting that, unlike the effect of HG on relaxation response in rat arteries, we have observed that the 24 h incubation with 44 mM D-glucose slightly attenuated BK-induced endothelium-dependent relaxation in PCA rings compared to control rings. The effect of HG was only endothelium-dependent because the SNP-induced endothelium-independent relaxing was not affected in PCA rings exposed to 44 mM D-glucose for 24 h compared to control rings. The differences in time exposure to HG and species used in experiments might explain different results in our work. Unfortunately, the HG-induced alteration of BK-mediated endothelium-dependent relaxation in PCA rings was too small. Therefore, the protective effect of gliflozin could not be examined. However, existing evidences in the literature support the presence of SGLTs in endothelium of blood vessels. Although studies regarding expression of SGLT2 and the effect of SGLT2 on blood vessels are still limited. Further studies are required to clarify the presence of SGLTs on endothelial cells, as well as their contribution on the level of oxidative stress induced by HG in endothelial cells in blood vessels in diabetes.

CHAPTER 6

CHARACTERISION OF HUMAN INTERNAL MAMMARY ARTERY FROM DIABETIC PATIENTS

6.1. Introduction

The association between diabetes and CVD has been widely published. It has been well documented that DM is frequently associated with more severe coronary artery disease, with involvement of a larger number of vessels and more lesions. Patients with diabetes have a two- to four-fold increased risk of coronary artery disease (CAD) over non-diabetic patients (). Diabetic patients with risk for coronary artery disease are more frequently requiring coronary artery bypass grafting (CABG) (Yamamoto, Hosoda et al. 2000). In fact, 12% to 38% coronary artery bypass grafting (CABG) patients are reported to be diabetic (Herlitz, Brandrup-Wognsen et al. 1997).

CABG is a procedure in which autologous mostly arteries are used as grafts to bypass coronary arteries that are partially or completely obstructed by atherosclerotic plaques (Alexander and Smith 2016). The most commonly used bypass conduits are the left internal mammary artery (IMA) (also known as the internal thoracic artery) and the saphenous vein. The IMA has become the gold standard in CABG, since its utilization yields with higher long-term patency rates than saphenous vein grafts (Alexander and Smith 2016); (Hirotani, Kameda et al. 1999). It can be explained by structural characteristics of each graft: the IMA has a discontinuous internal elastic lamina and a relatively thin media with multiple elastic laminae and absence of a significant muscular component, which explains a reduced tendency for spasm and the development of atherosclerosis. In contrast, the the saphenous vein graft has a thinner, more permeable endothelium and a thinner, less elastic and more muscular media (Taggart 2013). Consistently, it has also been reported that the use of an IMA graft during coronary bypass surgery showed to improve long-term clinical outcomes. In a 6.7-years follow-up study among 3,087 patients who received CABG, IMA grafts were shown to be associated with lower mortality, with hazard ratios of 0.77 (Hlatky, Shilane et al. 2011). In addition, in another study throughout a 15-year follow-up period, it has been reported that IMA grafts showed a survival advantage with a relative risk of dying of 0.73 as compared with saphenous-vein coronary bypass grafts (Cameron, Davis et al. 1996).

Since the proposed mechanism for this increased patency and subsequent improved clinical outcome is due to the inherent characteristics of IMA endothelium (Gurne, Chenu et al. 1999); (Glineur, Poncelet et al. 2007), one of our research goals is to characterize the endothelial function of IMAs from bypass patients. Amongs the cardiovascular risk factors, diabetes and hypertension are the most frequent factors presented in bypass patients. Therefore, we first aim to identify the relevance of diabetes, hypertension and combination of these two cardiovascular risk factors and the level of oxidative stress in IMAs. In addition, since eNOS is considered as one of potential sources of ROS in human blood vessels (Bouloumie, Bauersachs et al. 1997); (Forstermann and Munzel 2006); (Montezano and Touyz 2012) and the increased ROS has been shown to be induced by the local angiotensin system in endothelial cells (Pueyo, Arnal et al. 1998); (Sohn, Raff et al. 2000); (Touyz 2004). Therefore, our purpose is to determine the expression level of eNOS and components of the angiotensin system in IMA from diabetic patients in order to evaluate the alterations in the expression level of these proteins due to the presence of single or combined two main cardiovascular risk factors: diabetes and hypertension. Moreover, it has been reported that the cardiovascular risk factors lead to the excess production of superoxide anion $O_2^{\bullet -}$, which reacts with NO· to form peroxynitrite (OONO–) – a powerful oxidant. OONO–, in turn, can lead to eNOS uncoupling and enzyme dysfunction resulting in the enhanced generation of ROS (Forstermann and Munzel 2006); (Pacher, Beckman et al. 2007). Therefore, we examine the level of nitrotyrosine, an end product of OONO–, to assess the level of oxidative stress in human IMA. Finally, since some studies have been published that SGLT2 inhibitors have effect on reducing significantly cardiovascular events in diabetic patients (Zinman, Wanner et al. 2015); (Vasilakou, Karagiannis et al. 2013); (Dziuba, Alperin et al. 2014); (Neal, Perkovic et al. 2013). However, the expression of SGLTs on human blood vessels is unclearly known. For this reason, we investigate the expression level of SGLT1 and SGLT2 proteins in human IMA in order to clarify the role of SGLTs in D-glucose transport in diabetic patients, either alone or in association with hypertension.

6.2. Results

6.2.1. Clinical profile of bypass patients

The clinical profile of all studied patients was shown in Table 7. Totally 71 specimens of patients enrolled in our study. Segments of IMA as excess grafts were obtained from patients underwent CABG between July 2013 and January 2016 at the New Civil Hospital of Strasbourg. All patients were discharged from the hospital within about 7 days after surgery

and without complications. From total 71 samples: 13 samples were discarded because their abnormal structure and long time processing (> 24 h) (Figure 37).

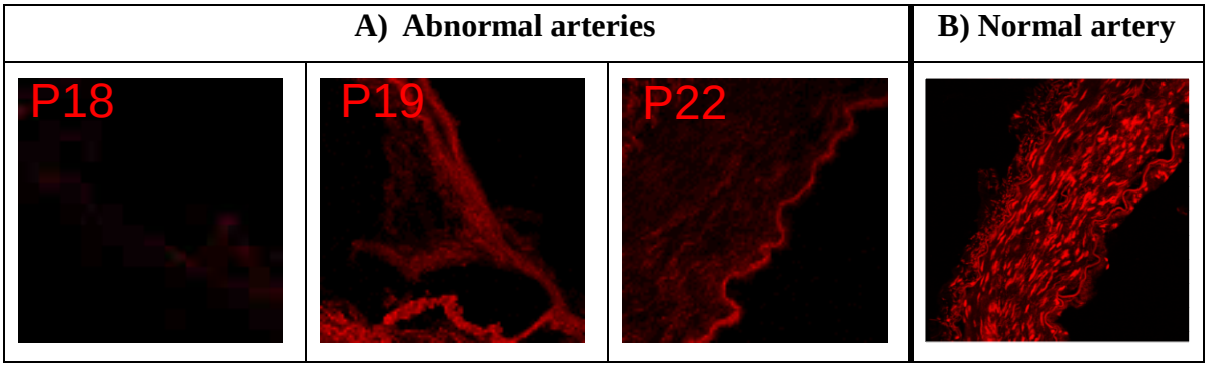

Figure 37. ***Comparison between structures of arterial wall of discarded segments abnormal IMA (A) and normal structure of investigated IMA (B).*** DHE staining of abnormal IMA (A) show a degradation of IMA walls from different patients (P18, P19 and P22), where none of DHE staining signals was observed. DHE staining of normal IMA (B) shows three layers (intima, media ans adventitia) of arterial wall with the DHE signals. DHE staining was performed to determine the presence of oxidative stress in IMA from bypass patients. Photos were taken by a Leica confocal laser-scanning microscope TSC SPE.

Subjects were divided into four groups according to the presence of diabetes, hypertension and the combination of both two risk factors. The control group (CONTROL, n = 13) includes the non-diabetic and normotensive patients; the hypertensive group (HT, n = 25) includes hypertensive patients; the diabetic group (DM, n = 3) and the diabetic and hypertensive group (DM & HT, n = 17) includes diabetic and hypertensive patients. It should be noted that about 70% of patients with diabetes also have hypertension (Lago, Singh et al. 2007); (Campbell, Leiter et al. 2009); (Sampanis and Zamboulis 2008); (Sowers, Epstein et al. 2001). Therefore, it is rare to obtain the diabetic patients without hypertension. In our study, we obtained IMA from only three diabetic patients without hypertension.

The clinical characteristics and preoperative treatments of the 58 patients were collected from database in hospital and were summarized in Table 15. Patients with the average of age in range 68 ± 1.21 (range, 46 to 85). These patients have received antianginal therapy (vasodilator, beta-blockers, or calcium channel antagonists), aspirin, statins, and antihypertensive, antidiabetic agents preoperatively.

Table 15. ***Clinical profile of the studied groups***. Values are mean ± SEM or percentage. Abbreviations: CONTROL: control group; HT: hypertensive group; DM: diabetic group; DM & HT: diabetic and hypertensive group; M, male; F, female; BMI, body mass index; ACE: angiotensin-converting enzyme; OADs: oral antidiabetic drugs.

General information	**CONTROL (n = 13) (Mean ± SEM)**	**HT (n = 25) (Mean ± SEM)**	**DM (n = 3) (Mean ± SEM)**	**DM & HT (n = 17) (Mean ± SEM)**
Age, years	68.4 ± 2.3	68.8 ± 1.9	58.3 ± 9.9	67.3 ± 1.8
Sex, male/female	12/1	20/5	3/0	16/1
BMI (kg/m^2)	26.7 ± 1.4	27.7 ± 0.9	26.4 ± 1.0	28.3 ± 3.2
Creatinine clearance (ml/min)	75.0 ± 6.4	77.1 ± 6.0	94.6 ± 39.8	129 ± 11
Dyslipidemia, n (%)	6 (46.15)	23 (92.00)	0 (0)	12 (70.6)
Smokers, n (%)	0 (0)	2 (8.00)	2 (66.7)	2 (11.8)
Ex-smokers, n (%)	5 (7.94)	13 (52.00)	1 (33.3)	5 (29.4)
Medication used, n (%)				
Treatment beta-blocker	6 (46.15)	23 (92)	3 (100)	13 (76.5)
Treatment aspirin	9 (69.23)	21 (84)	2 (66.7)	13 (76.5)
Treatment statin	7 (53.85)	22 (88)	3 (100)	10 (58.8)
Treatment ACE Inhibitor	4 (30.77)	20 (80)	3 (100)	11 (64.7)
Treatment diuretic	2 (15.38)	10 (40)	1 (33.3)	9 (52.9)
Treatment OADs	0 (0)	0 (0)	3 (100)	11 (64.7)

The patient groups were compared for mean age, sex, body mass index (BMI), creatinine clearance, and cardiovascular risk factors such as dyslipidemia, smoking and the preoperative drugs used (Table 15). The ANOVA tests showed no significant differences in the ages, genders, body mass index and creatinine clearance of the patients between the

groups. Notably, there were more men than women with CABG. It might be related to the fact that women with symptoms of coronary heart disease are being referred less often or later than men (Sheifer, Escarce et al. 2000); (Maas and Appelman 2010). In addition, it has been reported that women had less severe coronary artery disease than men in all age categories, including younger women (Vaccarino, Abramson et al. 2002).

In addition, the baseline characteristics of bypass patients showed that almost all hypertensive patients and diabetic patients with hypertension has dyslipidemia (Table 15). It is in complete agreement with the literature, which showed an increased prevelance of dyslipidemia in diabetes and hypertension (Elnasri and Ahmed 2008); (Dixit, Dey et al. 2014); (Dalal, Padmanabhan et al. 2012). It should be noted that the frequency of smoking or tobacco used tend to be increased in diabetes and hypertension in comparison with non-diabetic and normotensive patients (Table 15). The association between smoking and increased risk of coronary heart disease is well established in the general population. Besides, smoking is also strongly associated with an increased risk of coronary heart disease in hypertension and in diabetes (Fagard 2009); (Martin-Timon, Sevillano-Collantes et al. 2014); (Al-Delaimy, Manson et al. 2002).

6.2.2. Level of oxidative stress

The level of oxidative stress in IMA from patients with diabetes and/or hypertension was determined by using the redox-sensitive fluorescent probe dihydroethidine (DHE) is shown in Figure 38. We found a markedly increased fluorescent signal of DHE in patients with hypertension and both of diabetes and hypertension, which pointed out that there is an increased formation of ROS in IMA of patients with one or both of two cardiovascular risk factors (diabetes and hypertension) in comparison with the non-diabetic and normotensive patients. Moreover, there was no difference observed between HT and DM & HT groups.

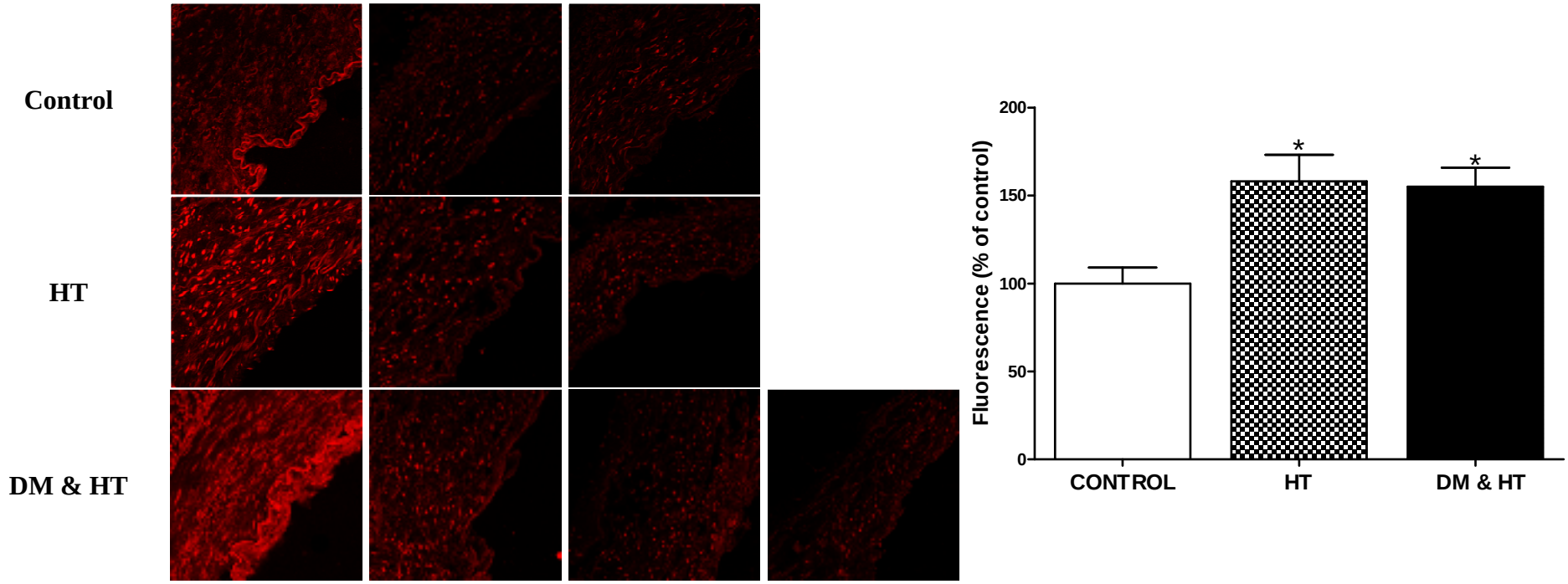

Figure 38. ***The increased level of oxidative stress in internal mammary artery sections from bypass diabetic and hypertensive patients.*** Left panels show representative photographs; right panels corresponding cumulative data. Oxidative stress was determined using the redox-sensitive fluorescent dye dihydroethidine (DHE). Results are shown as mean ± SEM of 3-4 different patients in each group. *p<0.05 indicates a significant difference of diabetic and/or hypertensive patients versus non-diabetic and normotensive patients. All micrographs were taken at the same magnification. Abbreviations: control: normotensive and non-diabetic patients; HT: hypertensive patients; DM & HT: diabetic and hypertensive patients.

In order to determine the nature of ROS, IMA sections from hypertensive patients and diabetic patients with hypertension were treated with different antioxidants such as N-acetyl-cysteine (NAC), superoxide dismutase (SOD), catalase (CAT), and membrane-permeant analogues of SOD and CAT (polyethylene glycol-superoxide dismutase (PEG-SOD), and polyethylene glycol-catalase (PEG-CAT)) for 30 min before DHE staining. The DHE fluorescence signal in IMA of hypertensive patients and diabetic patients with hypertension was markedly reduced by NAC, SOD, PEG-SOD, CAT and PEG-catalase, indicating the involvement of both superoxide anions and hydrogen peroxide in the high level of oxidative stress in IMA of hypertensive patients and diabetic patients with hypertension (Figure 39).

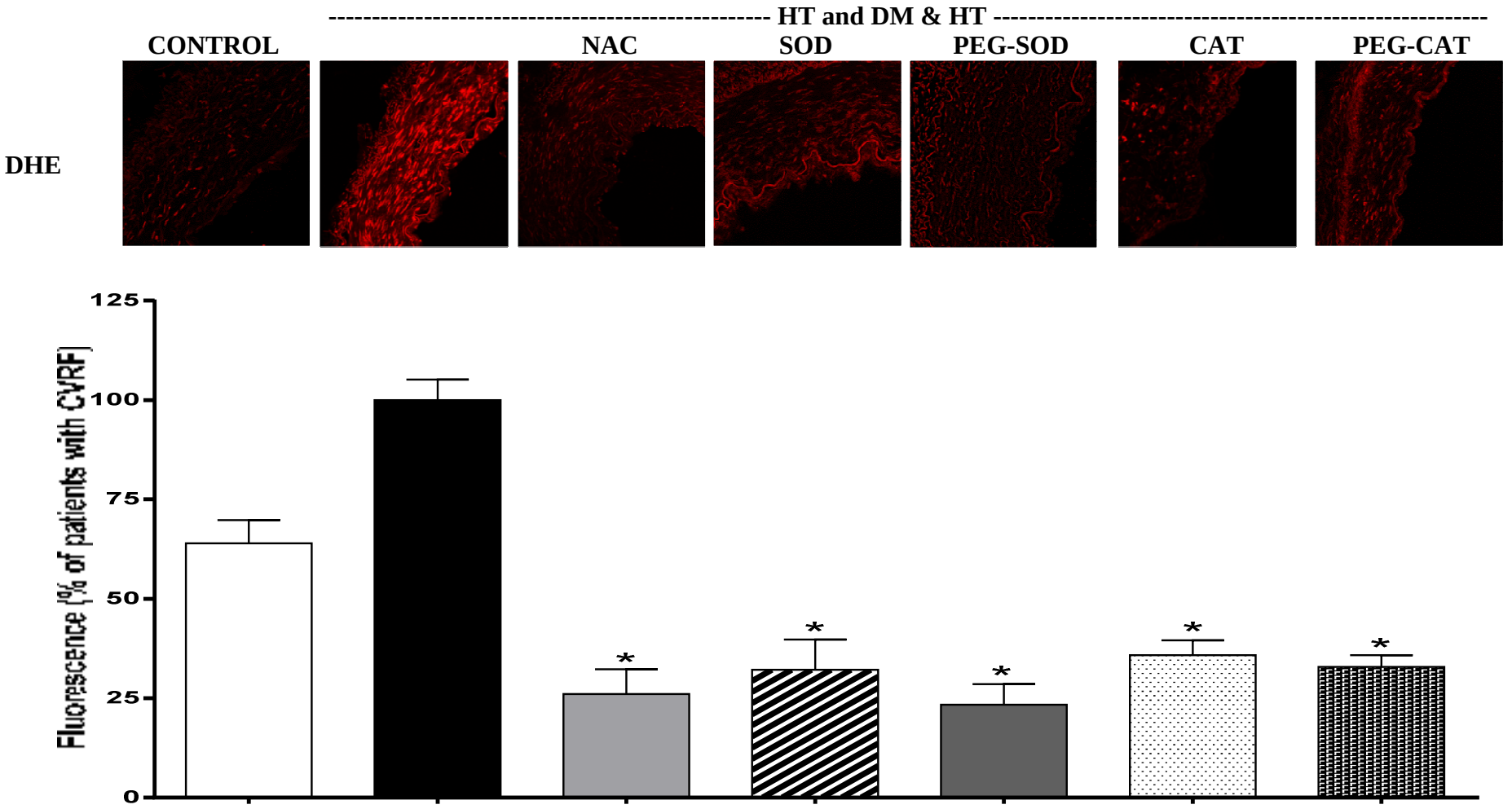

Figure 39. ***Role of superoxide anion and peroxide hydrogen in the induction of oxidative stress in internal mammary artery segments from bypass patients.*** Upper panels show representative photographs; lower panels corresponding cumulative data. Oxidative stress was determined using the redox-sensitive fluorescent dye dihydroethidine (DHE). Segments of internal mammary artery were exposed in

Figure 39. (*continued*) antioxidant N-acetyl-cysteine (NAC, 10 mM), superoxide dismutase (SOD, 500 U/mL), polyethylene glycol-superoxide dismutase (membrane-permeant superoxide dismutase, PEG-SOD, 500 U/mL), catalase (CAT, 500 U/mL), polyethylene glycol-catalase (membrane-permeant catalase, PEG-CAT, 500 U/mL) for 30 min before DHE staining. Results are shown as mean ± SEM of 3 non-diabetic and normotensive patients in control group (CONTROL) and 7 hypertensive (HT) and diabetic patients with hypertension (DM & HT). *$p<0.05$ indicates a significant difference of hypertensive patients and diabetic patients with hypertension versus non-diabetic and normotensive patients. All micrographs were taken at the same magnification.

In addition, IMA sections from bypass patients were also treated with N(ω)-nitro-L-arginine (L-NA, an eNOS inhibitor), indomethacin (a COX inhibitor), VAS-2870 (a NADPH oxidase inhibitor), sulfaphenazol (a cytochrome P450 inhibitor), and by a combination of inhibitors of the mitochondrial respiration chain (KCN, myxothiazol, and rotenone) in order to elucidate the contribution of each enzymatic system to the total intracellular ROS production. Similarly, we found that the increased DHE fluorescence signal was also markedly reduced by different inhibitors, indicating the involvement of several sources including uncoupled eNOS, COXs, NADPH oxidase, cytochrome P450, and the mitochondrial respiration chain in the enhanced formation of ROS in IMA from hypertensive patients and diabetic patients with hypertension (Figure 40).

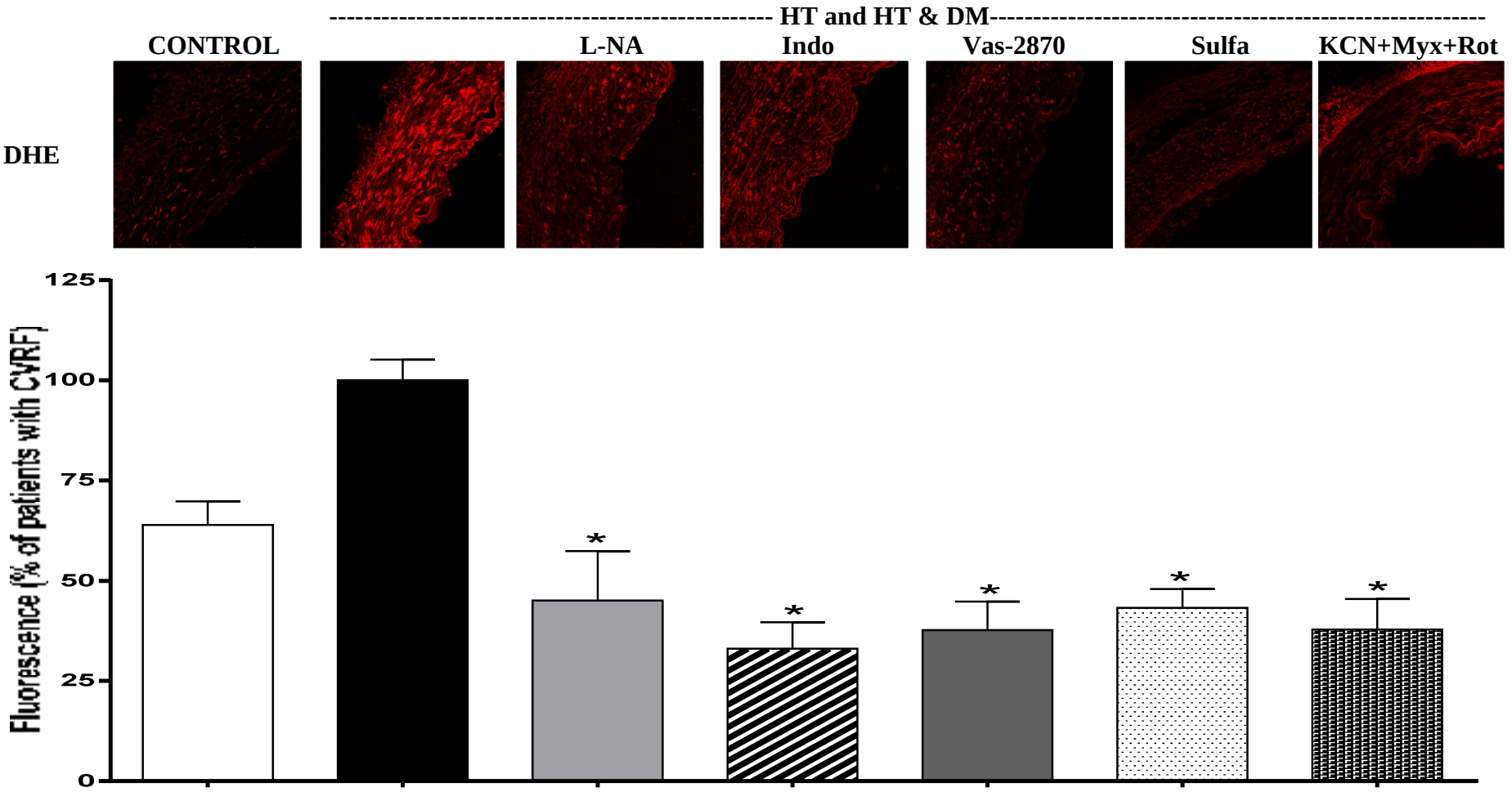

Figure 40. ***Enzymatic sources of ROS in internal mammary artery segments from bypass patients***. Upper panels show representative photographs; lower panels corresponding cumulative data. Oxidative stress was determined using the redox-sensitive fluorescent dye dihydroethidine (DHE). Segments of internal mammary artery were treated with N^{ω}-nitro-L-arginine (NO synthase inhibitor, L-NA, 300 μM),

Figure 40. (*continued*) indomethacin (cyclooxygenase inhibitor, INDO, 10 µM), VAS-2870 (NADPH oxidase inhibitor, VAS, 300 µM), sulfaphenazol (cytochrome P450 inhibitor, Sulfa, 100 µM) and inhibitors of the mitochondrial respiratory chain (combination of potassium cyanide (1 µM), myxothiazol (0.5 µM) and rotenone (1 µM), KCN+Myx+Rot) for 30 min before DHE staining. Results are shown as mean ± SEM of 3 non-diabetic and normotensive patients in control group (CONTROL) and 7 hypertensive (HT) and diabetic patients with hypertension (DM & HT). *$p<0.05$ indicates a significant difference of hypertensive patients and diabetic patients with hypertension versus non-diabetic and normotensive patients. All micrographs were taken at the same magnification.

6.2.3. The expression level of nitrotyrosine

In addition, the cardiovascular risk factors have been shown to be associalted with the excess production of superoxide anion $O_2^{\bullet -}$, which reacts with NO· to form peroxynitrite ($ONOO^-$) – a powerful oxidant. $ONOO^-$, in turn, can lead to eNOS uncoupling resulting in the enhanced level of oxidative stress (Forstermann and Munzel 2006); (Pacher, Beckman et al. 2007). Therefore, we examine the level of nitrotyrosine, an end product of $ONOO^-$, to assess the level of oxidative stress in IMA. As indicated in Figure 41 an increased signal of nitrotyrosine was observed in IMA from in hypertensive patients and diabetic patients with hypertension in comparison with the non-diabetic and normotensive patients, which indicates that $ONOO^-$ was largely produced in IMA from patients with one or both of two cardiovascular risk factors.

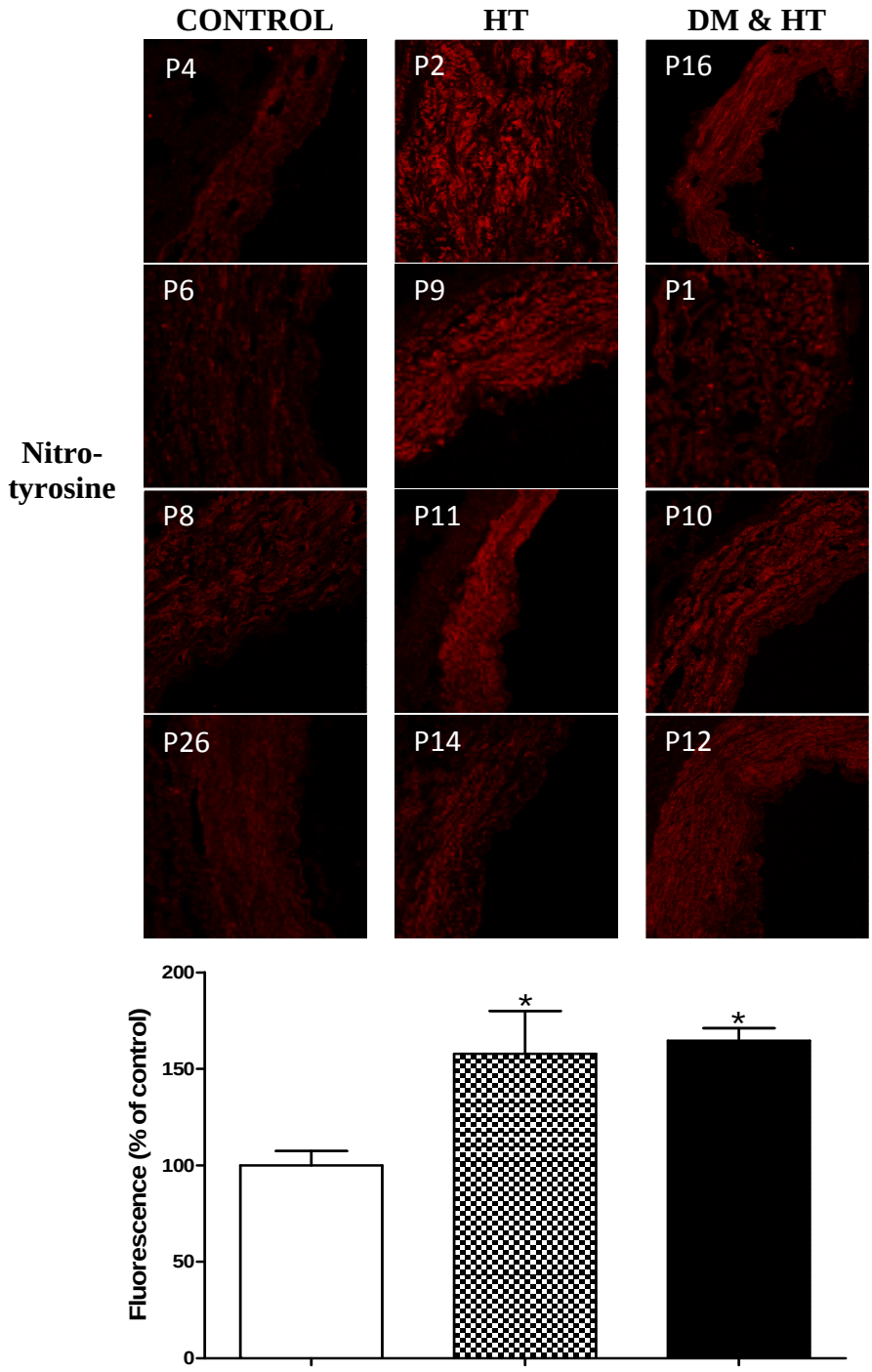

Figure 41. ***The pronounced formation of peroxynitrite in IMA from diabetic and hypertensive patients.*** The formation of peroxynitrite was determined by nitrotyrosine immunofluorescence staining. Upper panels show representative immunofluorescence staining; lower panels corresponding cumulative data. Results are shown as mean ± SEM of 4 different patients. *p<0.05 indicates a significant difference of hypertensive patients and diabetic patients with hypertension versus non-diabetic and normotensive patients. All micrographs were taken at the same magnification. Abbreviations: CONTROL: control group; HT: hypertensive group; DM & HT: diabetic and hypertensive group

6.2.4. The expression level of endothelial nitric oxide synthase

Since eNOS is considered as one of potential sources of ROS in human blood vessels (Bouloumie, Bauersachs et al. 1997); (Forstermann and Munzel 2006); (Montezano and Touyz 2012). Therefore, we determine the expression level of eNOS in IMA from diabetic patients in order to evaluate the alterations in the expression level of eNOS due to the presence of single or combined two main cardiovascular risk factors: diabetes and hypertension. Results of immunofluorescence of eNOS in IMA from bypass patients are shown in Figure 42, indicating the localization of eNOS at the luminal endotheliumm. We found a significantly increased expression level of eNOS in IMA of hypertensive patients and diabetic patients with hypertension compared with the non-diabetic and normotensive patients. However, the increased level of eNOS is most likely due to a more pronounced degradation of NO.

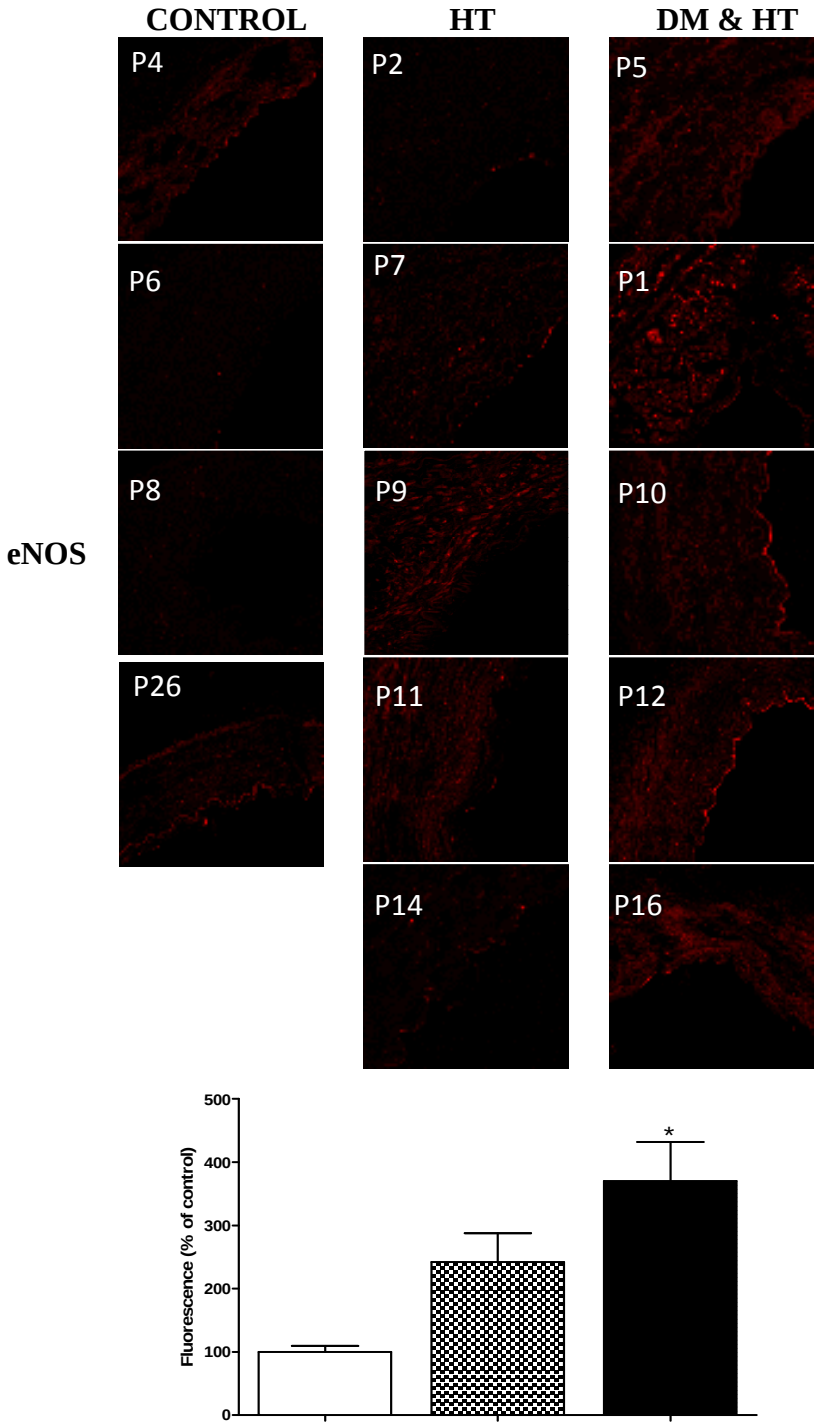

Figure 42. ***The increased expression level of eNOS in diabetic and hypertensive patients***. The expression level of eNOS in IMA segments was determined by immunofluorescence staining. Upper panels show representative immunofluorescence staining; lower panels corresponding cumulative data. Results are shown as mean ± SEM of 4-5 different patients. *p<0.05 indicates a significant difference of hypertensive patients and diabetic patients with hypertension versus non-diabetic and normotensive patients. All micrographs were taken at the same magnification. Abbreviations: CONTROL: control group; HT: hypertensive group; DM & HT: diabetic and hypertensive group.

Next, we investigate the relative abundance of eNOS protein in IMA of bypass patients using Western blot (**Figure 43**). Unfortunately, our results showed a high fluctuation. Among 5 non-diabetic and normotensive patients, there is an exception, patient A35, which has a lower expression level of eNOS compared to the one of other patients. The expression levels of eNOS of patients in diabetic group and hypertensive group show about only 62.1% and 54.2% of the one in control group, respectively. In hypertensive group, the expression level of eNOS is lower compared to the control; except for patient A21 who has about 15.3% higher than the control. Regarding the patients, who have both of diabetes and hypertenstion, the expression level of eNOS is approximately only 34.9% of the one in control group. Except for patient A20, who has about 35.3% higher expression level of eNOS compared to the control. Hence, from a total of 26 analyzed patients we found three exception patients, the rest of group shows a tendency of a decreased expression level of eNOS in diabetic and/or hypertensive patients compared to non-diabetic and normotensive patients.

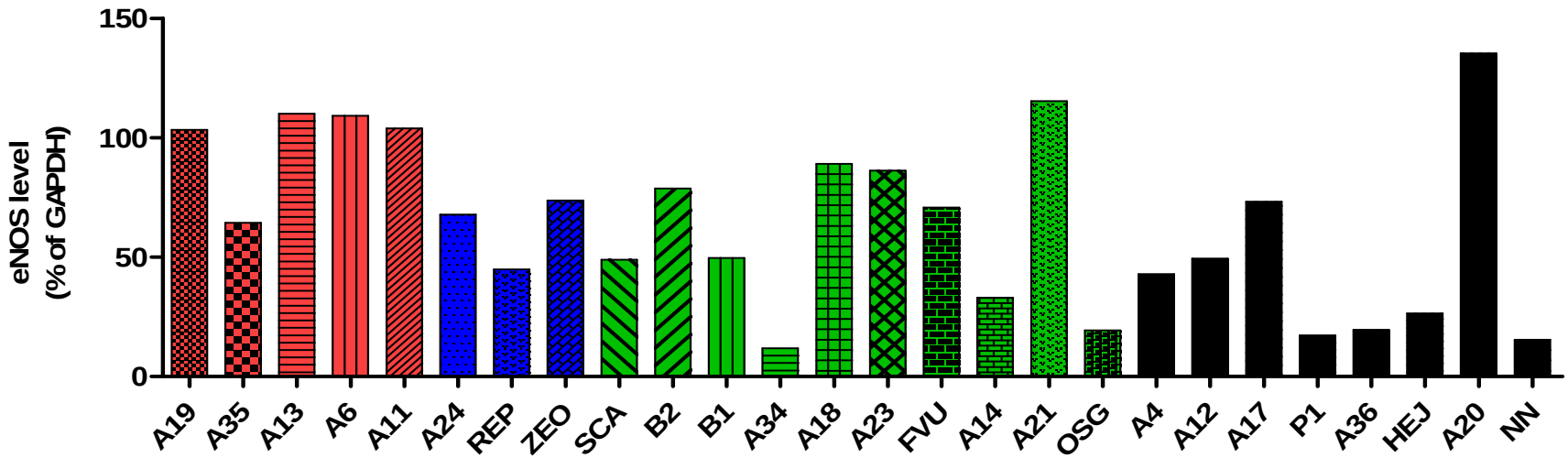

Figure 43. ***The expression level of endothelial nitric oxide synthase (eNOS) in IMA of bypass patients***. The expression level of eNOS in internal mammary artery segments was determined by Western blot. Upper panels show representative immunoblots; lower panels corresponding cumulative data. Results are shown as ratio eNOS/GADPH of each patient. Abbreviations: CONTROL: control group (red); DM: diabetic group (blue); HT: hypertensive group (green); DM & HT: diabetic and hypertensive group (black).

Besides, the NO production depends not only on the expression level of eNOS, but also on eNOS phosphorylation. Among the potential eNOS phosphorylation sites, the activation site at Ser1177 residue has been well documented as a sensor of shear stress (Tillery, Epperson et al. 2016); (Tanaka, Miyajima et al. 2015); (Yoon, Cho et al. 2010). Phosphorylation of eNOS at Ser1177 leads to a rise in its enzymatic activity and enhances NO production (Dimmeler, Fleming et al. 1999); (Fisslthaler, Dimmeler et al. 2000); (Li, Wang et al. 2009). Therefore, we investigate the expression level of phosphorylated eNOS at Ser1177 (peNOS Ser1177) in IMA of bypass patients using Western blot (Figure 44). Similar to the result of the expression level of eNOS, the expression level of peNOS shows a high fluctuation. The expression levels of peNOS-Ser1177 are almost similar among non-diabetic and normotensive patients; except for patient A11, whose expression level of peNOS-Ser1177 is about 18.5% less than others patients in control group. Among three diabetic patients, two patients show a similar expression level of peNOS-Ser1177 as the one in control group. However, the expression level of peNOS-Ser1177 in the third diabetic patient is only about 66.9% compared to control group. In hypertensive group, two thirds of group shows a reduced expression level of peNOS-Ser1177 in comparison with control group. There are two hypertensive patients, whose expression levels of peNOS-Ser1177 even are about twice less than the one in the control group. In the other hand, one third of hypertensive group has an increased expression level of peNOS-Ser1177, which is even increased about 2-4 times. Regarding the diabetic and hypertensive patients, there are three patients, whose expression levels of peNOS-Ser1177 are less than the control. The other five diabetic and hypertensive patients have about 2-4 times higher expression level of peNOS-Ser1177 compared to the non-diabetic and normotensive patients.

Taken together, unfortunately, our data of the expression levels of eNOS and peNOS show a high fluctuation. The reason for this variation remains still unclear. It migh be due to differences in sex, age, and disease status in human IMA from each patient. Besides, the limitation in a number of investigated samples can also cause the variation in the reults. Consequently, we could not see a clear message. Therefore, further experiments need to be performed with a larger number of patients to clarify the expression level of these proteins in human IMA.

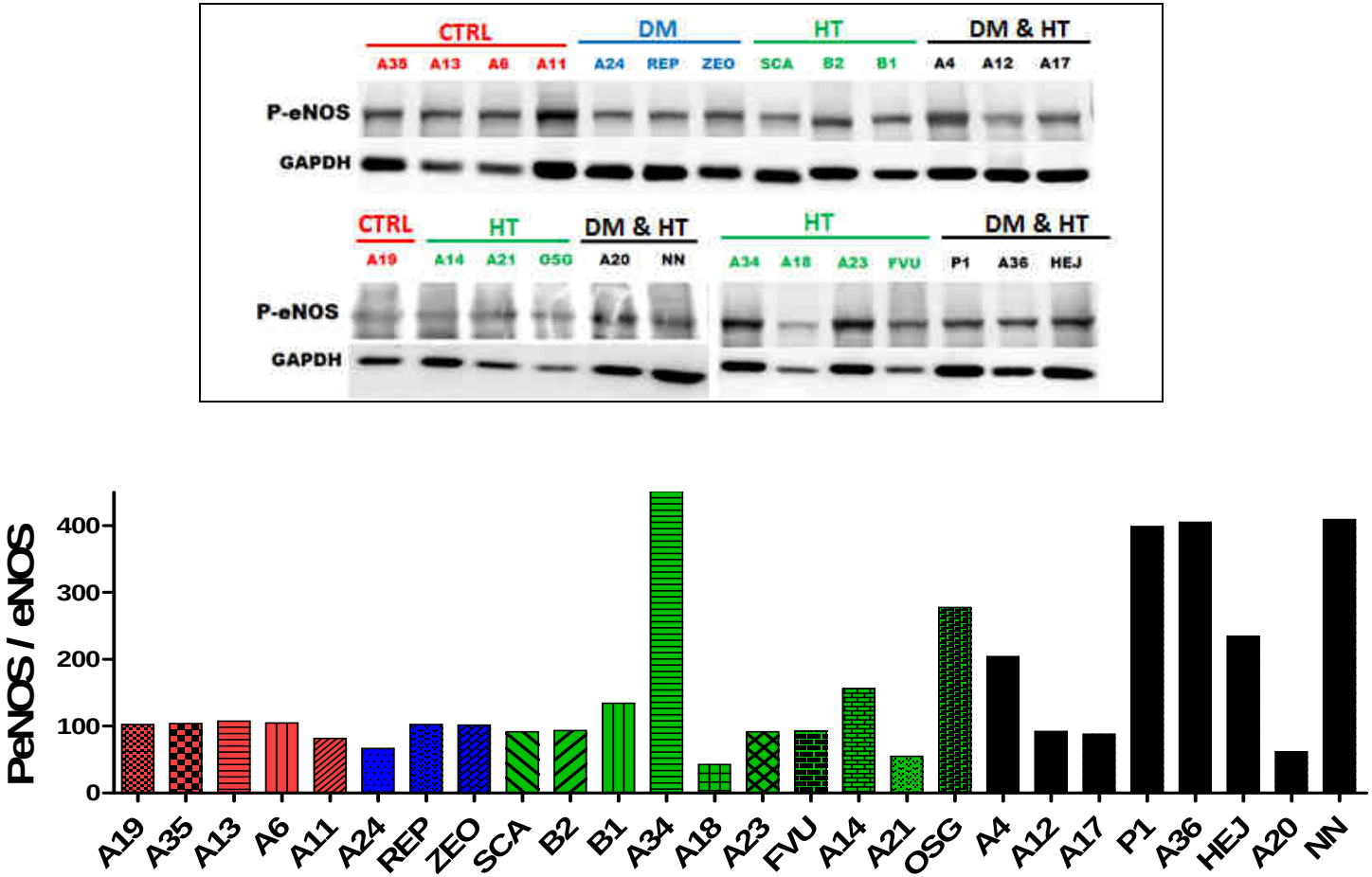

Figure 44. ***The expression level of phosphorylated-endothelial nitric oxide synthase (peNOS at Ser1177) in IMA of bypass patients***. The expression level of peNOS at Ser1177 in internal mammary artery segments was determined by Western blot. Upper panels show representative immunoblots; lower panels corresponding cumulative data. Results are shown as ratio peNOS/eNOS of each patients. Abbreviations: control group (red); DM: diabetic group (blue); HT: hypertensive group (green); DM & HT: diabetic and hypertensive group (black).

6.2.5. The expression level of angiotensin system

Since the increased ROS has been shown to be induced by the local angiotensin system in vasculature (Pueyo, Arnal et al. 1998); (Sohn, Raff et al. 2000); (Nguyen Dinh Cat, Montezano et al. 2013); (Touyz 2004). Therefore, we determine the expression level of angiotensin II (Ang II), AT_1 and AT_2 receptors in IMA from diabetic patients in order to evaluate the involvement of the angiotensin system in generation of ROS in IMA of patients having one or both of two main cardiovascular risk factors: diabetes and hypertension. Results of immunofluorescence of Ang II, AT_1 and AT_2 receptors in IMA from bypass patients are shown in Figure 45, 46 and 47 respectively. We found an increased signal of Ang II, AT_1 and AT_2 receptors in both hypertensive and diabetic with hypertension patients in comparison with non-diabetic and normotensive patients, which indicated the association of DM and hypertension and the enhanced expression level of angiotensin system in IMA.

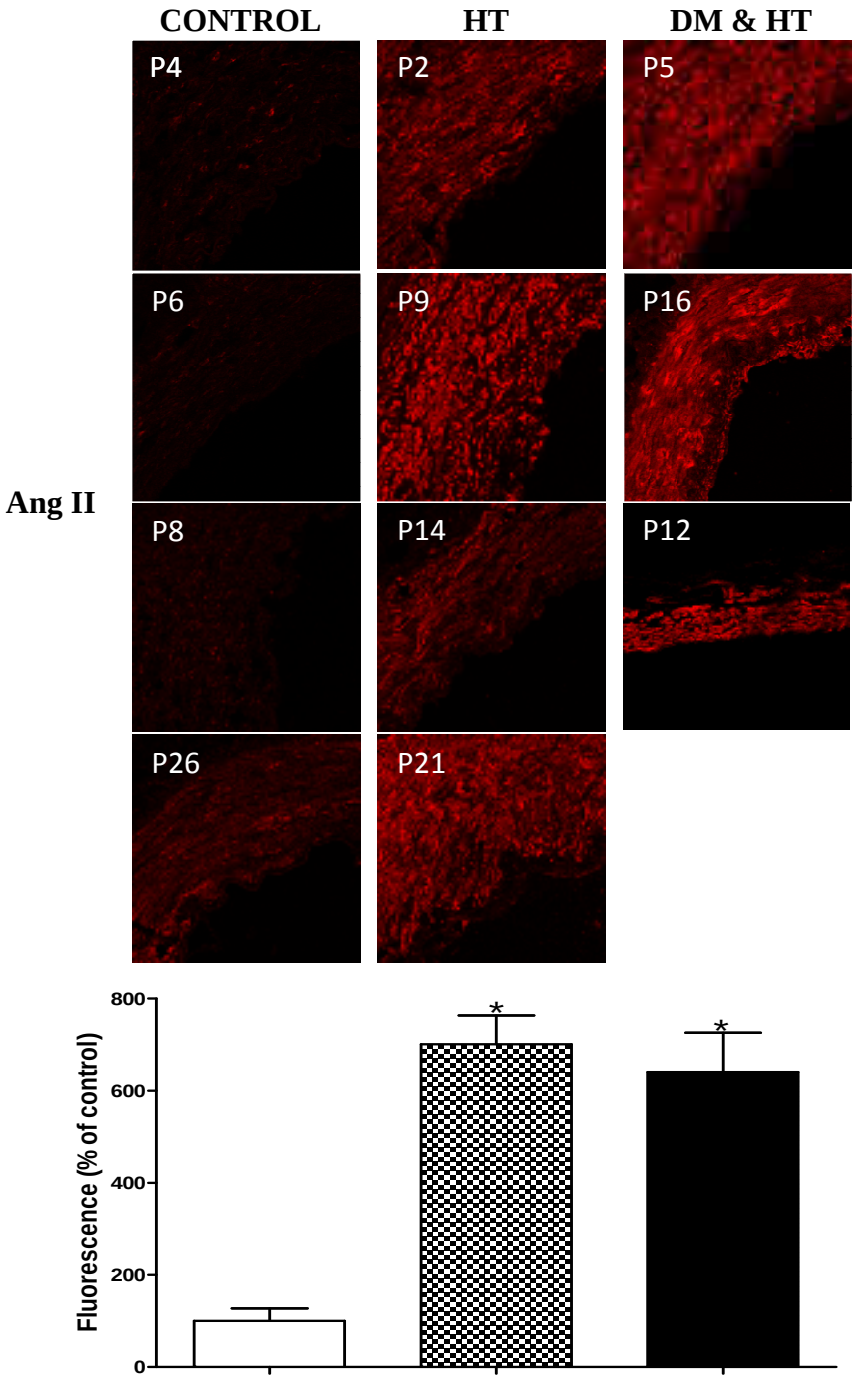

Figure 45. ***The pronounced expression level of angiotensin II in IMA of diabetic and hypertensive patients***. The expression level of angiotensin II was determined by immunofluorescence staining. Upper panels show representative immunofluorescence staining; lower panels corresponding cumulative data. Results are shown as mean ± SEM of 3-4 different patients. *p<0.05 indicates a significant difference of hypertensive patients and diabetic patients with hypertension versus non-diabetic and normotensive patients. All micrographs were taken at the same magnification. Abbreviations: CONTROL: control group; HT: hypertensive group; DM & HT: diabetic and hypertensive group

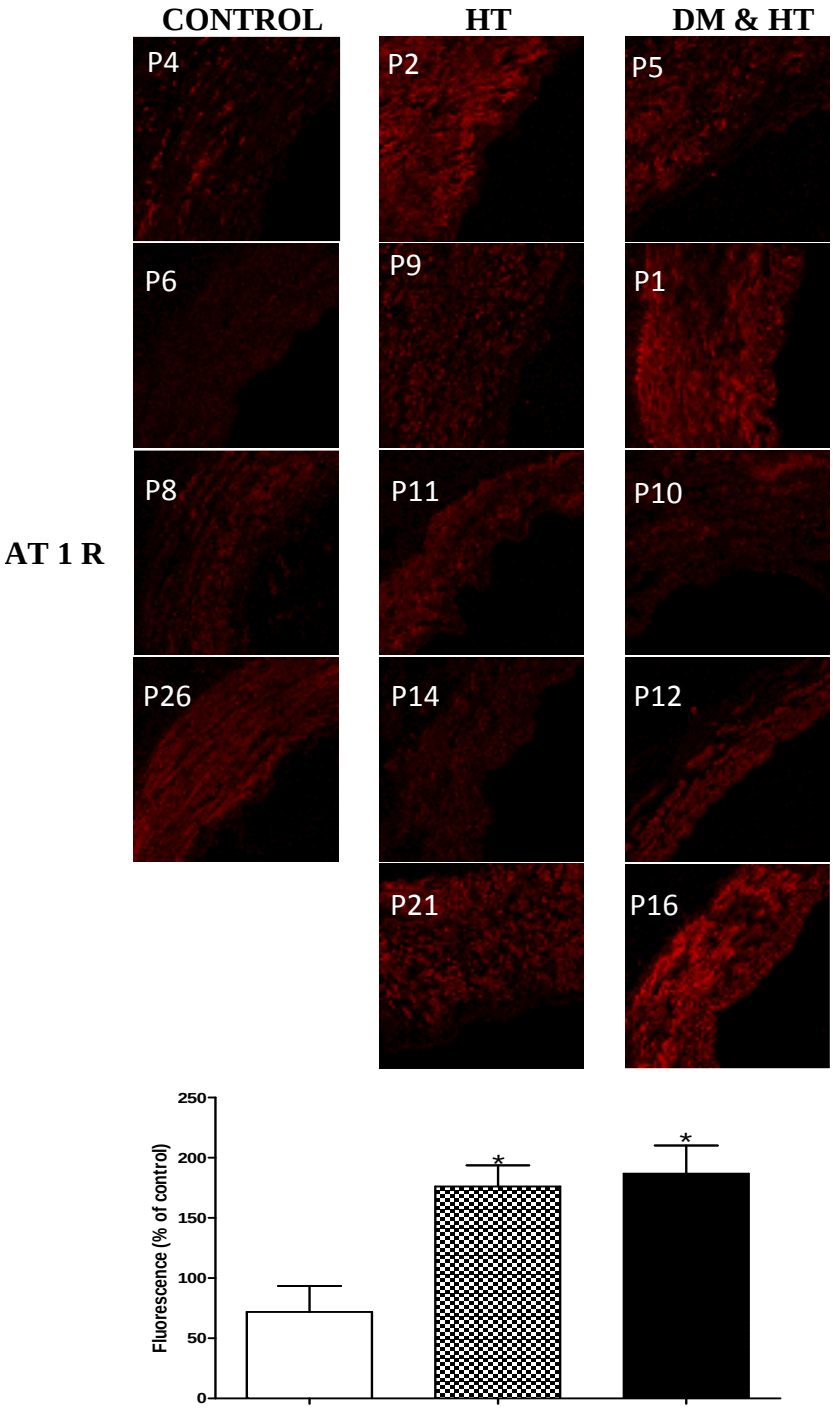

Figure 46. ***The increased expression level of angiotensin II receptor – AT1 receptor in IMA of hypertensive patients and diabetic patients with hypertension***. The expression level of AT1 receptor was determined by immunofluorescence staining. Upper panels show representative immunofluorescence staining; lower panels corresponding cumulative data. Results are shown as mean ± SEM of 4-5 different patients. *$p<0.05$ indicates a significant difference of hypertensive patients and diabetic patients with hypertension versus non-diabetic and normotensive patients. All micrographs were taken at the same magnification. Abbreviations: CONTROL: control group; HT: hypertensive group; DM & HT: diabetic and hypertensive group.

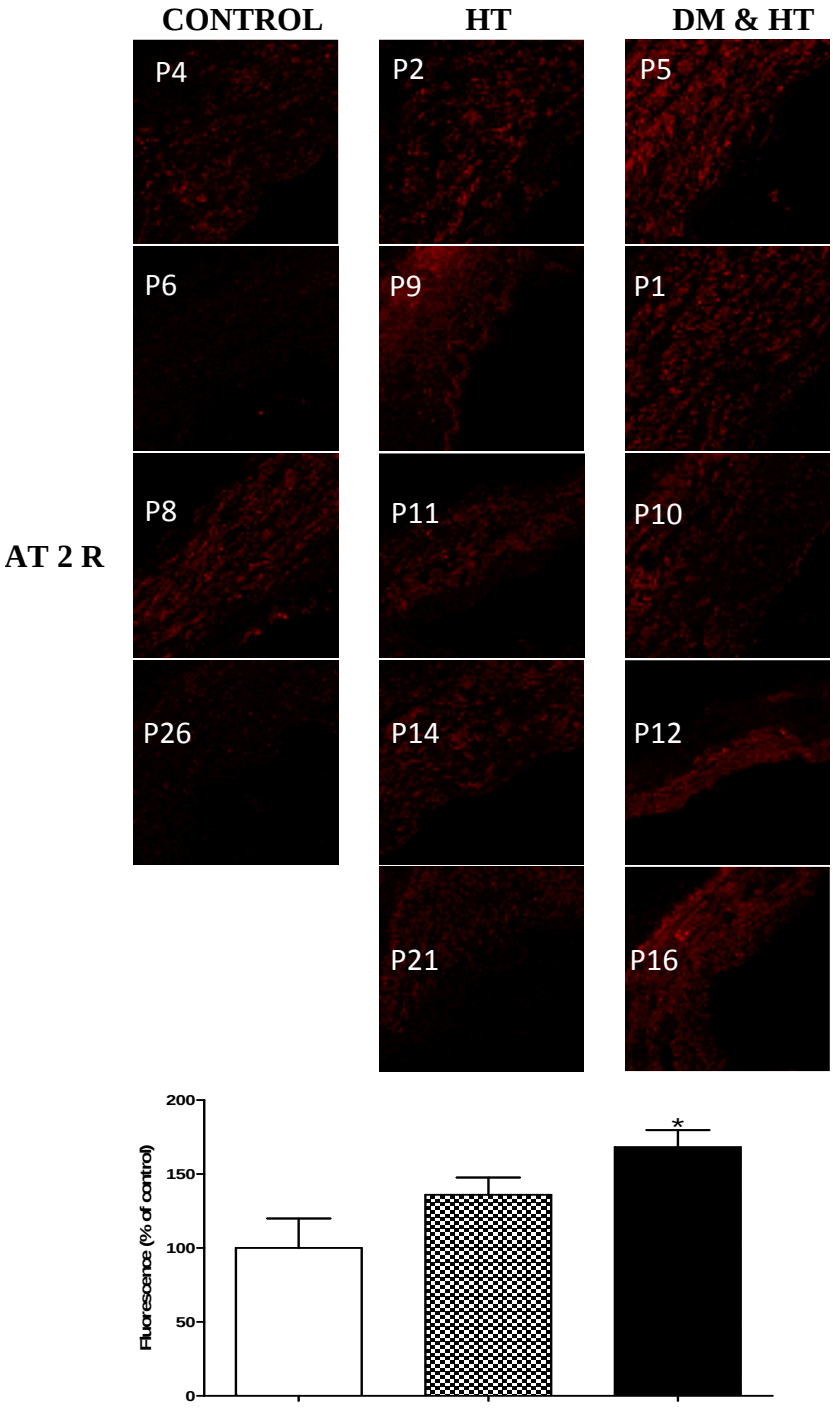

Figure 47. ***The pronounced expression level of angiotensin II receptor – AT2 receptor in IMA of hypertensive patients and diabetic patients with hypertension***. The expression level of AT2 receptor was determined by immunofluorescence staining. Upper panels show representative immunofluorescence staining; lower panels corresponding cumulative data. Results are shown as mean ± SEM of 4-5 different patients. **p*<0.05 indicates a significant difference of hypertensive patients and diabetic patients with hypertension versus non-diabetic and normotensive patients. All micrographs were taken at the same magnification. Abbreviations: CONTROL: control group; HT: hypertensive group; DM & HT: diabetic and hypertensive group.

6.2.6. The expression level of sodium-D-glucose cotransporters

Since some studies have been published that SGLT2 inhibitors have effect on reducing significantly cardiovascular events in diabetic patients (Zinman, Wanner et al. 2015); (Vasilakou, Karagiannis et al. 2013); (Dziuba, Alperin et al. 2014); (Neal, Perkovic et al. 2013). Nevertheless, the mechanisms behind these benefits are still not well understood. Besides, the study regarding the expression of SGLTs on blood vessels is unclearly known and the existing published papers still have their limitations. For this reason, we investigate the expression level of SGLT1 and SGLT2 proteins in IMA from diabetic patients, either alone or in association with hypertension by using Western blot (**Figure 48, 49**), respectively in order to clarify the presence of SGLTs in human IMA. The data indicates that the SGLT1 protein of human IMA is expressed at the lower level than the SGLT1 taken from the protein of rat kidney. In contrast, SGLT2 of both human IMA and rat kidney were found at the same level. The lower molecular weight of SGLT1 observed might be due to the cleavage and degradation of the protein, or the antibody is not specific for the human protein. Our results show a high fluctuation of both the SGLT1 and SGLT2 protein levels in bypass patients. Therefore, it is difficult to provide any conclusion. The reason for such heterogeneous SGLT1 and SGLT2 protein levels remains unclear. Additional experiments need to be performed such as the determination of the expression level of SGLT 1 & 2 mRNA to clarify the presence of these proteins in human blood vessels.

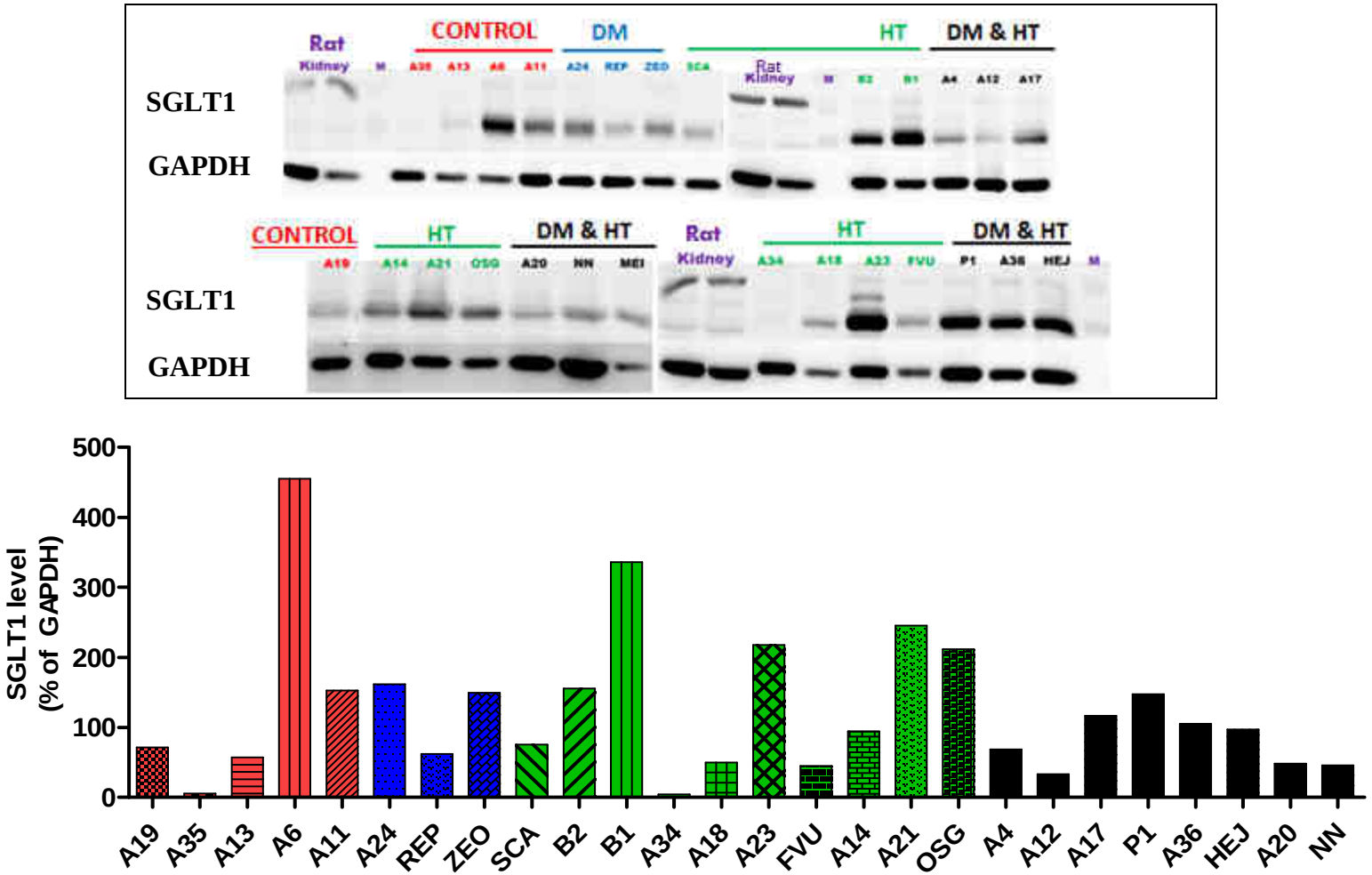

Figure 48. ***The expression level of Sodium-D-glucose cotranspoter 1 (SGLT1) in IMA of bypass patients***. The expression level of SGLT1 in internal mammary artery segments was determined by Western blot. Upper panels show representative immunoblots; lower panels corresponding cumulative data. Results are shown as ratio SGLT1/GADPH of each patient. Abbreviations: CONTROL: control group; HT: hypertensive group; DM & HT: diabetic and hypertensive group.

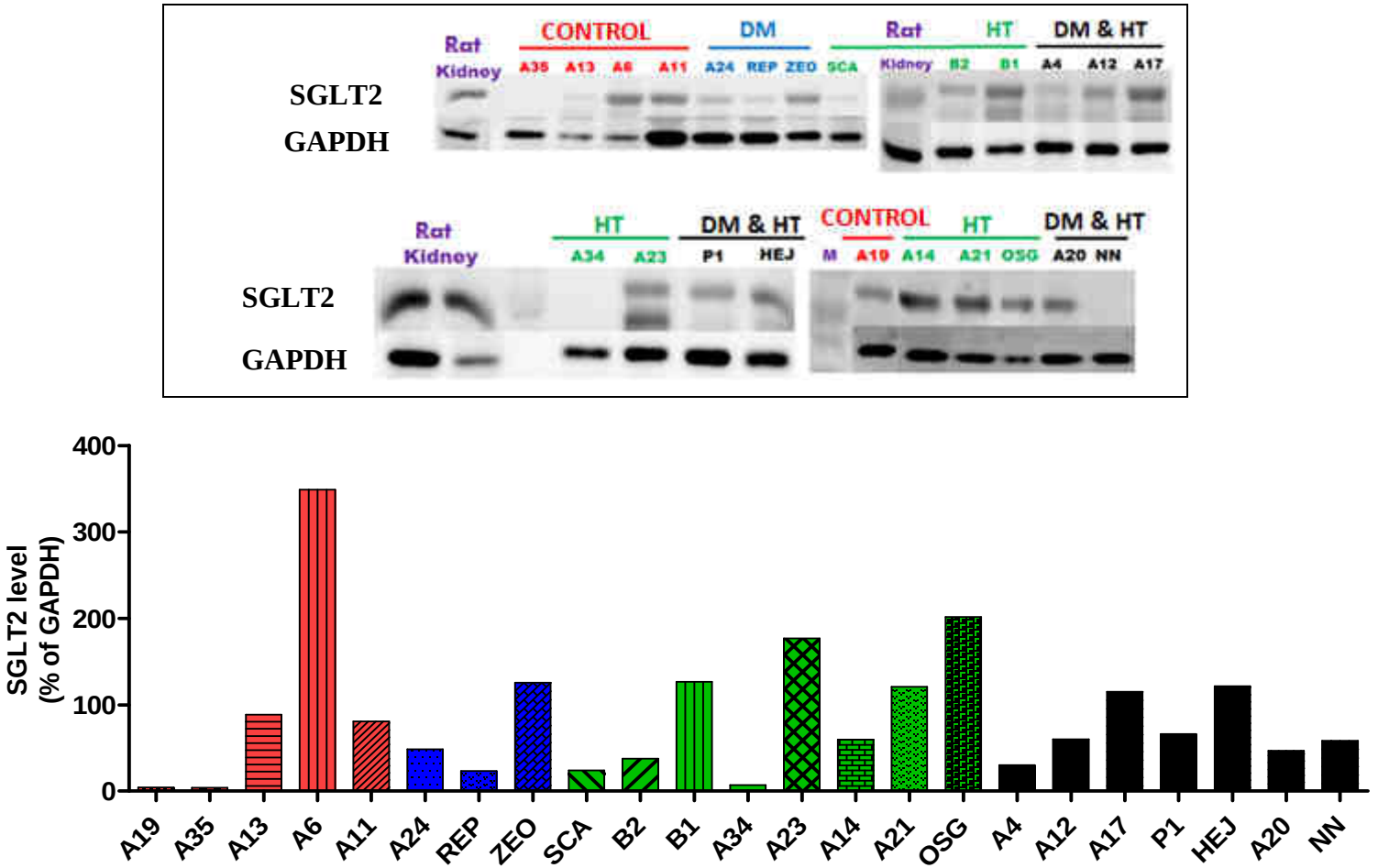

Figure 49. ***The expression level of sodium-D-glucose cotranspoter 2 (SGTL2) in IMA of bypass patients***. The expression level of SGLT2 in internal mammary artery segments was determined by Western blot. Upper panels show representative immunoblots; lower panels corresponding cumulative data. Results are shown as ratio SGLT2/GADPH of each patient. Abbreviations: CONTROL: control group; HT: hypertensive group; DM & HT: diabetic and hypertensive group.

6.3. Discussion

The striking result of this chapter was that we characterized the vascular properties in isolated IMAs from a population of cardiac patients passed coronary bypass surgery. Thus, despite considerable heterogeneity due to differences in sex, age, and disease status, our study in human materials represents a more relevant experimental model than blood vessels from animals. Based on the analyzed data of characteristics of patients involved in our study, the portion of diabetic patients among hypertensive patients is ~34% (20 over 58). An approximate proportion of diabetic patients was also found in the population with cardiac diseases from another studies (Barner, Sundt et al. 2001); (Okon, Chung et al. 2005). This observation confirmed the strong linkage between DM and hypertension, which has been well documented.

The principal findings of this chapter indicate the involvement of diabetes and hypertension in the augmentation of oxidative stress in isolated IMA from bypass patients. Besides, we have shown the important role of antioxidants (NAC) and antioxidant enzymes (SOD and CAT) in reducing the enhanced vascular ROS. At the same time, we also indicated that uncoupled eNOS, NADPH oxidase are the most major enzymatic sources of ROS in human IMA.

The reduction in ROS production in IMA from diabetic patients after 30 min incubation with L-NA suggests that the net effect of eNOS activity in diabetic vessels is ROS production rather than NO production. Our findings confirm previous ones in regard to the association between high level of oxidative stress and endothelial dysfunction in diabetes. Several studies have highlighted the potential importance of dysfunctional eNOS regulation in vascular disease states (Hink, Li et al. 2001); (Maier, Cosentino et al. 2000). Huraux and colleagues have suggested that in human IMAs, the endothelium is an important source of superoxide anion production (Huraux, Makita et al. 1999). The group of Guzik firstly found that the significantly increased superoxide anion production in human IMA from diabetic patients is mediated by upregulated eNOS uncoupling. Furthermore, they suggested that the increase possibly was mediated by PKC signaling, in the pathophysiology of ED in human diabetes mellitus (Guzik, Mussa et al. 2002).

At the same time, we found that VAS-2870 also significantly reduced the fluorescent signal of DHE, which indicates that reduced NADPH oxidase is a major source of $O_2^{\bullet-}$ production in IMA from diabetic and/or hypertensive patients. Our data are consistent with previous ones in the literature, which show the association between diabetes and increased activity of NADPH oxidase in the vascular wall of human IMA (Guzik, West et al. 2000);

(Guzik and Harrison 2006). Guzik and colleagues also found an abundance of NADPH oxidase protein and its elevated activity in IMA from diabetic patients compared with non-diabetic ones (Guzik, Mussa et al. 2002). Similarly, in another study, it has been observed that type 2 diabetes is related to increased membrane translocation of the cytosolic subunits p47phox and Rac1 of NADPH oxidase, explaining the activation of vascular NADPH oxidase in these patients.

In addition, we found that cyclooxygenases (COX), cytochrome P450 and mitochondria are also involved in the elevated level of ROS in IMA in diabetic and/or hypertensive patients compared with the non-diabetic and normotensive patients. Mitochondria and cytochrome P450 have been considered as a potential source of ROS in vascular wall (Fleming, Michaelis et al. 2001); (Zangar, Davydov et al. 2004), but data in IMA from diabetic patients are rare. The inducible form of COX, named COX-2, is not expressed under physiological conditions but it can be rapidly expressed under pathological conditions. COX-2 is a rate-limiting enzyme that catalyzes the conversion of free arachidonic acid into prostaglandin H_2, the first step in the biosynthesis of prostaglandin and thromboxane (Davidge 2001). Our findings are in line with previous studies in the literature showing that cyclooxygenases is important sources of ROS in human vessels. Indeed, it has been shown that COX-2 expression is higher in IMA from diabetic patients compared with non-diabetic ones (Gordillo-Moscoso, Ruiz et al. 2013). However, the group of Guzik has documented that inhibition of cyclooxygenase had minor effect on the formation of ROS in comparison with inhibitors of eNOS and NADPH oxidase. Besides the contradictory results, the scientific evidence regarding the contribution of COX, cytochrome C and mitochondrial chain reaction in oxidative stress in human IMA is still limited in comparison to eNOS and NADPH oxidase, therefore, further investigations are needed.

Our data also indicates the enhanced formation of peroxynitrite (OONO–), which plays a key role in the pathogenesis of the cardiovascular complications of diabetes (for reviews, see section 1.2.3.2). Peroxynitrite is a strong oxidant, which reacts at a relatively slow rate with various biomolecules, leading to cardiovascular dysfunction via multiple mechanisms. One of these pathways involves DNA strand breakage and consequent activation of the nuclear enzyme poly(ADP-ribose) polymerase (Stadler 2011); (Soriano, Virag et al. 2001); (Szabo, Zanchi et al. 2002); (Pacher, Liaudet et al. 2002). Besides, OONO–-mediated oxidation of tetrahydrobiopterin, a critical cofactor for eNOS, may represent a pathogenic cause of uncoupling of eNOS, leading to production of $O_2^{\bullet -}$ rather than NO (Forstermann and Munzel 2006); (Pacher, Beckman et al. 2007). Our results are consistent with previous one in literature. Du et al showed that the incubation of human endothelial cells with HG led to an

increased formation of $ONOO^-$ formed from NO and $O_2^{\bullet-}$ (Du, Edelstein et al. 2001). They suggested that $ONOO^-$ are the mediators of the cytotoxic effects of HG on EC. In addition, the group of Guzik has characterized human IMA, saphenous vein, and radial artery obtained from bypass by evaluating the relationships and interactions between $O_2^{\bullet-}$ production, NO bioavailability, and $ONOO^-$ formation. They quantified the $ONOO^-$ production by using luminol-enhanced chemiluminescence and found matched increases in $O_2^{\bullet-}$ and NO production resulting in increased $ONOO^-$ formation (Guzik, West et al. 2002). These findings suggest that the release of $O_2^{\bullet-}$ from human blood vessels is modulated not only by total $O_2^{\bullet-}$ production, but also by NO production. Therefore, our findings support the involvement of the powerful oxidant $ONOO^-$ in the augmentation of oxidative stress in isolated IMA from bypass patients. Moreover, oxidative stress is more pronounced in diabetic and/or hypertensive patients.

In regared to the expression levels of eNOS and peNOS, unfortunately, our data show a high fluctuation. Besides, experiments by IHC and Western blot show a contrary. The reason for this variation still remains unclear. It migh be due to differences in sex, age, and disease status in human IMA from each patient. Besides, the limitation in a number of investigated samples can also causes the variation in the reults. Consequently, we could not see a clear message. Therefore, further experiments need to be performed with a larger number of patients to clarify the expression level of these proteins in human IMA.

In addition, we found an increased expression level of the angiotensin system by performing IHC in IMA from bypass patients. Both Ang II and its two receptors AT_1 and AT_2 were found significantly increased in IMAs from diabetic and/or hypertensive patients compared to the control group. Since Ang II is a known factor in the pathogenesis of diabetic cardiovascular complications, which can induce $O_2^{\bullet-}$ formation, leading to the increased level of oxidative stress (Nguyen Dinh Cat, Montezano et al. 2013); (Touyz 2004). Our results are in line with previous findings in the literature (Berry, Hamilton et al. 2000; Giacchetti, Sechi et al. 2005). Indeed, Berry and colleagues have shown that Ang II can increase $O_2^{\bullet-}$ production in human arteries and that this effect is AT_1 receptor dependent, since it was blocked by an AT_1 receptor antagonist (losartan) (Berry, Hamilton et al. 2000). Similarly, our data indicates that the expression level of AT_1 receptor is significantly increased in IMA of diabetic and/or hypertensive patients. Besides, our result also shows the significant enhanced expression level of AT_2 receptor in IMA from hypertensive patients and diabetic patients with hypertension. It has been reported that Ang II activates the AT_2 receptor, resulting in the increased production of BK and NO (Gohlke, Pees et al. 1998); (Siragy, Inagami et al. 1999).

NO, in turn, might react with $O_2^{\bullet-}$ leading to augmented formation of $ONOO^-$, consequently, enhances the level of oxidative stress. Moreover, van de Wal and colleagues have found that AT2 receptor mRNA is present in human IMA, however, the AT_2 receptor stimulation did not mediate vasodilation in these arteries (van de Wal, van der Harst et al. 2007). Taken together, our findings have confirmed a pronounced upregulation of the angiotensin system in IMA from diabetic and/or hypertensive patients. Beside, unlike eNOS, which was highly expressed only in the endothelium of IMA, the expression of angiotensin system was found enhanced both in endothelium and in SMC. Our observation is consistent with others studies in vitro (Griendling, Minieri et al. 1994) as well as in human (Berry, Hamilton et al. 2000). This suggests that vascular SMCs may be an important source of $O_2^{\bullet-}$ generation besides the uncoupled eNOS in endothelium.

Finally, based on our data of the expression level of SGLTs, we were unable to conclude about the expression level of SGLTs in human IMA due to the high fluctuations of receiving results. Besides, it is also due to the fact that the molecular weight of SGLT1 observed in IMA is lower than the one in rat kidney, which can be explained by the cleavage and degradation of the protein, or the antibody is not specific for the human protein. We propose to evaluate the expression level of SGLTs mRNA in human IMA for further study in order to characterize the expression level of these D-glucose transporters in blood vessels.

Taken together, our findings in this chapter suggest the involvement of superoxide anion and peroxynitrite in the increased level of oxidative stress in IMA from diabetic and/or hypertensive patients. In addition, further experiments performed on a larger number of patients may be able to clarify more precisely the expression level of eNOS and local angiotensin system in IMA from patients with diabetes and/or hypertension. Besides, the expression levels of SGLTs in IMA from bypass patients still need to be clarified by evaluating the expression level of SGLTs' mRNA.

There were certain limitaions in our work:

- The difficulty in collecting samples and their small size. We obtained only a small amount of proteins from human IMA; therefore, we are limited to study all interesting markers.
- A small number of subjects in non-diabetic and normotensive group raises a difficulty for us to conclude a precise message.
- The difficulty in controlling of the integrity of the endothelium, it is hard to distinguish the received results come from the association with diabetes or losing the

integrity of the endothelium. To be more precise, we need to evaluate the samples in both conditions: with and without endothelium (endothelium could be denuded mechanically). However, the limited size of received samples could not allow us have all the conditions.

- The high fluctuation among the patients. The variation between groups may be due to the heterogeneous clinical characteristics of bypass patients. Such patients have differences in their genetic backgrounds, their atherosclerotic risk factors, their disease duration and severity, and their drug therapy.
- The variation between groups also might be related to the fact that the NO and $O_2^{\bullet -}$ balance may be different in vessels between *ex vivo* and *in vivo* study because of tonic activation of endothelial NO production by shear stress and because of other sources of NO.

GENERAL DISCUSSION AND CONCLUSIONS

The first main purpose of our study was to establish an *ex vivo* model of HG-induced endothelial dysfunction in different types of isolated arteries from male Wistar rat and in porcine coronary artery. The literature has reported that HG attenuated the ACh-mediated endothelium-dependent relaxation in isolated blood vessels of non-diabetic animal, such as in rabbit aorta (Tesfamariam, Brown et al. 1990; Tesfamariam, Brown et al. 1991; Goel, Thor et al. 2008), in rat aorta (Wang, Xiong et al. 2005; Qian, Wang et al. 2010; Goel, Zhang et al. 2007); and rat mesenteric artery (Salheen, Panchapakesan et al. 2015; Taylor and Poston 1994). However, our *ex vivo* model of short-term incubation (3 – 6 h) of isolated arteries from male Wistar rat (such as aorta, main mesenteric artery, renal, carotid and femoral arteries) with HG (25 and 44 mM) did not show any alteration of ACh-mediated endothelial relaxation. Our unexpected results raised the question regarding the contribution of oxidative stress to the attenuation of endothelial relaxation in blood vessels. However, we have found that the oxidative stress induced by 30 min of artery rings incubation with pyrogallol (10 and 30 µM) significantly enhanced the contractile response to PE and markedly attenuated endothelium-dependent relaxation response to ACh in aortic rings from male Wistar rat. Our findings are in line with the previous ones in the literature, which show that the auto-oxidation of pyrogallol induces oxidative stress resulting in the impaired endothelium-dependent relaxation response to ACh in the rat aorta (Jin, Qian et al. 2009; Demirci, McKeown et al. 2008; Qian, Fu et al. 2012; Yeh-Siang, Subramaniam et al. 2011). Hence, although oxidative stress induced by pyrogallol caused the endothelial dysfunction in our rat aortic rings, the level of oxidative stress induced by HG might be too small to alter the endothelium-dependent relaxation of healthy rat arteries in our *ex vivo* model. Therefore, it could not be detected. It might also possibly be related to certain factors, such as a gender, age, strain, species of studied animals, and conditions of housing of animals. The literature has also reported the fluctuation of HG-induced impaired endothelium-dependent relaxation between male and female animals (Goel, Zhang et al. 2007; Goel, Thor et al. 2008), as well as between different strains (such as Sprague Dawley and Wistar rats) and species (such as rabbits and rats) (Rama Krishna Chaitanya and Chris 2013; Dhar, Dhar et al. 2010). Besides, the literature also indicates that exposure to the same time and same glucose concentration can modulate endothelial function in different way, depending on the vascular bed. Thus, the individual vascular beds exhibit differences in vascular reactivity; therefore, HG may affect differentially relaxation and

contraction responses. Moreover, the impairment of endothelial relaxation induced by HG in the same type of artery still differ to some extent between publications (Qian, Wang et al. 2006; Zhang, Yang et al. 2004; Wang, Xiong et al. 2005). In addition, it might also be related to the different baseline of the health status of the studied animals due to the differences in animal age, food, water supplied, conditions of caging or housing system such as the temperature, humidity, and cleanliness of stored room or the behavior of animals due to the different level of stress caused during the performance of experiments. Overall, such reasons might explain the differences between our results and previous ones.

Since the effect of HG on the ACh-mediated endothelium-dependent relaxation might be too small in our studied healthy rats, we evaluated the effect of HG on endothelium-dependent relaxation in aortic rings with a partially impaired endothelial function, which was induced by a low concentration of pyrogallol (3 µM). Unexpectedly, it has been shown that 6 h co-incubation of aortic rings with elevated D-glucose (25 mM) and pyrogallol (3 µM) did affect neither contractile nor relaxation responses. The lack of effect of HG in endothelium-dependent relaxation might be related to the fact that the level of oxidative stress induced by HG in short time incubation was too small in these aortic rings from male Wistar rats. It should be noted that almost all *ex vivo* models of HG-induced endothelial dysfunction were performed in aorta from Sprague Dawley rats. Besides, the literature has shown that HG induced a higher level of impairment of endothelium-dependent relaxation in aortic rings from Sprague Dawley rats than from Wistar rats with the same incubation conditions. The different susceptibility among rat strains could contribute to the variation of effect of HG-induced endothelial dysfunction. Genetic factors between animal strains might possibly explain the differences in strain-dependent susceptibility to high glucose. Therefore, we suggest to use aortic rings from Sprague Dawley rats instead of Wistar rats to evaluate the effect of HG on endothelium-dependent relaxation.

In addition, we suggest that the lack of effect of HG in endothelium-dependent relaxation response in aortic rings might be related to the fact that the combination of HG and pyrogallol induced the enhanced formation of H_2O_2, which can act as an EDHF, and hence would compensate for the loss of NO. As Matoba's group has shown that catalase (CAT), which dismutates H_2O_2 to form water and oxygen, inhibited ACh-induced endothelium-dependent relaxation and hyperpolarization attributed to EDHF in mouse small mesenteric artery (Matoba, Shimokawa et al. 2000). Moreover, CAT, when inactivated at its peroxide-binding site by aminotriazole, lost its inhibitory effect on the EDHF-mediated relaxation. It also has been suggested that H_2O_2 may fully compensate for the loss of NO in arterioles

isolated from patients with coronary artery disease via an EDHF-type mechanism (Phillips, Hatoum et al. 2007). These findings suggested that the reduced NO bioavailability in diabetes may unmask the effect of EDHF as a back-up mechanism because under control conditions NO suppresses the action of EDHF (Cai 2005; Shi, Ku et al. 2006). Thus, further studies are required to use CAT and PEG-CAT to block the effect of the extra- and intracellular of H_2O_2 on relaxation responses in order to evaluate the effect of HG on NO-mediated endothelium-dependent relaxation in rat isolated vessels.

Next, since the short time incubation might be one of the reasons why HG did not affect the endothelium-dependent relaxation in aortic rings from Wistar rats, we have evaluated the effect with HG on endothelium-dependent relaxation response after long-term incubation of rat aorta and porcine coronary artery (PCA). Unexpectedly, 15 h and 22 h incubation of aortic rings in DMEM media with HG (25 mM) depressed the PE-induced contractile response. Therefore, we were unable to evaluate the effect of HG on endothelium-dependent relaxation in long-term period in aortic rings from male Wistar rats. It could be explained by the release of inducible NOS and COX induced by proinflammatory cytokines. Although, polymixin B (10 µg/ml) was already added to the media to neutralize the effect of lipopolysaccharides-induced production of proinflammatory cytokines such as TNF-α, IL-1 and IL-6 (Patterson, Dahlquist et al. 2009; Paneni, Beckman et al. 2013), it seems that polymixin B did not fully inactivate the action of proinflammatory cytokines or the concentration of polymixin B used in our study was insufficent. Therefore, besides polymixin B, peniciline and streptomycin were supplemented to DMEM media. We suggest that adding a combination of 1400W and indomethacin (inhibitors of inducible NOS and COX, respectively) to DMEM media may possibly improve the contractile response to PE in aortic rings after long time incubation.

In contrast, porcine heart closely resembling the human heart from the point of view of size, physiology and anatomy (Hearse and Sutherland 2000) and PCA with a larger intima-media thickness compared to rat aorta (Zoumi, Lu et al. 2004) may avoid the deterioration of endothelial and smooth muscle cells' functions during long-term incubation. PCA rings were incubated with 44 mM D-glucose for 24 h in RPMI 1640 media supplemented by penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 µg/mL) and polymixin B (10 µg/ml). RPMI 1640, a less nutrient-rich medium, contains less calcium and more phosphate in comparison to DMEM media. Moreover, RPMI 1640 was also supplemented by polymixin B (10 µg/ml) - an inhibitor of lipopolysaccharides. Therefore, RPMI 1640 media may possibly reduce the release of pro-inflammatory factors, which might be the reasons for the depression

of PE-induced contractile response in rat aorta after long period incubation (as reported in Chapter 3). Thus, our results have shown that a 24 h incubation of PCA rings with 44 mM D-glucose in RPMI 1640 media induced a small impairment of BK-mediated endothelium-dependent relaxation, but did not affect the SNP-mediated endothelium-independent relaxation. Besides, the 6 h incubation of PCAs in the same conditions did not alter the BK-mediated endothelium-dependent relaxation. In addition, our results showed that gliflozin did not affect the small impairment of endothelium-dependent relaxation induced by HG in PCAs. Our findings also indicate that the long-term incubation (24 h) of HG (44 mM) induced only a small impairment of endothelium-dependent relaxation to BK in PCAs. We suggest increasing the time incubation of PCAs in HG (44 mM) from 24 h to 36 and 48 h to evaluate the effect of high glucose on endothelium-dependent relaxation in longer period.

In addition, although the *ex vivo* model has a number of advantages as it is the most direct method to measure the reactivity of blood vessels to chemical and physical stimulis, it also has some limitations. Firstly, endothelium – the most inner layer of isolated arteries is very thin and extremely sensitive, it can be easily damaged during preparations due to technical problem. Secondly, there is a difficulty in reproducing the exact environment for the arteries as inside the body. Moreover, isolated arteries will not last indefinitely and their response may not be identical between *ex vivo* and *in vivo* conditions. Hence, besides using *ex vivo* models, we can use the *in vivo* diabetic models to assess the hyperglycemic effect on endothelium-dependent relaxation. Normally diabetic models are classified into genetic or spontaneously induced models and non-genetic or experimentally induced models. Some of the most commonly used *in vivo* diabetic models are outlined in Table 16. There are certain genetic models such as Zucker diabetic fatty rat, ob/ob and db/db mice which develop obesity and diabetes spontaneously resembling human T2D by mutations in either the leptin gene (*Lep*) or its receptor (*Lepr*) (Leptin is a hormone that regulates appetite, and induces unregulated feeding). However, the development of diabetes in these genetic models is predominantly genetically determined unlike in humans (Shafrir 2010). Moreover, these animals are expensive and are not easily available for the investigative purposes as well as regular screening experiments. A number of non-genetic models have been developed in last three decades for diabetes research including alloxan (AX) /streptozotocin (STZ)-induced diabetes, high-fat diet fed, fructose-fed, combination of high-fat diet-fed and STZ, partial pancreatectomy, and nicotinamide–STZ models (Table 16).

Table 11: List of most commonly *in vivo* diabetic models used to modulate the contribution of hyperglycemia in the pathogenesis of diabetes. Modified from (King 2012).

		Induction mechanism	Models	Features
Type 1 diabetic models		Chemically induction	- Alloxan - Single high-dose STZ - Multiple low-doses STZ	Simple model of hyperglycemia
Type 2 diabetic models	**Obese**	High-fat diet High-fructose diet		
		Combination of chemical induction and high-fat diet	**Single low-dose STZ and high-fat diet feeding**	Obesity-induced hyperglycaemia
		Obese models (monogenic)	- ob/ob mouse - db/db mouse - Zucker fatty rat	Obesity-induced hyperglycaemia
	Non obese	Surgical diabetic animals	Partial pancreatictomized animals e.g. dog, primate, pig & rats	Hyperglycaemia induced by insulin deficiency
		Nicotinamide-STZ induction		

Most commonly used chemicals agents used to induce diabetes are alloxan (AX) and streptozotocin (STZ). Both STZ and AX with β-cell toxicity abilities have been used for producing chemically induced T1DM models by administration with a high-dose or repeated low-doses for several days (30-40 mg.kg-1). STZ and AX enter the pancreatic β-cells via the GLUT2 glucose transporter and destroy them through oxidative stress mechanisms. More specifically, AX is a uric acid derivative and STZ is a natural antibiotic produced by the bacterial species Streptomyces achromogenes (Szkudelski 2001). However, the use of alloxan nowadays is significantly lower compared to STZ due to its lesser efficacy and some proven side effects in animals e.g., liver and kidney damage. STZ with alkylating properties causes alkylation and thus fragmentation of DNA, modifies biological macromolecules and finally destroys β-cells. The development of hyperglycemia in STZ-induced diabetes model is primarily due to the direct pancreatic β-cells destruction, and resulting insulin deficiency rather than the consequence of insulin resistance; therefore, it imitates mostly T1D (Bolzan and Bianchi 2002).

On the other hand, T2D is a very heterogeneous disease involving hyperglycemia, insulin resistance, β-cells dysfunction and liver dysfunction. However, none of the above-mentioned models combine all these clinical pathogeneses of T2D. Although high-fat or high-fructose diet models can induce insulin resistance as well as impaired glucose tolerance. However, they take significantly longer time to develop the complete pathogenesis of T2D

which is not suitable for most of the researchers. The STZ–nicotinamide model was originally developed by Masiello and colleagues (Masiello, Broca et al. 1998). Masiello et al developed this model by injecting (i.p.) a dose of 230 mg/kg BW nicotinamide 15 min prior to a STZ injection (i.p.) at a dose of 65 mg/kg BW in 3-month-old male Wistar rats. The STZ–nicotinamide injection induced T2D is based on fact that the STZ-induced DNA damage stimulates DNA repair mechanisms that consume large quantities of nicotinamide adenine dinucleotide. The supplementation of nicotinamide serves as a partial protection against excessive pancreatic beta cell damage caused by STZ. This model resembles a non-obese non-genetic model of T2D. In addition, partial pancreatectomy model is another non-obese model, which induces T2D in rats by removal of 85–90% of the pancreas to reduce pancreatic β-cell masses. These animals displayed mild to moderate hyperglycemia from day 4 post-surgery and was maintained for a further 6-week period. Nevertheless, this model has not become the popular method in research due to the regeneration of pancreatic cells. Moreover, advanced surgical skill is required to develop this model. At present time, the combination of high-fat or high-fructose diet with a diabetic inducer particularly STZ is considered as a best choice for the development of T2D models. Srinivasan et al. developed a high-fat diet-fed and STZ-injected rat model by a high-fat diet containing 58% calories from lard to Sprague Dawley rats for 2 weeks prior to STZ injection (i.p.) with a dosage of 35 mg/kg BW and continued ad libitum feeding of high-fat diet throughout the experimental period (Srinivasan, Viswanad et al. 2005). The high-fat diet-fed and STZ-injected rat model showed frank hyperglycemia, significantly elevated total serum cholesterol, and serum triglycerides. Serum insulin concentration was significantly lower in this group compared to the rats fed only a high-fat diet. To date, it is the most suitability model for T2D for either rapid or routine pharmacological screening of antidiabetic drugs and natural products.

Once an *ex vivo* hyperglycemic model or *in vivo* diabetic model are successfully developed, we can assess the effect of high glucose on the endothelial relaxation of blood vessels. Following the data published in the literature, high glucose induces attenuation of the endothelium-dependent relaxation and does not affect the endothelium-independent relaxation. In case we find the impaired endothelium-dependent relaxation in blood vessels, we can evaluate the effect of SGLT1/2 inhibitors on this impairment. Such data will clarify the role of SGLT1/2 in the transport of glucose in blood vessels. Besides, since the studies regarding expression of SGLT1/2 and their effect on blood vessels are still limited, further study will need to be performed to clarify the presence of SGLT1/2 as well as their

contribution on the level of oxidative stress induced by HG in endothelial cells in blood vessels in diabetes.

To sum up, our findings have led us to conclude that:

- The auto-oxidation of pyrogallol induces oxidative stress resulting in a significant enhancement of the contractile response to PE and attenuation of endothelium-dependent relaxations to ACh in rat aortic rings.
- The effect of short-term incubation with HG on the ACh-mediated endothelium-dependent relaxation is too small in our studied healthy rats. Therefore, it could not be detected. It might possibly be related to certain factors, such as a gender, age, strain, species of studied animals, and conditions of housing of animals.
- The long-term incubation of aortic rings with HG depressed the PE-mediated contractile response
- The long-term incubation of PCAs with HG induced a small impairment of BK-mediated endothelium-dependent relaxation. Although this alteration is too small, therefore, we could not examine the protective effect of gliflozin on HG-induced the impairment of endothelial relaxation.

We suggest to :

- Use isolated blood vessels from male Sprague Dawley rats instead of Wistar rats to evaluate the effect of HG on endothelium-dependent relaxation.
- Add a combination of 1400W and indomethacin (inhibitor of inducible NOS and COX, respectively) to RPMI 1640 media to improve the contractile response to PE in aortic rings after long time incubation.
- Use CAT and PEG-CAT to block the effect of the extra- and intracellular of H_2O_2 on relaxation responses in order to evaluate the effect of HG on NO-mediated endothelium-dependent relaxation in rat isolated vessels.
- Increase the time incubation of PCAs in HG (44 mM) from 24 h to 36 and 48 h to evaluate the effect of high glucose on endothelium-dependent relaxation in longer period.
- Develop an *in vivo* diabetic model by a high-fat diet containing 58% calories from lard to Sprague Dawley rats for 2 weeks prior to STZ injection (i.p.) with a dosage of 35 mg/kg BW
- Determine the expression level of SGLT1/2 in blood vessels in normal and high glucose conditions.

In the second study with human artery, we investigated the internal mammary arteries (IMA), which were taken from patients underwent coronary bypass surgery. It should be mentioned that diabetic patients have a 2 – 4 fold increased risk of coronary artery disease over non-diabetic patients (Al-Nozha, Ismail et al. 2016). Therefore, coronary artery bypass grafting seems to be particularly important in diabetic patients. In fact, 12% to 38% of the patients that undergo coronary artery bypass surgery are diabetics (Herlitz, Brandrup-Wognsen et al. 1997). However, the influence of diabetes on the results of coronary bypass surgery has not been well documented. Hence, we characterized the vascular properties in isolated IMA from diabetic patients, who underwent coronary bypss surgery in the absence or presence of main cardiovascular risk factors such as hypertension and age. Thus, despite considerable heterogeneity due to differences in sex, age, disease status, and treatments, the study of human IMA represents a more pertinent model of blood vessels than those from animals. In our investigated population, we found that the presence of about ~34% (20 over 58) diabetic patients among all hypertensive patients, which supports the strong linkage between DM and hypertension. As an approximate proportion of diabetic patients was also found in the population with cardiac diseases from another studies (Barner, Sundt et al. 2001); (Okon, Chung et al. 2005).

Since the oxidative stress plays a pivotal role in the development of diabetes as well as CVD, we first aimed to identify the relevance of the two main cardiovascular risk factors (including diabetes and hypertension) and the level of oxidative stress in IMAs from bypass patients. We found that the level of oxidative stress in IMA from hypertensive patients and patients with diabetes and hypertension was significantly higher than the one in non-diabetic and normotensive patients. The characterization of the vascular level of oxidative stress has indicated the involvement of several sources including uncoupled eNOS, COXs, NADPH oxidase, cytochrome P450, and the mitochondrial respiration chain in the enhanced formation of ROS in IMA from hypertensive patients and diabetic patients with hypertension.

In addition, we also found an enhanced formation of $ONOO^-$ and an increased expression level of eNOS in the diabetic and hypertensive group compared to non-diabetic and normotensive patients. These findings suggest the involvement of an uncoupled eNOS in generation of $O_2^{\bullet-}$, which, in turn, reacts rapidly with NO, reducing NO bioactivity and leading to augmented formation of $ONOO^-$. Our results are in line with previous ones in the literature. Indeed, Huraux and colleagues have reported that in human IMAs, the endothelium is an important source of $O_2^{\bullet-}$ (Huraux, Makita et al. 1999). The group of Guzik found the

matched increases in $O_2^{\bullet-}$ and NO production resulting in increased $ONOO^-$ formation in human IMA from diabetic patients is mediated by upregulated eNOS uncoupling (Guzik, Mussa et al. 2002; Guzik, West et al. 2002). Therefore, our findings support the involvement of the powerful oxidant $ONOO^-$ in the augmentation of oxidative stress in isolated IMA from bypass patients. Moreover, oxidative stress is more pronounced in diabetic and/or hypertensive patients. Although, we could not confirm these findings by examination the expression level of eNOS by Western blot. Our Western blot data of the expression levels of eNOS and peNOS show a high fluctuation. It migh be due to differences in clinical profile of each patient, such as the differences in age, sex, disease status and treatments of bypass patiens. Besides, the limitation in a number of investigated samples can also cause the variation in the results. Moreover, the contrary results between IHC staining and Western blot might be related to the fact that IHC staining and Western blot were not performed in the same group of patients; because the limited size of obtained sections of IMA from bypass patients did not permit us to perform each type of experiments using the same samples. Hence, the expression level of eNOS in IMAs from hypertensive and diabetic patients is still questionable. Further experiments need to be performed with a larger number of patients to clarify the expression level of eNOS and peNOS in human IMA. In addition, we suggest also performing the vascular reactivity study to clarify the role of eNOS in endothelium of IMA from bypass patients. Results from vascular reactivity study might give us the link between the relaxant responses in human IMAs and the main cardiovascular risk factors such as diabetes, hypertenstion, smoking, and age).

Moreover, the increased level of ROS has been shown to be induced by the local angiotensin system in endothelial cells (Pueyo, Arnal et al. 1998; Sohn, Raff et al. 2000; Touyz 2004). Therefore, we determined the expression level of components of the angiotensin system in IMA from diabetic patients in order to evaluate the alterations in the expression level of the local angiotensin system in the presence of single or combined two main cardiovascular risk factors: diabetes and hypertension. We found an increased expression level of angiotensin system by performing IHC in IMA from bypass patients. Both Ang II and its two receptors AT_1 and AT_2 were found significantly increased in both endothelium and smooth muscle cells of IMAs from hypertensive patients and diabetic patients with hypertension compared with non-diabetic and normotensive patients. Our results are in line with previous findings in the literature. Indeed, Berry and his colleagues have firstly demonstrated that Ang II can increase $O_2^{\bullet-}$ production in human IMA (Berry, Hamilton et al. 2000). It also should be noted that, the expression of angiotensin system was found enhanced

both in the endothelium and in the smooth muscle cells. Our observation was consistent with others studies *in vitro* (Griendling, Minieri et al. 1994) as well as in human IMA (Berry, Hamilton et al. 2000). This suggests that vascular smooth muscle cells may be an important source of $O_2^{\bullet-}$ generation besides uncoupled eNOS in endothelium. Nevertheless, further experiments need to be performed with a larger number of patients to clarify the expression level of angiotensin system in human IMA by Western blot to confirm the results from immunofluorescence.

Finally, recent studies have shown the benefits of SGLTs inhibitors on cardiovascular outcome in diabetic patients (Zinman, Wanner et al. 2015; Vasilakou, Karagiannis et al. 2013; Dziuba, Alperin et al. 2014; Neal, Perkovic et al. 2013) existing the evidences regarding the expression level of SGLT1 in endothelial cells (Taubert, Rosenkranz et al. 2004; Jin, Yi et al. 2013; Han, Cho et al. 2015). However, the literature related to the expression level of SGLT2 on blood vessels is poorly documented. We have evaluated the expression level of SGLT1/2 in IMA from bypass patients by Western blot in order to clarify the presence of SGLT1/2 in endothelial cells in IMA from diabetic patients. However, we could not to conclude about the expression level of SGLT1/2 in human IMAs due to the pronounced fluctuations in the results as well as a technical problem. As the SGLT1 was observed at a lower molecular weight in comparison with the one in the positive control, we suggest using another positive control such as human kidney to clarify the molecular weight of SGLT1 in human IMAs. In addition, we suggest evaluating the expression level of SGLT1/2 mRNA in human IMA in order to characterize the expression level of these glucose transporters at the molercular level in human IMA.

Taken together, our study in human IMA has highlighted that:

- Clinical profile of patients shows that diabetes and hypertension frequently occur together.
- The involvement of the $O_2^{\bullet-}$, H_2O_2 and $ONOO^-$ in the augmentation of oxidative stress in isolated IMA from bypass patients.
- Several sources including uncoupled eNOS, COXs, NADPH oxidase, cytochrome P450, and the mitochondrial respiration chain are involved in the enhanced formation of ROS in IMA from hypertensive patients and diabetic patients with hypertension.
- IHC staining indicates a significant increased expression level of both eNOS in endothelial cells and angiotensin system in endothelium and SMC. However, further

studies are needed to be performed to confirm these findings by other methods such as examination the expression level of these proteins by Western blot.

- The expression level of SGLT1/2 are unclear. Further studies are required to have a clear message regarding the expression level of SGLTs in human IMA.

We suggest to

- Increase the number of patients
- Perform the vascular reactivity study to clarify the role of eNOS in endothelium of IMA from bypass patients
- Determine the expression level of the angiotensin system by Western blot to confirm the results from immunofluorescence
- Use another positive control such as human kidney to clarify the molecular weight of SGLT1/2
- Determine the expression level of SGLT1/2 mRNA to clarify their presence in human IMA.

Appendix

Table 1. ***Clinical profile of all patients studied.*** Abbreviation: BMI: body mass index; BB: beta blocker; ACE: angiotensin-converting enzyme; AT2: angiotensin receptor 2; ARA II: antagonist receptor of angiotengin II; OAD: oral antidiabetic drugs; 0 = no, 1 = yes.

Experiment	Patients	Time taking sample	Sex	Age	BMI kg/m2	Creatinin clearance (ml/min)	Hospital stay duration (days)	Hyper-tension	Dia-betes	Smokers	Tobacco used	dyslipi-demia	Treatment
IHC staining: DHE, sources of ROS and proteins	**P1**		M	65	32,7	109,8	6	1	1	0	0	1	diuretic, statin, AT2 inhibitor, metformin, aspinin
	P2		M	71	26,9	71	7	1	0	0	0	1	nitres, BB, statin, calcium bloker, aspirin
	P4		M	63	22,6	71,3	7	0	0	0	0	1	aspirin
	P5		M	65	27,3	61,8	7	1	1	0	0	1	insulin, BB, statin, aspirin
	P6		M	61	22,2	75,2	5	0	0	0	0	0	BB, statin, aspirin, ACE
	P7		M	74				1	0				BB,statin Aspirin,ACE
	P8		M	70	23,6	87,2	7	0	0	0	0	0	sur poids, inhibitor de Ca^{2+}, aspirine
	P9		M	78	23,1	66	6	1	0	0	1	1	vasten, lovenox, prednisolone, moduretic, amlor, inhibitor Ca, diuretic, statin,

Experiment	Patients	Time taking sample	Sex	Age	BMI kg/m2	Creatinin clearance (ml/min)	Hospital stay duration (days)	Hyper-tension	Dia-betes	Smokers	Tobacco used	dyslipi-demia	Treatment
													aspirine
	P10		M	70	37,5	129	6	1	1	0	0	1	inhibitor Ca, Nitres, ACE, Oral + insulin, Statin, aspirine, Ticlid/plavix/others
	P11		M	77	24,6	58,7	7	1	0	0	1	1	Inh Ca, BB, ACE, Aspirine, statine,
	P12		M	60	32,4	82,8	7	1	1	1	1	1	aspirine, statines, OAD, BB, antiarrythm
IHC staining DHE	**p14**	11/12/2013	M	65	32,9	108,9	6	1	0	1	1	1	aspirine, HBPM, AVK, statine, BB, ACE, diuretiques
	p16	12/12/2013	M	81	31,1	41,9	11	1	1	0	1	0	notrés; staines, aspirine, OAD, BB, ACE
	p21	17/01/2014	M	61	25,3	78	6	1	0	0	0	1	aspirine, plavix, statine, BB, ACE
	p26	06/02/2014	M	57	26	61,8	23	0	0	0	1	0	BB, statines, aspirin
WB (2014) attempt	**P30 (Ao)**	25/04/2014	M	81	25,3	51,7	6	1	1	0	0	1	coaprovel, loxen, atenolol, alpress, amarel, metformine
	P31(A1)	05/05/2014	M	80	30,9	63,7	10	0	0	0	0	1	detensiel, kardegic, lescol

Experiment	Patients	Time taking sample	Sex	Age	BMI kg/m2	Creatinin clearance (ml/min)	Hospital stay duration (days)	Hyper-tension	Dia-betes	Smokers	Tobacco used	dyslipi-demia	Treatment
	P32(A2)	24/05/2014	M	78	30,3	73,9	7	0	0	0	1	0	plavix, detensiel
WB (2016)	**no name (NN)**	10/10/2014	M	66	24,2	19,3	4	1	1	0	0	0	BB, calcium inhibitor, aspirin, ACE, diuretic, OAD
	A4	20/11/2014	M	79	28,1	59,4	6	1	1	0	0	0	aspirin, diuretic, BB, fluindone, ADO, insuline
	A5	17/12/2014	M	78	27,9	63,9	35	1	0	0	0	1	aspirin, BB, statin, sartan
	A6	16/01/2015	M	69	37,7	83	32	0	0	0	0	1	BB, ACE, diuretic, aspirin, clopidogrel, statins
	A11	30/01/2015	M	62	23,2	69,2	6	0	0	0	0	1	statins
	A12	30/01/2015	F	67	23	75,9	10	1	1	0	0	1	BB, ACE, calcium channels blocker, statin, aspirin, OAD
	A13	04/02/2015	F	63	25,1	91,2	7	0	0	0	1	0	BB, statin, aspirin, clopidogrel, ACE
	A14, A15	05/02/2015	M	71	26,7	80,8	5	1	0	0	0	1	BB, ACE, aspirin,statin,
	A16	10/02/2015	M	65	24,1	68,7	6	1	0	0	1	1	BB, statin
	A17	16/02/2015	M	61	30,2	186,7	7	1	1	0	1	1	BB, nitric, ACE, diuretics, oral antidiabetic, statin, aspirin

Experiment	Patients	Time taking sample	Sex	Age	BMI kg/m2	Creatinin clearance (ml/min)	Hospital stay duration (days)	Hyper-tension	Dia-betes	Smokers	Tobacco used	dyslipi-demia	Treatment
	A18	16/02/2015	M	58	25,6	114,5	7	1	0	0	1	1	calcium channels inhibitor, BB, ACE, aspirin
	A19	06/03/2015	M	72	26,7	68,1	7	0	0	0	1	0	BB, ACE, aspirin
	A20	09/03/2015	M	75	32	77,6	7	1	1	0	1	1	calcium channels inhibitor, BB, aspirin, sartan, nitric, diuretics
	A21	02/04/2015	M	78	33,7	136,9	7	1	0	0	1	1	BB, diuretics, statin, clopidogrel
	A22	09/04/2015	M	85	32,7	66	7	0	0	0	0	1	nitric, diuretics, central antihypertension, aspirin, statin
	A23	09/04/2015	F	53	32	11,6	12	1	0	1	1	1	BB, ACE, aspirin, statin, fluindione
	A24	27/04/2015	M	78	28,4	54,8	10	0	1	0	1	0	BB, ACE/ ARA II, diuretics, OAD, statin, fluindione= AVK anticoagulant
	A25	28/04/2015	M	62	20,8	miss		1	0	0	0	1	no treatment
	B1	29/01/2015	M	76	29,3	80,4	7	1	0	0	1	1	BB, ACE, potassium channels activator, statin, aspirin
	B2	29/01/2015	M	68	25,5	78,8	6	1	0	0	1	0	BB, fluindione AVK =

Experiment	Patients	Time taking sample	Sex	Age	BMI kg/m2	Creatinin clearance (ml/min)	Hospital stay duration (days)	Hyper-tension	Dia-betes	Smokers	Tobacco used	dyslipi-demia	Treatment
													anticoagulant
	MEF	30/01/2015	M	66	19,5	-	6	0	0	0	1	1	BB, statin, aspirin
	MMA	30012015	M	58	34	109,4	6	1	1	0	1	1	BB, ACE, potassium chanels activator, statin, clopidogrel = anticoagulant
	OSG	09/02/2015	M	65	24,1	68,7	6	1	0	0	1	1	BB, statin
	BRC	09/02/2015	M	57	39,4	149,8	7	1	0	0	0	1	aspirin, diuretic, sartan, statin, BB
	REP	04/02/2015	M	51	26	-	8	0	1	1	-	-	BB, ACE, diuretics, insulin, statin, aspirin
	ZEO	06/02/2015	M	46	24,9	134,3	6	0	1	1	0	0	nitirc, BB, ACE, insulin, statin, aspirin
	SCA	13/02/2015	M	84	31,2	59,1	17	1	0	0	0	1	BB, diuretics, statin, aspirin
	FVU	19/02/2015	M	84	27,4	60,7	7	1	0	0	0	1	nitric, BB, sartan, diuretics, statin, aspirin
	HEJ	02/02/2015	M	84	-	-		1	1	-	-	-	calcium channels inhibitor, BB, ACE, OAD, aspirin
PCR/ WB	**A31**	02/10/2015	F	78	29,3	76,6	7	1	0	0	1	1	diuretiques, statines; aspirin
	A32	13/11/2015	F	58	32,7	96,5	6	1	0	0	0	0	Ca inh, BB, ARA

Experiment	Patients	Time taking sample	Sex	Age	BMI kg/m2	Creatinin clearance (ml/min)	Hospital stay duration (days)	Hyper-tension	Dia-betes	Smokers	Tobacco used	dyslipi-demia	Treatment
													II, aspirin
	A33	13/11/2015	M	65	31,6	75,2	7	1	1	0	0	1	Ca inh, BB, ACE, diuretiques, aspirin
	P1	01/12/2015	M	59	24	68,2	7	1	1	1	1	0	Ca inh, BB, ACE, statin, aspirin
	P2	01/12/2015	M	55	33,2	109	8	1	0	0	0	1	Ca inh, ACE, statin, aspirin
	A34	14/12/2015	F	76	27,1	55	6	1	0	0	0	1	BB, ACE, diuretiques, statine, aspirin
	A35	15/12/2015	M	63	26,9	89	7	0	0	0	0	0	-
	A36	19/01/2016	M	61	29,4	129	7	1	1	0	0	1	BB, Ca inhibitor, ACE,
WB in HG	**A26**	29/05/2015	M	62	23,9	82,6	7	1	0	0	0	1	statin, ACE, aspirin, BB
	A27	05/06/2015	F	53	20,9	32,5	8	1	0	0	1	1	statin, ACE, BB, diuretic
	A28	12/06/2015	M	78	28,1	55,1	7	1	1	0	0	1	statin, ACE, BB, diuretic, aspirin, OAD - NO insulin)
	A29	03/07/2015	M	73	22,2	65,9	7	1	0	0	0	1	BB, ARA II, aspirin
	A30	03/07/2015	M	76	23,9	53,4	6	1	0	0	1	1	aspirin, Statin, ARA II, i.v nitrate, inhaled Corticoid and bronchodilators

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Phuong Nga NGUYEN

Caractérisation de la fonction vasculaire dans les vaisseaux sanguins isolés en réponse au glucose élevé et de l'artère mammaire interne de patients diabétiques

Résumé en français

D'abord, nous avons visé à établir un modèle *ex vivo* de la dysfonction endothéliale induite par le glucose élevé (HG) dans les artères isolées de rat Wistar mâle et porc. Notre but était de clarifier le rôle des SGLT1/2 dans les cellules endothéliales dans les conditions HG pour évaluer l'effet protecteur des gliflozines sur la fonction endothéliale. Cependant, HG n'a pas affecté la relaxation dépendant de l'endothélium. L'absence d'effet d'HG pourrait être liée aux facteurs, tels que les conditions d'incubation, le genre, l'âge, la souche, l'espèce d'animal et les conditions de logement. Enfin, nous avons caractérisé les artères mammaires internes humaines (AMI) de 58 patients ayant subi un pontage coronarien au Nouvel Hôpital Civil de Strasbourg. Nous avons trouvé l'association du diabète et de l'hypertension au niveau accru du stress oxydatif dans les AMI humaines. Les niveaux d'expression de la eNOS, des SGLT et du système d'angiotensine local doivent être étudiés plus en détail.

Mots clés :
Le glucose élevé, la dysfonction endothéliale, le stress oxydatif, SGLT, les artères mammaires internes humaines.

Résumé en anglais

Firstly, we aimed to establish an ex vivo model of high glucose (HG)-induced endothelial dysfunction in isolated arteries from male Wistar rat and porc. Then, our goal was to clarify the contribution of SGLT1/2 in endothelial cells under HG conditions to evaluate the protective effect of gliflozins on the endothelial function. However, HG did not affect the endothelium-dependent relaxation response in all tested types of artery. The lack of effect of HG might be related to certain factors, such as the incubation conditions, gender, age, strain, species of studied animals, and conditions of housing of animals. Secondly, we characterized human internal mammary arteries (IMA) of 58 patients underwent coronary artery bypass grafting in the New Civil Hospital of Strasbourg. We found the association of diabetes and hypertension in the enhanced level of oxidative stress in human IMA. The expression levels of eNOS, SGLTs and the components of angiotensin system need to be further investigated.

Keywords :
High glucose, endothelial dysfunction, oxidative stress, SGLT, human internal mammary arteries.