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Protection vasculaire par la nouvelle formulation d'omega3 : Rôle de la NO synthase endothéliale

Par

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Dedication

This thesis is dedicated to my family, my lovely wife Hajer and my adorable daughter Heba for their love, patience and understanding.

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PUBLICATIONS

1. Faraj ZGHEEL, Mahmoud ALHOSIN, Sherzad K. RASHID, Cyril AUGER & Valérie B. SCHINI-KERTH. The highly purified EPA:DHA 6:1 product-evoked endothelium-dependent NO-mediated relaxation in the coronary artery involves a copper-dependent event triggering the redox-sensitive PI3-kinase/Akt pathway to activate eNOS by phosphorylation at Ser 1177, PLOS ONE August 2014 ;9 (8) : e105102

2. Faraj ZGHEEL, Cyril AUGER, Stéphanie PERRIER, Jean-Philippe MAZZUCOTELLI, Olivier MOREL and Valérie B SCHINI-KERTH. The optimized EPA:DHA 6:1 formulation inhibits aggregating platelet-induced 5-HT-mediated contractions in porcine coronary artery and human internal mammary artery rings: role of endothelial NO. En préparation.

ORAL PRESENTATION

Faraj ZGHEEL, Mahmoud ALHOSIN, Cyril AUGER & Valérie B. SCHINI-KERTH. The highly purified EPA:DHA 6:1 product evokes potent endothelium-dependent relaxations of porcine coronary artery rings via the redox-sensitive PI3-kinase/Akt-dependent phosphorylation of eNOS. European Society of Cardiology (ESC) Congress 2012, Munich, Allemagne, 25-29 août 2012. Eur. Heart J. 33 (Suppl.), 512, 2012

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3. Faraj ZGHEEL, Mahmoud ALHOSIN, Cyril AUGER & Valérie B. SCHINI-KERTH. The highly purified EPA:DHA 6:1 product evokes potent endothelium-dependent relaxations of porcine coronary artery involves a copper-dependent event triggering the redox-sensitive PI3-kinase/Akt-dependent to activate eNOS by phosphorylation. European Society of Cardiology (ESC) Congress 2013, Amsterdam, Pays-Bas, 31 aout – 4 septembre 2013.

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
AC	Adenylyl cyclase
ACE	Angiotensin Converting Enzyme
ACh	Acetylcholine
ADP	Adenosine Di-Phosphate
ALA	Alpha-Linolenic Acid
ADM	Adenosine Monophosphate
AT1R	Angiotensin II Type 1 Receptor
AT2R	Angiotensin II Type 2 Receptor
ATP	Adenosine Tri-Phosphate
BH4	Tetrahydrobiopterin
cAMP	Cyclic Adenosine-3',5'-Mono-Phosphate
CBDL	Common Bile Duct Ligation
COX	Cyclooxygenases
CRP	C-reactive protein
GLA	Gamma-Linolenic Acid
DHA	Docosahexaenoic Acid
DPA	Docosapentaenoic Acid
EFA	Essential Fatty Acids
EPA	Eicosapentaenoic Acid
EC	Endothelial Cell
EDCF	Endothelium-Derived Contracting Factor
EDH	Endothelium-Dependent Hyperpolarization
EDHF	Endothelium-Derived Hyperpolarizing Factor
EDRF	Endothelium-Derived Relaxing Factor
EETs	Epoxyeicosatrienoic Acids
EFA	Essential Fatty Acids
eNOS	Endothelial Nitric Oxide Synthase
ER	Estrogen Receptor
GPx	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
HDL	High Density Lipoprotein
HO-1	Hemeoxygenase-1
IK_{Ca}	Intermediate conductance calcium-activated potassium channel
iNOS	Inducible Nitric Oxide Synthase
LA	Linoleic Acid
LDL	Low Density Lipoprotein
LPC	Lysophosphatidylcholine
LPX	Lipoxygenases
LTs	Leukotrienes
L-NA	N^{ω}-Nitro-L-arginine
L-NAME	N^{ω}-Nitro-L-Arginine Methyl Ester
LPS	Lipopolysaccaride
MCP-1	Monocyte Chemoattractant Protein-1
MGJ	Myoendothelial Gap Junction
MnTMPyP	Mn (III) Tetrakis(1-methyl-4 pyridyl) porphyrin, Superoxide

	Dismutase Mimetic
MUFA	Mono-unsaturated Fatty Acids
NADH	Nicotinamide Adenine Dinucleotide
MMPs	Matrix Metalloproteinase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
nNOS	Neuronal Nitric Oxide Synthase
O^{2-}	Superoxide Anions
$OH^{\cdot -}$	Hydroxyl Groups
OA	Oleic Acid
PC	Phosphatidylcholine
PDGF	Platelet-Derived Growth Factor
PEG-SOD	Polyethylene Glycol-Superoxide Dismutase
PGE	Prostaglandins E2
PGH_2	Prostaglandins H_2
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
RAAS	Renin-Angiotensin-Aldosterone-System
ROS	Reactive Oxygen Species
SKca	Small conductance calcium-activated potassium channel
sGC	Soluble Guanylyl Cyclase
SMC	Smooth Muscle Cell
SOD	Superoxide Dismutase
TGF-B1	Transforming Growth Factor-B1
TNF-α	Tumor Necrosis Factor-alpha
TXA2	Thromboxane A_2
VEGF-A	Vascular Endothelial Growth Factor A
VEGFR	Vascular Endothelial Growth Factor Receptor

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RÉSUMÉ

L'endothélium, une monocouche cellulaire recouvrant la lumière des vaisseaux sanguins, est un organe complexe, fortement spécialisé et métaboliquement actif, qui joue un rôle central dans le maintien de la perméabilité et de l'homéostasie vasculaire. L'endothélium exerce ses fonctions, au moins en partie, en formant et libérant de nombreux facteurs agissant sur la tonicité vasculaire. Les facteurs majeurs ayant une activité vasorelaxante sont le monoxyde d'azote (NO), l'hyperpolarisation dépendante de l'endothélium (endothelium-dependent hyperpolarization, EDH) et la prostacycline (PGI_2). Les facteurs vasoconstricteurs endothéliaux comprennent le thromboxane A_2 (TXA_2), l'angiotensine II, prostaglandine H_2 (PGH_2), les espèces réactives de l'oxygène (ROS), et les endothélines.
Les facteurs nutritionnels joueraient un rôle important dans la modulation de la fonction endothéliale. Ainsi, l'influence de l'alimentation sur la santé vasculaire (structure et fonction vasculaires) a fait l'objet de nombreuses études épidémiologiques et interventionnelles. Ces études ont rapporté une amélioration de la fonction endothéliale associée à la consommation de plusieurs aliments dont les fruits, les légumes, les poissons gras, les thés et le vin rouge, et de plusieurs micronutriments incluant les acides gras n-3 (omega-3), les vitamines C, D et E, et les caroténoïdes.

De même, plusieurs études ont montré une corrélation inverse entre l'incidence des maladies cardiovasculaires et la consommation d'acides gras oméga-3, tels que les acides eicosapentaénoïque (EPA, C20:5 n-3) et docosahéxanéoïque (DHA, C22:6 n-3), que l'on retrouve en grande quantité dans les poissons gras (saumon, truite, hareng, sardines, maquereaux…). Les effets bénéfiques de la supplémentation en acides gras oméga-3 au niveau cardiovasculaire comportent notamment la diminution des survenues d'arythmies, des diminutions des taux plasmatiques en triglycérides, des diminutions de la pression artérielle et des diminutions de l'aggrégation plaquettaire, l'ensemble conduisant à une diminution du risque de mortalité cardiovasculaire chez des patients souffrant de maladies cardiovasculaires.
De plus, les effets protecteurs des acides gras oméga-3 pourraient aussi être dus à leur capacité à améliorer les relaxations des artères contrôlées par l'endothélium via la stimulation de la formation des facteurs vasoprotecteurs endothéliaux NO et EDH.

Le but du présent travail de thèse est en premier lieu d'évaluer le potentiel de divers produits à base d'oméga-3 (EPA, DHA, divers ratio et degré de pureté) à induire des relaxations

dépendante de l'endothélium dans des artères coronaires de porc, de déterminer le rôle des composantes vasorelaxantes de l'endothélium NO et EDH, ainsi que les voies de transduction conduisant à l'activation de la eNOS (NO synthase endothéliale). Le second but du travail est d'évaluer l'effet vasculaire bénéfique des acides gras oméga-3 sur la contraction des artères coronaires en réponse aux plaquettes activées, et de caractériser les mécanismes sous-jacents, notamment le rôle de la sérotonine et des prostanoïdes vasoconstricteurs.

La première étude a évalué le rôle de la formulation en acides gras oméga-3 sur la capacité à induire des relaxations dépendantes de l'endothélium. Les effets des divers produits à base d'acides gras oméga-3 ont été étudiés soit sur des anneaux d'artère coronaire de porc soit sur des cultures primaires de cellules endothéliales d'artère coronaire de porc.
Les deux acides gras oméga-3 majeurs, EPA et DHA, ont induit des relaxations dépendantes de la concentration dans des anneaux d'artère coronaire pourvus d'un endothélium fonctionnel, alors que ces relaxations étaient absentes dans les anneaux sans endothélium. La relaxation en réponse à l'EPA à 0,4 % (v/v) était légèrement mais significativement plus prononcée que celle à la DHA (77.8±10.3 et 64.7±12.8%, respectivement). L'évaluation du potentiel de mélange d'acides gras oméga-3 à divers ratios à induire des relaxations dépendantes de l'endothélium a montré que les mélanges EPA:DHA aux ratios 6:1 ou 9:1 induisaient des relaxations significativement supérieures à celles observées pour les ratios 3:1, 1:1, 1:3, 1:6, et 1:9.
De même, l'évaluation du rôle du degré de pureté du ratio 6:1 a mis en évidence qu'un ratio avec une grande pureté d'acide gras oméga-3 EPA:DHA (694:121 mg/g) induisait une relaxation dépendante de l'endothélium significativement plus forte qu'un ratio de pureté plus faible (352:65 mg/g).
Cette relaxation dépendante de l'endothélium induite par la formulation optimisée d'acide gras oméga-3 EPA:DHA (694:121 mg/g) était significativement diminuée en présence de N^{ω}-nitro-L-arginine (inhibiteur de la eNOS) et pas affectée par la présence des inhibiteurs de la réponse EDH (TRAM34 plus apamine), suggérant que la formulation optimisée EPA:DHA 6:1 induit la relaxation principalement par l'activation de la eNOS. De plus, la relaxation dépendante du NO en réponse à la formulation EPA:DHA 6:1 était significativement inhibée en présence des inhibiteurs du stress oxydant et de Src kinase, PI3-kinase, p38 MAPK, JNK et MEK, suggérant que la formulation EPA:DHA 6:1 induirait l'activation de la eNOS via une activation redox-sensible des voies de signalisation MAPKs et PI3-kinase/Akt.

Sur des cultures primaires de cellules endothéliales, la formulation EPA:DHA 6:1 a induit une phosphorylation des kinases p38 MAPK, ERK, JNK, Src, Akt et de la eNOS qui a été significativement réduite par le MnTMPyP et la PEG-catalase, confirmant ainsi que la formulation EPA:DHA 6:1 induit une activation redox-sensible de la eNOS via les voies de signalisation MAPKs et Src/PI3-kinase/Akt. De plus, la formulation EPA:DHA 6:1 a induit dans les cellules endothéliales en culture une formation d'espèces réactives de l'oxygène sous forme d'anions superoxydes et de peroxyde d'hydrogène.

En conclusion, cette étude a montré que la capacité des acides gras oméga-3 à stimuler la formation endothéliale de NO est dépendante de la formulation en termes de ratio et de degré de pureté. De plus, cette formation endothéliale de NO en réponse aux acides gras oméga-3 se fait par une voie de signalisation originale impliquant une activation redox-sensible des voies MAPKs et Src/PI3-kinase/Akt aboutissant à une augmentation de l'activité de la eNOS.

La seconde étude a évalué le potentiel de la formulation optimisée en acides gras oméga-3 EPA:DHA 6:1 à prévenir la vasoconstriction induite par les plaquettes, et déterminé les mécanismes sous-jacents. L'addition de plaquettes humaines lavées sur des anneaux d'artères coronaires de porc a induit une contraction dépendante de la concentration. Le fait que cette contraction ait été significativement inhibée par la kétansérine, un antagoniste du récepteur de la sérotonine 5-HT_{2A}, suggère que la contraction induite par les plaquettes est due principalement au relargage de sérotonine par les plaquettes activées. De plus, la contraction induite par les plaquettes était abolit après une exposition à court terme des anneaux à la formulation optimisée EPA:DHA 6:1, cette abolition n'étant pas observée après exposition à la formulation EPA:DHA 1:1. La formulation optimisée EPA:DHA 6:1 inhibait aussi de façon dépendante de la dose les contractions d'anneaux d'artère coronaire de porc en réponse à la sérotonine, mais n'avait aucun effet sur celles en réponse à un analogue de thromboxane A_2 (U46619). L'effet préventif de la formulation optimisée EPA:DHA 6:1 sur la contraction induite par les plaquettes était abolit soit en l'absence d'un endothélium fonctionnel, soit en présence d'un inhibiteur de eNOS, ce qui suggère que la formulation optimisée EPA:DHA 6:1 exerce son effet préventif via une augmentation de la formation endothéliale de NO.

Des résultats similaires ont été observés dans des anneaux d'artères mammaires interne humaines, la formulation optimisée EPA:DHA 6:1 prévenant significativement la contraction induite par la sérotonine, et induisant des vasorelaxation, par des mécanismes impliquant le NO.

L'ensemble de ces résultats indiquent que la formulation optimisée EPA:DHA 6:1 est capable de prévenir la contraction due à la sérotonine induite par les plaquettes dans les anneaux d'artères coronaires de porc et d'artères mammaires internes humaines via un mécanisme dépendant de l'endothélium impliquant le NO.

En conclusion, le présent travail a évalué le potentiel de formulations EPA:DHA à protéger la fonction endothéliale *ex vivo*. La capacité des formulations EPA:DHA à induire des relaxations dépendantes de l'endothélium est affecté à la fois par le ratio et le degré de pureté des formulations, avec un effet maximal obtenu pour la formulation EPA:DHA 6:1 avec un haut degré de pureté. La relaxation dépendante de l'endothélium induite par la formulation optimisée EPA:DHA 6:1 est principalement due à une activation redox-sensible des voies de signalisation PI3-kinase/Akt et/ou MAPKs qui mène à la phosphorylation activatrice de eNOS. De plus, la formulation optimisée EPA:DHA 6:1 est capable de fortement inhiber les contractions induites par les plaquettes impliquant la sérotonine dans des anneaux d'artères coronaires de porc, mais n'a aucun effet sur la contraction en réponse au thromboxane A_2. L'effet inhibiteur de la formulation optimisée EPA:DHA 6:1 sur la contraction en réponse à la sérotonine dans les artères coronaires porcines et mammaires internes humaines est principalement dû à une augmentation de la formation endothéliale de NO. L'ensemble de ces résultats indique que la formulation optimisée EPA:DHA 6:1 est capable d'exercer un puisant effet bénéfique sur le système cardiovasculaire via l'activation de la fonction endothéliale. La formulation optimisée en acides gras oméga-3 EPA:DHA 6:1 semble donc être une approche innovante et prometteuse dans la prise en charge de la dysfonction endothéliale, caractérisée entre autre par une diminution de la formation endothéliale de NO, qui est associée au développement des maladies cardiovasculaires.

ABSTRACT

The endothelium lining the inner wall of blood vessels is a complex, highly specialized and metabolically active organ, which plays a pivotal role in the maintenance of vessel wall permeability and blood homeostasis. The endothelium exerts its function, at least in part, by releasing several factors that control vascular tone. The key endothelium-derived relaxing factors are the nitric oxide (NO), the endothelium-dependent hyperpolarization (EDH) and prostacyclin (PGI_2). Endothelium-derived vasocontracting factors include thromboxane A_2 (TXA_2), angiotensin II, prostaglandin H_2 (PGH_2), reactive oxygen species (ROS), and endothelins.

Evidence suggests that dietary factors play an important role in the modulation of the endothelial function. Hence, relations between diet and vascular structure and function (vascular health) have been the subject of several studies, both epidemiological and interventional. Improvements of endothelial function have been associated with several kind of foods and beverages including fruit, vegetables, fatty fish, tea and red wine, and with micronutrients such as omega-3 fatty acids, vitamins C, D and E, and carotenoids.

In addition, several studies have indicated an inverse correlation between the risk of cardiovascular diseases and the increased consumption of omega-3 fatty acids, such as eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), that are found mainly in fatty fish (e.g., salmon, trout, herring, sardines, and mackerel). The beneficial cardiovascular effects of dietary supplementation with omega-3 fatty acids include decreased arrhythmias, decreased triglycerides plasma concentrations, decreased blood pressure, and decreased platelet aggregation, all leading to reduced risk of cardiovascular mortality in patients with cardiovascular diseases. In addition, the protective effect of omega-3 fatty acids could also be explained by their ability to improve endothelium-dependent relaxation of the arteries by stimulating the activation of the endothelium vasoprotective factors NO and EDH.

The aim of the present work was firstly to evaluate the role of the formulation of omega-3 fatty acids on the induction of endothelium-dependent relaxations, the contribution of both NO- and EDH-mediated relaxations, and to determine the underlying mechanisms. The second aim of the study was to evaluate the beneficial vascular effect of omega-3 fatty acids on the platelet-induced coronary artery contraction, and to characterize the role of serotonin and vasoconstricting prostanoids.

The first study assessed the role of the formulation of omega-3 fatty acids on the induction of endothelium-dependent relaxations. The effects of different omega-3 fatty acid products were studied either on porcine coronary artery rings or in cultured porcine coronary artery endothelial cells.

EPA and DHA, the major omega-3 fatty acids, induced concentration-dependant relaxations in coronary artery rings with functional endothelium, whereas these relaxations were absent in rings without endothelium. The relaxation in response to EPA was slightly but significantly greater than those to DHA at 0.4% (v/v) (77.8±10.3 and 64.7±12.8%, respectively). Then, the ability of optimized omega-3 ratios to induce endothelium-dependent relaxation was determined and the results showed that EPA:DHA at ratio of either 6:1 or 9:1 induced relaxations significantly more potent than ratio of 3:1, 1:1, 1:3, 1:6, and 1:9.

Similarly, the role of the purity of the EPA:DHA ratio was determined. The endothelium-dependent relaxation in porcine coronary artery rings in response to a product with a high purity of omega-3 EPA:DHA (694:121 mg/g) was significantly greater than those in response to a product with a lower purity of omega-3 EPA:DHA (352:65 mg/g).

The endothelium-dependent relaxation induced by the high purity omega-3 EPA:DHA 6:1 optimized formulation was slightly but significantly inhibited by N^{ω}-nitro-L-arginine (eNOS inhibitor) but not affected by the EDH inhibitors (TRAM 34 plus apamin), suggesting that EPA:DHA 6:1 exerts its vasorelaxant properties mainly through the activation of eNOS.

Moreover, the NO-mediated relaxation was significantly reduced by inhibitors of either oxidative stress, Src kinase, PI3-kinase, p38 MAPK, JNK or MEK, indicating that EPA:DHA 6:1 induces NO activation through a redox-sensitive activation of PI3-kinase/Akt and MAPKs pathways.

In cultured endothelial cells, EPA: DHA 6:1 induced a time-dependent phosphorylation of p38 MAPK, ERK, JNK, Src, Akt and eNOS that were markedly reduced by MnTMPyP and PEG-catalase, confirming that EPA:DEA 6:1 induces the redox-sensitive activation of eNOS through PI3-kinase/Akt and MAPKs pathways. Indeed, EPA:DHA 6:1 induced the formation of ROS in cultured endothelial cells including both superoxide anions and hydrogen peroxide.

In conclusion, the ability of omega-3 fatty acids to stimulate the endothelial formation of NO is dependent on the ratio and amount of the EPA:DHA formulation and it involved an original pathway triggered by the intracellular oxidative stress leading to the activation of the Src/PI3-kinase/Akt and MAPKs pathways to increase ultimately eNOS activity.

The second study evaluated the potency of the optimized EPA:DHA 6:1 formulation to prevent the platelet-induced vasocontraction, and determine the underlying mechanisms. In porcine coronary artery rings, human washed platelets induced dose-dependent contractions that were significantly inhibited by the 5-HT_{2A} receptor antagonist ketanserin, suggesting that the platelet-induced contraction is mainly due to the release of serotonin from activated platelets. Moreover, the short-term incubation of the rings with the EPA:DHA 6:1 optimized formulation, but not with the EPA:DHA 1:1 formulation, resulted in the abolition of the platelet-induced contraction. The EPA:DHA 6:1 formulation also dose-dependently prevented the 5-HT-induced contraction, whereas it was without effect on that induced by the thromboxane A_2 analogue (U46619). The fact that the prevention of the platelets-induce contraction by EPA:DHA 6:1 0.4% (v/v) was abolished both in absence of a functional endothelium and in presence of an eNOS inhibitor suggests that the EPA:DHA 6:1 exert its inhibitory effects through an increased endothelial formation of NO.

Moreover, similar results were observed in human internal mammary artery rings, where the EPA:DHA 6:1 formulation at 0.4 % (v/v) significantly prevented the 5-HT-induced contraction and induced vasorelaxation through NO-mediated mechanisms.

Altogether, these findings indicate that the optimized EPA:DHA 6:1 formulation is able prevents the 5-HT-mediated platelet-induced contraction in porcine coronary and human internal mammary artery rings through endothelium-dependent NO-mediated mechanisms.

In conclusion, the present work has assessed the potency of EPA:DHA formulations to protect the endothelial function *ex vivo*. The ability of EPA:DHA formulations to induce endothelium-dependent relaxations is dependent on both the ratio and the purity of the formulation, with the maximal effect obtained with a highly purified EPA:DHA 6:1 ratio. The endothelium-dependent relaxation induced by the optimized EPA:DHA 6:1 formulation is mainly mediated by a redox-sensitive activation of PI3-kinase/Akt and/or MAPKs pathways leading to the activating phosphorylation of eNOS. Moreover, the optimized EPA:DHA 6:1 formulation is able to effectively inhibit the platelet-induced serotonin-mediated contraction of porcine coronary rings, but has no effect on the thromboxane A_2-mediated contraction. The inhibition by the optimized EPA:DHA 6:1 formulation of the 5HT-induced contraction in both porcine coronary artery and human internal mammary artery rings is mainly due to an increased endothelial formation of NO. Taken together, the present findings indicate that the optimized EPA:DHA 6:1 formulation is able to exert potent beneficial cardiovascular effects through the activation of the endothelial function. The omega-3 EPA:DHA 6:1 optimized

formulation seems to be a promising novel strategy against the endothelial dysfunction characterized, at least in part, by a reduced endothelial formation of NO, and associated with the development of cardiovascular diseases.

Chapter one:

Physiology of the endothelium

Introduction

1. Cardiovascular diseases

Cardiovascular diseases are a group of several pathologies including coronary heart diseases (CHD such as myocardial infarction), cerebrovascular diseases (stroke), hypertension, peripheral artery diseases, rheumatic heart diseases, congenital heart diseases and heart failure. Cardiovascular diseases are the leading cause of mortality and morbidity worldwide, with an estimated 17 million annual deaths (WHO 2011). Amongst cardiovascular diseases, CHD accounts for 7.2 million deaths and 5.7 million are due to stroke. It is now well established that endothelial dysfunction is an early hallmark of major cardiovascular diseases, which is thought to contribute to the initiation and the development of these diseases.

1.1 The endothelium

Blood vessels, including arteries, capillaries, and veins are part of the circulatory system with the heart. The vessel's wall, except for the smallest, consists of three individualized layers: the intima, the media and the adventitia (Figure 1). The intima (tunica interna) is the innermost layer, which is characterized by the presence of the endothelium, a monocellular layer located at the interface between the blood flow and the vessel wall, supported by connective tissue and the internal elastic lamina. The media (tunica media) is mainly build of smooth muscle cells with an elastic fiber layer, the later increasing with the size of the artery. The adventitia (tunica externa or tunica adventitia) is the outermost layer containing collagen and elastin, fibroblastes, macrophages, *vasa vasorum* and fibers which protect the blood vessels (Mulvany 1990).

The endothelium, which covers the luminal surface of all blood vessels, plays a pivotal role in the regulation of blood flow and of blood homeostasis, in part through the synthesis and secretion of various active molecules, including vasodilatating factors and vasoconstricting factors controlling vascular tone.

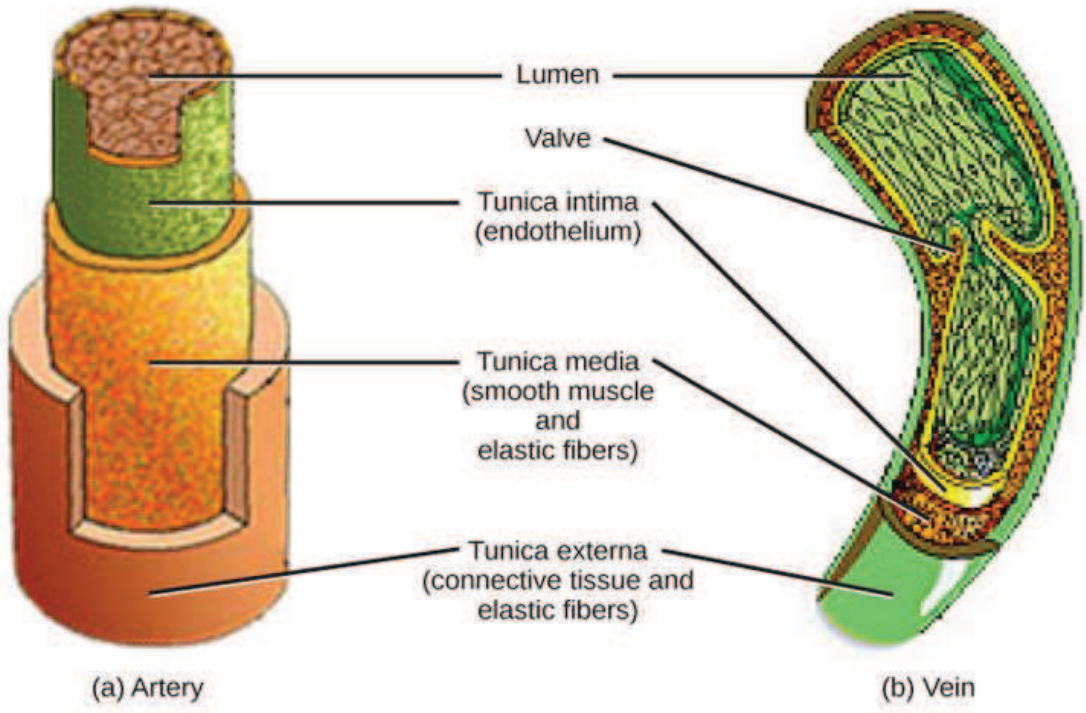

Figure 1 . Structure of the blood vessel (adapted from Boston University School of Public Health).

1.2 Control of vascular tone by the endothelium

In 1980, Furchgott and Zawadzki reported that acetylcholine induced relaxation in an arterial ring through the release of an endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki 1980). Thereafter, it was shown that EDRF induces the relaxation of the vascular smooth muscle cells through the stimulation of the soluble guanylyl cyclase (Ignarro et al. 1986), and that EDRF is inactivated by superoxide anions (Gryglewski et al. 1986; Rubanyi and Vanhoutte 1986). EDRF was later identified as the free radical gas nitric oxide (NO). After this discovery, it became clear that endothelial cells have a pivotal role in the regulation of vascular homeostasis, endothelial permeability, smooth muscle cell proliferation, as well as of the immune response (Santiago-Delpin 2004). The regulation of vascular homeostasis mainly consists in the ability of the endothelium to maintain a balance between vasodilatation and vasoconstriction (Figure 2), and to modulate the proliferation and migration of smooth muscle cells, thrombosis and fibrinolysis, through the release of various factors (Davignon and Ganz 2004). Endothelial cells also respond to physical and chemical stimuli such as pressure, shear stress, and pH (Feletou and Vanhoutte 2006) and to numerous

physiological stimuli such as circulating hormones, by releasing various factors, known as endothelium-derived vasoactive factors, which include both endothelium-derived relaxing factors and endothelium-derived contraction factors (Moncada and Higgs 2006). The main endothelium-derived vasorelaxing factors include nitric oxide (NO), endothelium-dependent hyperpolarization (EDH) and prostacyclin (PGI_2). The endothelium-derived contracting factors include reactive oxygen species (ROS), thromboxane A_2 (TxA_2) angiotensin II, prostaglandin H_2 (PGH_2), and endothelins (Feletou and Vanhoutte 2006; Mombouli and Vanhoutte 1999).

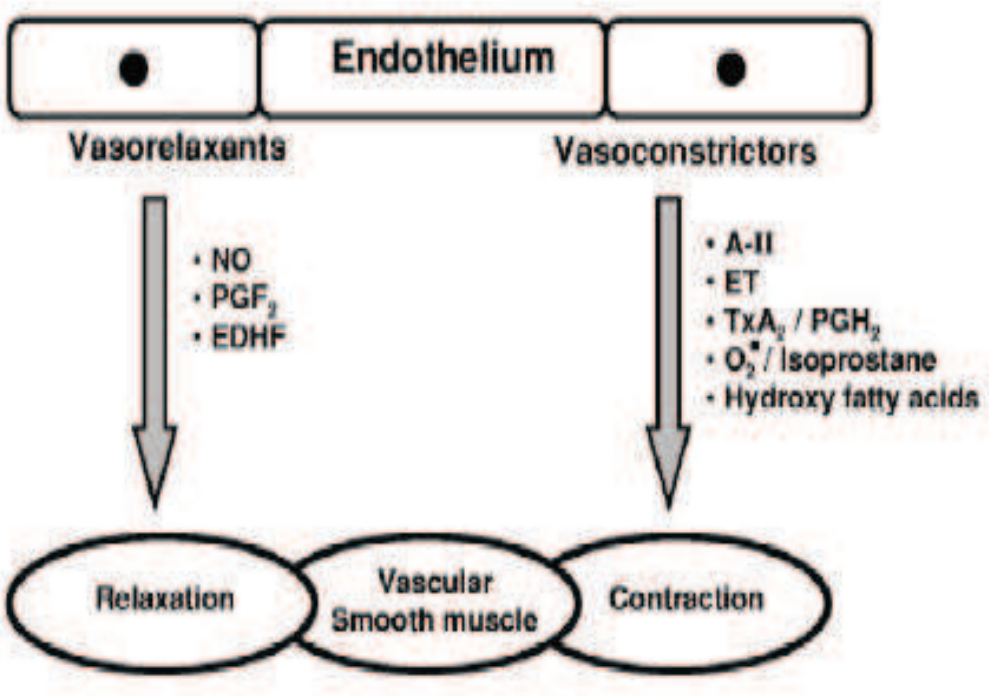

Figure 2. Endothelium-dependent release of vasocontracting and vasorelaxing factors. NO, nitric oxide; PGI_2, prostacyclin; EDHF, endothelium-derived hyperpolarizing factor; A-II, angiotensin II; ET, endothelin; TxA_2/PGH_2, thromboxaneA_2/prostaglandin H_2O_2, hydrogen peroxide (Abeywardena and Head 2001).

1.2.1 The endothelium-derived vasorelaxing factors

1.2.1.1 Nitric oxide (NO)

The endothelium-derived nitric oxide, or NO, is produced by the endothelial NO synthase (eNOS) from L-arginine, and plays a critical role in normal vascular biology and pathophysiology. There are three different isoforms of NO synthase; the neuronal NOS (NOS1 or nNOS), the inducible NOS (NOS2 or iNOS) and the endothelial NOS (NOS3 or eNOS). The two isoforms nNOS and eNOS are being constitutively expressed whereas iNOS is an inducible isoform (Mombouli and Vanhoutte 1999; Stuehr 1999). NO diffuses towards the underlying smooth muscle cells to active soluble guanylyl cyclase by converting GTP into cyclic guanosine 3'-5'monophosphate (cGMP) leading to smooth muscle cells relaxation (Figure 3). In addition, NO exerts several vasoprotective and anti-atherogenic effects including inhibition of platelets aggregation, monocyte adhesion, vascular smooth muscle cell migration and proliferation, oxidation of LDL, expression of pro-inflammatory and pro-atherothrombotic mediators such as monocyte chemoattractant protein-1 (MCP-1), adhesion molecules and tissue factor (Dimmeler et al. 1997; Hermann et al. 1997; Tsao et al. 1996). The eNOS enzyme can be activated either by receptor-dependent or receptor-independent mechanisms, leading to an increase of the intracellular concentration of cytosolic free calcium ($[Ca^{2+}]_i$) and the subsequent formation of the Ca^{2+}/calmodulin (CaM) complex that binds to eNOS (Fleming et al. 2001). Indeed, both the NO formation and the subsequent vasorelaxation are abolished by the removal of calcium from the extracellular space as well as by calmodulin antagonists (Fleming et al. 2001).

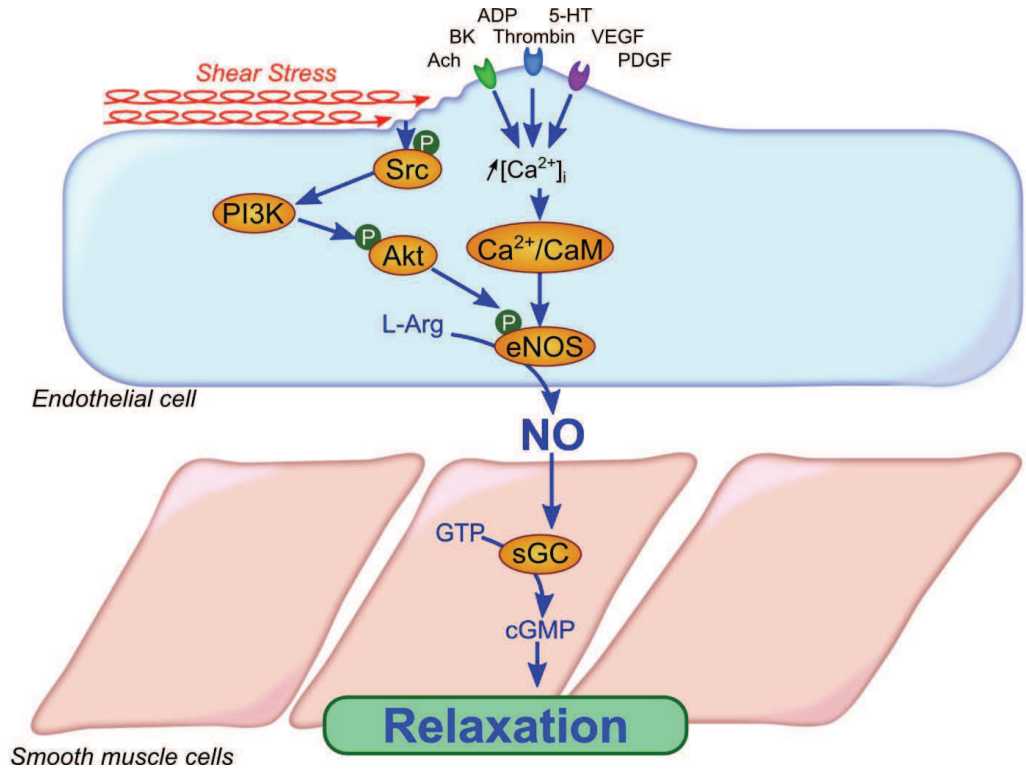

Figure 3. Nitric oxide synthesis pathway in the endothelial cell and its actions in the vascular smooth muscle cell. ACh, acetylcholine; BK, bradykinin; ADP, adenosine diphosphate; 5-HT, serotonin; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; Src, Sarcoma-family kinases; PI3/kinase, phosphoinositide 3-kinase; Akt, Protein kinase B; Ca/CaM, calcium calmodulin; L-Arg, L-arginine; eNOS, endothelial Nitric Oxide Synthase; NO, nitric oxide; sGC, soluble guanylyl cyclase; GTP, Guanosine 5'-Triphosphate; cGMP, Cyclic guanosine 3'-5' monophosphate.

eNOS can also be regulated in endothelial cells at a post-translational level primarily through protein/protein interactions and multisite phosphorylations. The main sites of phosphorylation on eNOS are at the residue Ser1177 and Ser 615 (activating sites), and the residue Thr497 and Ser114 (Bauer et al. 2003; Bohm et al. 2002; Dimmeler et al. 1997). Moreover, eNOS has been shown to be regulated by the interaction with positive and negative protein modulators such as caveolin-1 (Cav-1) and heat shock protein 90 (Garcia-Cardena et al. 1998; Ju et al. 1997; Pritchard et al. 2001). In the basal state, the majority of eNOS appears to be bound to caveolin-1 with its enzymatic activity being repressed in the caveolae (Michel et al. 1997). This tonic inhibition of eNOS can be released by displacing caveolin-1 with Ca^{2+}/CaM in response to Ca^{2+}-mobilizing agonists (Ju et al. 1997). In addition to these modulators, phosphorylation of eNOS at key regulatory sites plays an important a role in the regulation of enzyme activity in response to several physiological stimuli (Ju et al. 1997). It has been shown that phosphorylation of eNOS at Ser1177 is associated with an increased enzyme activity (Gallis et al. 1999; McCabe et al. 2000). Akt, one of the major regulatory targets of

PI3-kinase, has been shown to directly phosphorylate eNOS at Ser117 and activate the enzyme in response to vascular endothelial growth factor (VEGF), sphingosine-1-phosphate, and estrogens (Dimmeler et al. 1997; Fulton et al. 1999). In addition, eNOS can be also activated on Ser1177 by AMP-activated protein kinase (Busse et al. 2002), protein kinase A (PKA), and protein kinase G (PKG).

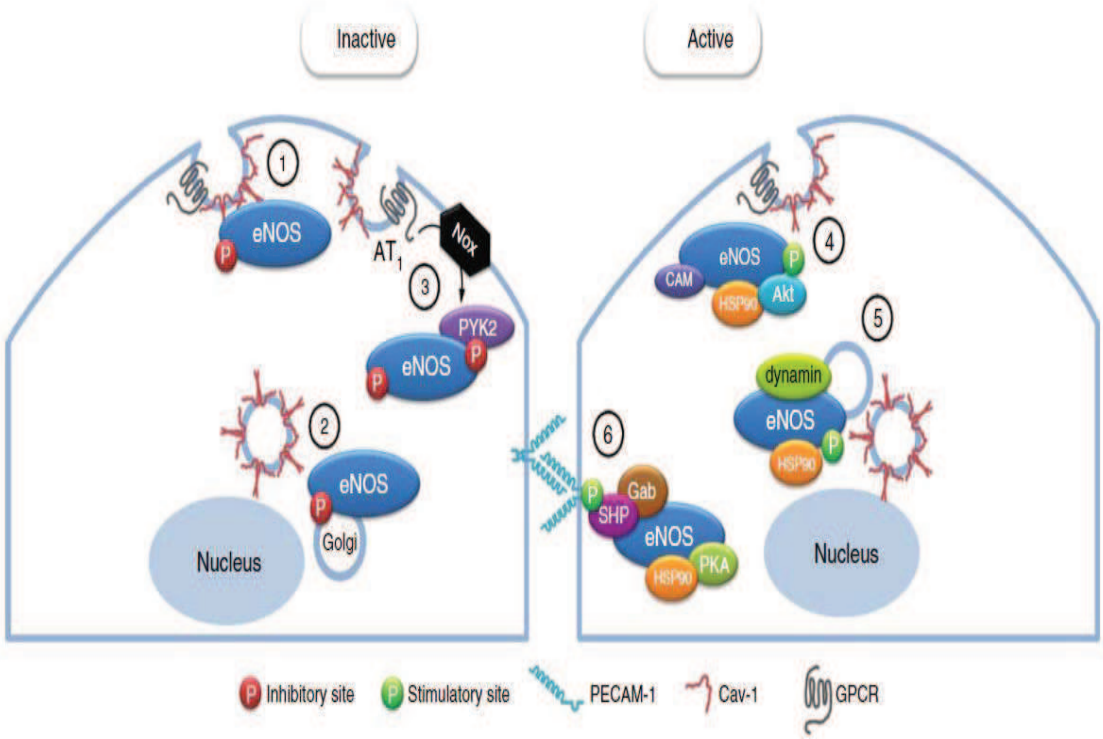

Figure 4. Regulation of eNOS activity. (1) At rest, the eNOS is coupled to cav-1 (caveolin-1, a structural protein of caveolae) that decreases its activity. (2) eNOS is constitutively phosphorylated at Thr 495 preventing its activation by the Ca^{2+}/CaM. (3) eNOS may be inhibited in response to oxidative stress by tyrosine phosphorylation by PYK2 (proline-rich tyrosine kinase). (4) eNOS can be activated by both Ca^{2+}/CaM (calcium / calmodulin) and phosphorylation of Ser1177. Hsp90 (heat shock protein) facilitates the recruitment of Akt responsible for the phosphorylation of eNOS (Fleming 2010).

The activity of eNOS can also be regulated by post-translational modification. As eNOS is constitutively expressed, phosphorylation plays an important role in regulating the activity of the enzyme. eNOS can be phosphorylated on serine, threonine and tyrosine residues leading to eNOS activation or inactivation. There are various putative phosphorylation sites, but the most extensively studied eNOS residues, the phospho-status of which determines enzyme activity, are a serine residue (human eNOS sequence: Ser1177; bovine sequence Ser1179) in the reductase domain, which positively regulates NO production, and a threonine residue (human eNOS sequence: Thr495; bovine sequence Thr497) within the CaM-binding domain (Boo et al. 2002). Another eNOS regulator is the ischemia-reperfusion injury which leads to eNOS phosphorylation at Ser1177 and Ser 633 through the activation of PKA pathway (Li et

al. 2010). Moreover, there are various kinases reported to be involved in the phosphorylation of eNOS following cell activation by different stimuli such as shear stress, vascular endothelial growth factor (Butt et al. 2000), hypoxia (Chen et al. 2008; Michell et al. 1999), including extracellular signal-regulated kinase 1/2 which alters eNOS protein expression and activity (Ramasamy et al. 1998).

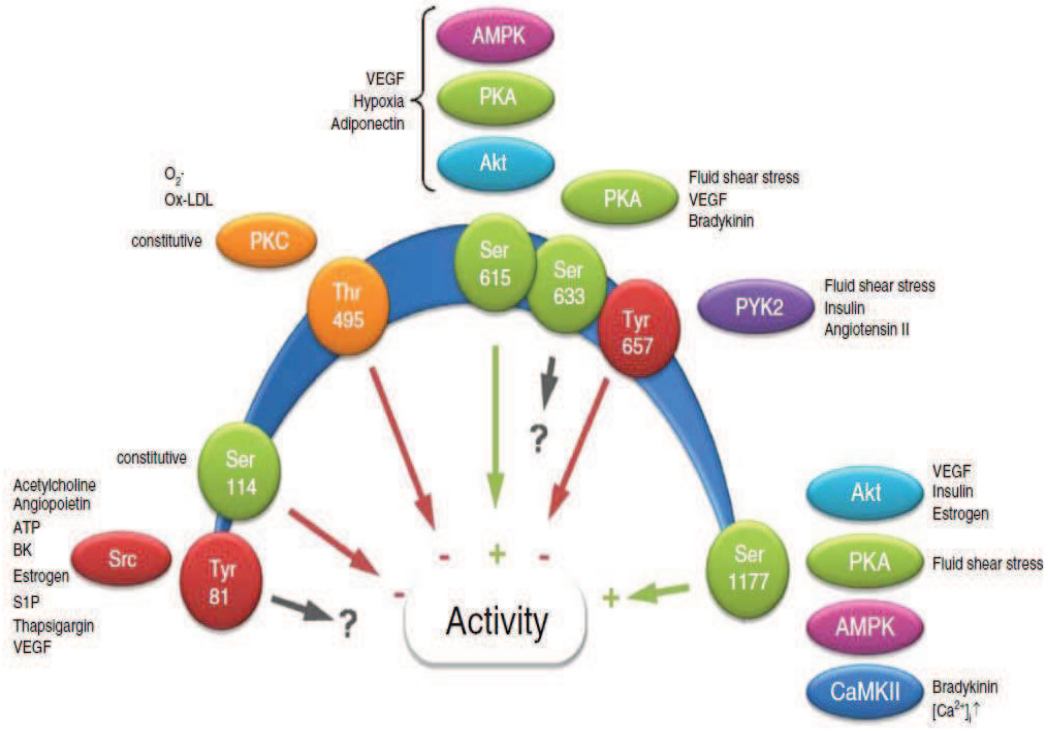

Figure 5. The activation and inhibition sites of eNOS. (Green arrows for activation, red arrows for inhibition, black arrow for no direct effect on enzyme activity). The numbers refer to the human sequence (Fleming 2010).

1.2.1.2 The endothelium-dependent hyperpolarization (EDH)

The EDH was previously known as the Endothelium-Derived Hyperpolarizing Factor (EDHF) and is another important mechanism of endothelium-dependent relaxation mostly observed in medium to small calibre resistance arteries, small arteries and arterioles such as second and third-branch mesenteric artery (Feletou and Vanhoutte 1996), as well as in coronary arteries. Indeed, the role of EDH is more important in resistance vessels than that of NO and prostacyclin (Nakashima et al. 1993; Shimokawa et al. 1996).

The mechanism of EDH involves the hyperpolarization of endothelial cells induced by activation of both small and intermediate conductance calcium-activated potassium channels (SK_{Ca} and IK_{Ca}, respectively), leading to the exit of potassium ions from the intracellular

compartment to the extracellular one (Edwards et al. 1998). Thereafter in smooth muscle cells, the higher concentration of potassium ions in the extracellular space can activate the inwardly rectifying K^+channel (K_{IR}) and Na^+/K^+-ATPase to cause hyperpolarization of smooth muscle cells through entry of potassium ions (Edwards et al. 1998; Feletou and Vanhoutte 2006). Moreover, Edwards et al. have reported that a direct transfer of the hyperpolarization from endothelial cells to smooth muscle cells can also occur via myoendothelial gap junctions (Edwards et al. 1998). The factors involved in EDH seems to differ depending on the species of the animal and the vascular bed considered, and have been reported to include hydrogen peroxide H_2O_2 (Matoba et al. 2002), and arachidonic acid-derived metabolites including epoxyeicosatrienoic acids (Quilley and McGiff 2000).

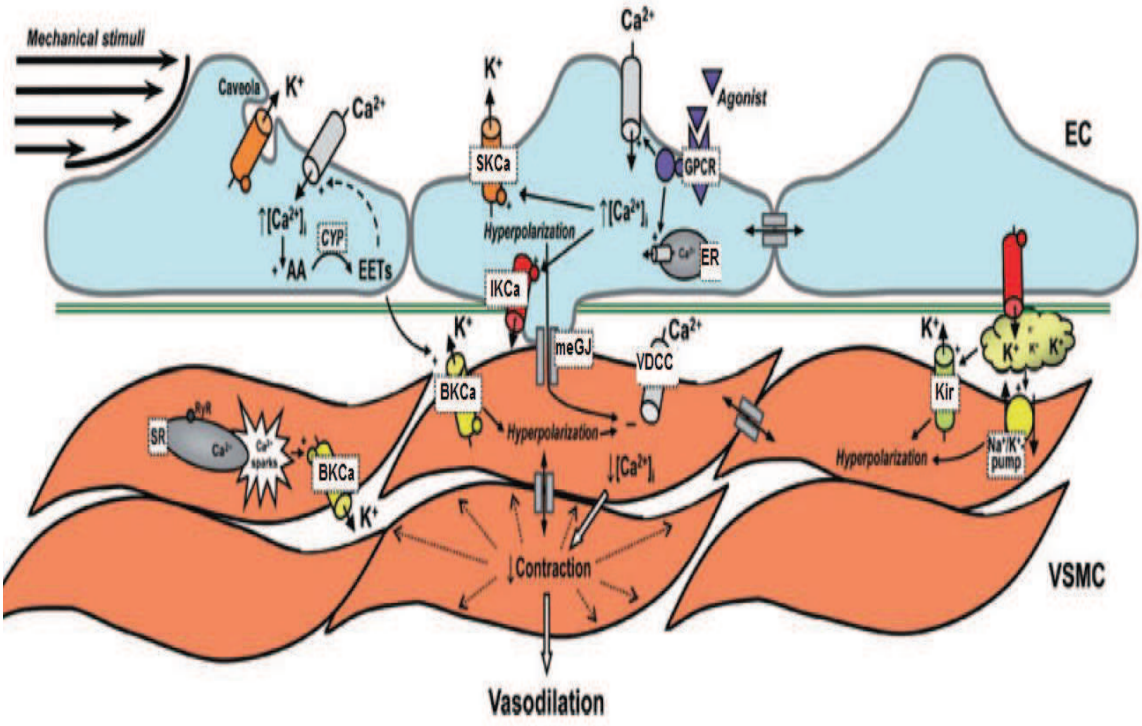

Figure 6. Hypothesis describing the endothelium-derived hyperpolarizing pathway.
AA, arachidonic acid; ACh, acetylcholine, $[Ca^{2+}]i$, intracellular calcium concentration; CYP, cytochrome P450 epoxygenase; EC, endothelial cell; EDHF, endothelium-derived hyperpolarizing factor; EETs, epoxyeicosatrienoic acids; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; BK_{Ca}, large conductance Ca^{2+}-activated K+ channel; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channel subtype 3; IK_{Ca}, intermediate-conductance Ca^{2+} -activated K+ channel; Kir, inwardly rectifying K^+ channel; meGJ, myo-endothelial gap-junction; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; VDCC, voltage-dependent Ca^{2+} channel; VSMC, vascular smooth muscle cell (Grgic et al. 2009).

1.2.1.3 Prostacyclin (PGI_2)

Prostacyclin (PGI_2) is a metabolite of arachidonic acid generated by vascular cells and endothelial cells. Arachidonic acid is converted to prostaglandin H_2 (PGH_2) by the action of cyclooxygenases, and PGH_2 is then further metabolized into PGI_2 by the prostacyclin synthase (Moncada and Vane 1979). PGI_2 a potent vasodilator that binds the prostaglandin receptor (IP), a G-protein coupled receptor on the vascular smooth muscle cells, leading to an increase in cAMP levels (Coleman et al. 1994). Thereafter cAMP can activate the protein kinase A leading to a reduction in the cytosolic Ca^{2+} level in smooth muscle cells through Ca^{2+} uptake by the endoplasmic reticulum (Kukovetz et al. 1979). In addition, PGI_2 is a potent inhibitor of platelet aggregation and adhesion, whereas the inhibition of PGI_2 synthesis promotes thrombus formation in the microcirculation (Smith 1981). Moreover, it has been reported that PGI_2 can enhance the release of NO in endothelial cells (Mustard et al. 1980; Shimokawa et al. 1988a).

1.2.2 Endothelium-derived contracting factors (EDCFs)

Endothelium-dependent relaxing factors are important for the regulation of vascular homeostasis. However, under various physical and chemical stimuli such as noradrenaline, thrombin, hypoxia, increased transmural pressure and mechanical stretch and circulating hormones (De Mey and Vanhoutte 1982, 1983; Harder 1987; Katusic et al. 1987; Shimokawa et al. 1988a), endothelial cells can release contracting factors, which include thromboxane A_2 (TXA_2), prostaglandin H_2 (PGH_2), reactive oxygen species (ROS), endothelin-1 (ET1), and angiotensin II (Taddei et al. 2003; Vanhoutte et al. 2005).

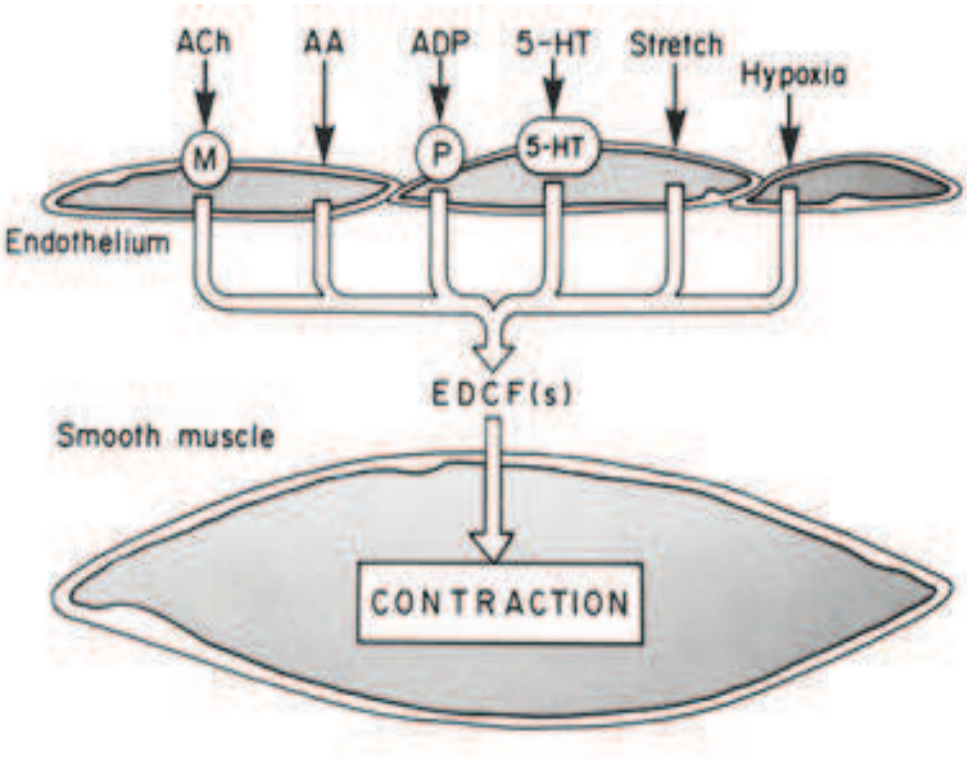

Figure 7. Physicochemical stimuli can evoke endothelium-derived contractile factors (EDCFs) in certain blood vessels, Ach; acetylcholine , AA; arachidonic acid, ADP; adenosine diphosphate, 5-HT; serotonin (Vanhoutte et al. 2005).

1.2.2.1 Reactive oxygen species (ROS)

ROS are produced and released by vascular cells during pathophysiological conditions like hypertension, diabetes mellitus, atherosclerosis and in acute and chronic inflammatory diseases (Eisenberg and Ghigliotti 1999). Several enzymes have been implicated in the production of ROS in the vascular wall, including cytochrome P450, cyclooxygenases (COX-1 and COX-2), lipoxygenases, NADPH oxidase, xanthine oxidase and the mitochondrial respiratory chain complexes (Griendling and Ushio-Fukai 1997). The major species of ROS involved in vascular oxidative stress include superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet-}$) and hydrogen peroxide (H_2O_2), which can promote vasoconstriction by facilitating the mobilization of cytosolic Ca^{2+} and/or by promoting Ca^{2+} sensitization of the contractile elements (Jin et al. 1991). In addition, ROS and in particular superoxide anions ($O_2^{\bullet-}$) can also act directly or indirectly as potent contracting agents via the reduction of the NO bioavailability or by activating COX_S in vascular smooth muscle cells (Hibino et al. 1999), leading to attenuated endothelium-dependent relaxations (Aubin et al. 2006; Liu et al.

2007). Moreover, ROS can also impair EDH-mediated endothelium-dependent relaxations through the reduction of calcium-activated potassium channels activity (Kusama et al. 2005) or by modifying the transmission of the hyperpolarization from endothelial cells to the underlying smooth muscle cells through myoendothelial gap junctions (Griffith et al. 2005). Several studies have shown the beneficial effects of antioxidants on the deleterious effect of oxidative stress on the endothelial function (Aubin et al. 2006; Kanani et al. 1999; Liu et al. 2007).

1.2.2.2 Angiotensin II (Ang II)

Angiotensin II is the major bioactive peptide of the renin–angiotensin system (RAS) and is a multifunctional hormone responsible for the regulation of blood pressure and cardiovascular homeostasis. The liver-synthetized precursor peptide angiotensinogen is cleaved by the kidney-produced enzyme renin, leading to angiotensin I. Angiotensin I, a biologically inactive peptide is further converted by angiotensin-converting enzyme (ACE) to the biologically active octapeptide Ang II. Then, Ang II can be converted to the vasorelaxant peptide Angiotensin (1-7) by plasma or neutral endopeptidases (Schindler et al. 2007). Ang II exerts its diverse actions mainly via two G-protein-coupled receptors, Ang II type 1 (AT1R) and type 2 (AT2R) receptors (Touyz and Schiffrin 2000). These effects include vasocontraction, induction of growth, apoptosis, fibrosis, matrix metalloproteinase expression and extracellular matrix degradation (Griendling et al. 2000; Tomita et al. 1998). It is well-known that increased Ang II formation is associated with vasoconstriction and vascular structural changes (Gibbons 1997). Such effects are observed in hypertension, atherosclerosis, and portal hypertension (Kane et al. 2010). The angiotensin II type 1 receptor (AT1R) is a G protein receptor coupled to Gq. Upon the binding of Ang II, AT1R induces the activation of phospholipase C, the mobilization of intracellular Ca^{2+} and the activation of mitogen-activated protein kinase in vascular smooth muscle cells, leading to contraction and vascular remodeling (Griendling and Ushio-Fukai 1997). Such effects may involve production of ROS, particularly $O_2^{\bullet-}$ and H_2O_2 (Berry et al. 2000).

1.2.2.3 Vasocontracting prostanoids

In endothelial cells, the prostaglandin H_2 (PGH_2) is generated by the metabolism of arachidonic acid (AA) by COX-1 and COX-2. PGH_2 is further converted to thromboxane A_2 (TXA_2) by the action of the thromboxane A synthase in particular in activated platelets. TXA_2 is a potent inducer of platelet aggregation. Moreover, binding of TXA_2 to thromboxane-

prostanoid (TP) receptors induces smooth muscle cell contraction and proliferation via increased intracellular Ca^{2+} levels (Coleman et al. 1994; Vanhoutte and Tang 2008). The maintenance of vascular homeostasis needs a fine regulation of the TXA_2/PGI_2 ratio, due to their opposing functions. The ratio has been suggested to be more important than the individual levels of both prostanoids in pulmonary vascular disease (Caughey et al. 2001).

1.2.2.4 Endothelins (ET)

Endothelins are a family of peptides, which are potent contracting factors of blood vessels and they can raise blood pressure (Schiffrin 2005). ET-1 is released by endothelial cells, following the proteolytic process of its precursor, big-endothelin, by endothelin converting enzyme-1 (Agapitov and Haynes 2002). Several cytokines, including interleukin-1 and TNF-α, increase the production and release of ET-1, whereas it is inhibited by NO and PGI_2 (Alonso and Radomski 2003). In addition, the activation on smooth muscle cells of endothelin receptors ET_A or ET_{B2} causes the opening of Ca^{2+} channels, leading to the entry of extracellular Ca^{2+} and subsequent contraction (Agapitov and Haynes 2002). In contrast, the activation of ET_{B1} receptors on endothelial cells causes vasodilatation by inducing the release of NO and PGI_2 (Cardillo et al. 2000). Moreover, studies have shown that a decreased expression of ET_{B1} receptors on endothelial cells and/or an increased expression of ET_{B2} receptors on smooth muscle cells contribute to the development of endothelial dysfunction (Bohm et al. 2002).

1.2.2.5 Arachidonic acid (AA)

Arachidonic acid is metabolized by three distinct enzyme systems including cyclooxygenases, lipoxygenases and cytochrome P450-dependent epoxygenases (Fitzpatrick and Murphy 1988). There are two isoforms of cyclooxygenase, each having the capacity to catalyze the conversion of arachidonic acid to prostaglandins. The first isoform, COX-1, is constitutively expressed in most tissues, whereas the second, COX-2, is inducible (Figure 8). Studies have shown that the two isoforms are involved in the generation of EDCF (Miller and Vanhoutte 1985; Tang and Vanhoutte 2009). Moreover, a study suggests that COX-1, but not COX-2, is responsible for the cyclooxygenase-derived endothelium-dependent contraction in the spontaneously hypertensive rats (Tang et al. 2005).

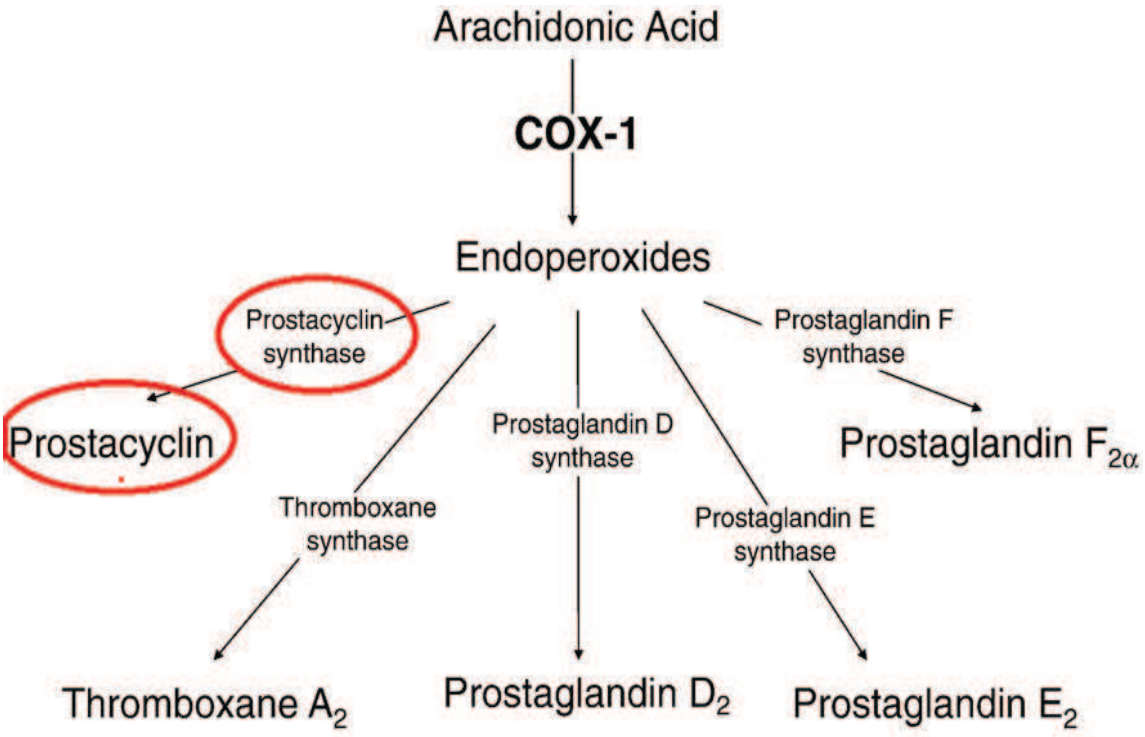

Figure 8. Arachidonic acid metabolism pathways (Tang and Vanhoutte 2009).

1.3 Endothelial dysfunction

Endothelial dysfunction is a pathophysiological condition characterized by impaired endothelium-dependent relaxation and vascular responses (Abraham and Distler 2007). The endothelial dysfunction involves an imbalance between the formation of the endothelium-derived vasodilatating and vasoconstricting substances (Deanfield et al. 2005; Schini-Kerth et al. 2011), as well as growth-promoting and growth-inhibiting factors and also of pro-atherogenic and anti-atherogenic factors (Quyyumi 1998). Endothelial dysfunction has been associated with an impairment of endothelium-dependent relaxations involving a reduced bioavailability of NO in major CV diseases such as hypertension, atherosclerosis, chronic renal failure, and diabetes (Griendling and FitzGerald 2003; Rush et al. 2005). The mechanism underlying endothelial dysfunction has been linked to increased oxidative stress which is associated with a reduced NO bioavailability and the formation of inflammatory mediators such as vascular cell adhesion molecular-1 (VCAM-1) expression (Khan et al. 1996; Libby 2002). In addition, different enzymes have been involved in the arterial oxidative stress involving NADPH oxidases, xanthine oxidases, COX-1 and COX-2, cytochrome P450 monooxygenases, enzymes of the mitochondrial respiratory chain, and eNOS uncoupling. ROS ($O^{2\cdot-}$) can react with NO to form the radical peroxynitrite (Koppenol et al. 1992),

leading to the oxidation of the eNOS cofactor tetrahydrobiopterin (BH_4) and the subsequent uncoupling of eNOS thereby further promoting oxidative stress (Cai and Harrison 2000).

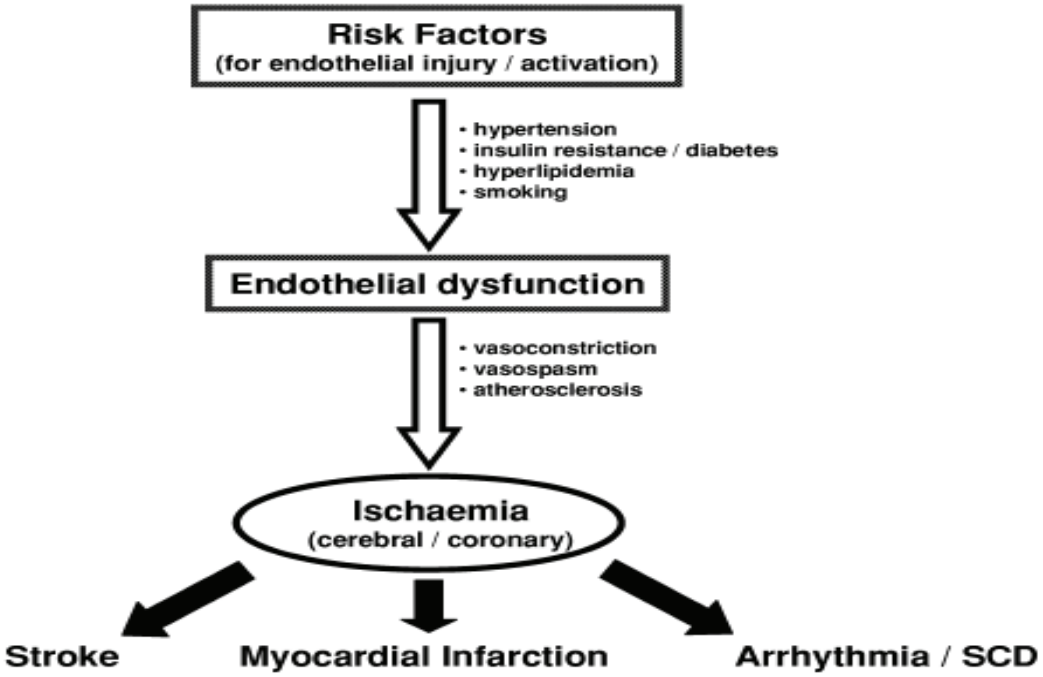

Figure 9. Endothelial dysfunction in cardio- and cerebro-vascular diseases. Risk factors associated with endothelium dysfunction are hypertension, hyperlipidemia, diabetes mellitus, chronic smoking (Abeywardena and Head 2001).

1.3.1 Hypertension

Hypertension is a chronic medical condition that affects more than 30% of the adult population in developed countries. This condition is characterized by a disruption of the balance between the endothelium-mediated vasodilatation and vasoconstriction towards the predominance of vasoconstriction (Nadar et al. 2004). Many studies using animal models and hypertensive subjects have demonstrated that hypertension is associated with endothelial dysfunction. Moreover, in spontaneously hypertensive rats (SHR), endothelial dysfunction is associated with an increased vascular oxidative stress, due to an increased expression and activity of NADPH oxidases and uncoupling of eNOS (Sanchez et al. 2006). In addition, Ang II-induced hypertension is associated with an over-production of superoxide anions in both endothelial cells and smooth muscle cells (Lee et al. 2011; Rajagopalan et al. 1996). Moreover, the accumulation of Ang II in the vascular wall has been observed mostly due to an overexpression of Angiotensin-converting enzyme (ACE) in blood vessels of hypertensive animals (Harrison 1997).

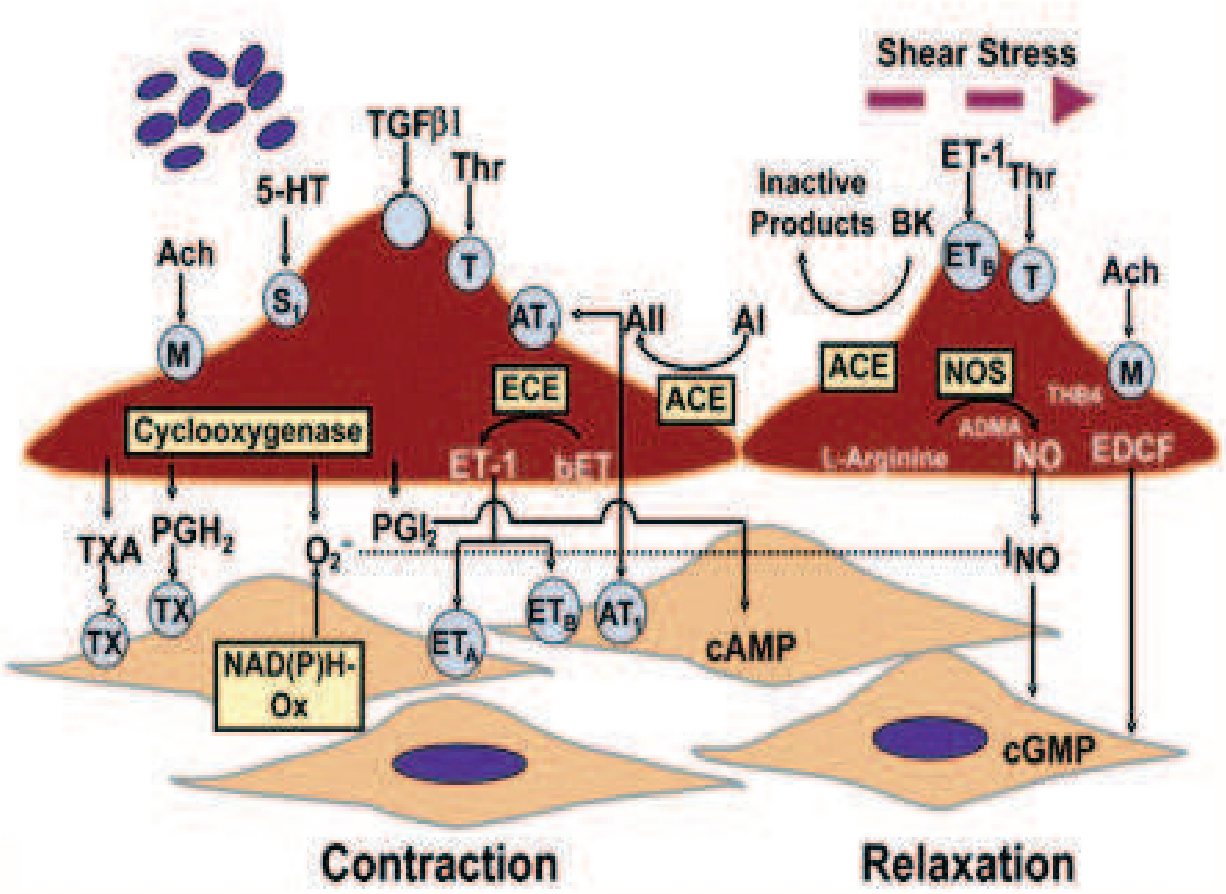

Figure 10. Endothelium-derived contracting factors (EDCF) pathways in hypertension. 5-HT, serotonin; ACE, angiotensin-converting enzyme; Ach, acetylcholine; ADMA, asymmetric dimethyl arginine; AI, angiotensin I; AII, angiotensin II; AT1, angiotensin receptor type 1; bET, big endothelin; BK, bradykinin; cAMP, cyclic adenosine 3',5' monophosphate; cGMP, cyclic guanosine monophosphate; ECE, endothelin-converting enzyme; EDCF, endothelial-derived contracting factors; ET-1, endothelin-1; ETA, endothelin receptor-A; ETB, endothelin receptor-B; M, muscarinic receptor; NADPH-Ox, NADPH oxidase; NO, nitric oxide; NOS, nitric oxide synthase; O_2, oxygen; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; S_1, serotonin receptor type 1; T, thrombin receptor; TGFβ1, transforming growth factor-β1; THB4, tetrahydrobiopterin; Thr, thrombin; TXA_2, thromboxane A_2. (Spieker et al. 2006).

1.3.2 Diabetes Mellitus

Diabetes Mellitus (DM) is a major health problem worldwide, associated with morbidity and mortality. DM is characterized by persistent elevation of the blood glucose level (Monesi et al. 2012). There are two types of diabetes: the type 1 diabetes (T1DM), which occurs due to the absence of the formation of insulin, and type 2 diabetes (T2DM), which is characterized by insulin insensitivity as a result of insulin resistance usually

associated with metabolic syndrome and obesity (Sharma et al. 2012). Several clinical studies with both types 1 and type 2 diabetic patients have shown the presence of an endothelial dysfunction (McVeigh et al. 1992; Nathan et al. 2003). In addition, studies report that endothelial dysfunction appears early in the development of DM, which may suggest a role of impaired endothelium-dependent vasodilatation in the initiation and development of both macro-vascular and micro-vascular complications of diabetes. Moreover, impaired endothelium-dependent relaxations are also observed in healthy-non diabetic subjects who are first degree relatives of type 2 diabetic patients, and associated with an increased plasma level of markers of endothelial cell activation and systemic pro-inflammatory markers (Balletshofer et al. 2000; Caballero et al. 1999; Tesauro et al. 2007). Increased vascular oxidative stress is another important mechanism of the development of endothelial dysfunction in diabetes, and involves both superoxide anions and hydrogen peroxide, predominantly due to an up-regulation of NADPH oxidase, and possibly also to an uncoupling of eNOS (Gao and Mann 2009). The increased vascular oxidative stress impairs NO bioavailability in the vascular wall and EDH-mediated relaxation, in part, by reducing the expression the calcium-activated potassium channels (IK_{ca} and SK_{ca}) (Urso and Caimi 2011). Moreover, the COX-derived arachidonic acid metabolites have been associated with endothelium-dependent contractions in diabetes (Feletou et al. 2010). Taken together, these findings suggest that the endothelial dysfunction associated with diabetes mellitus could be a main contributor to the increased risk of cardiovascular diseases in diabetic patients.

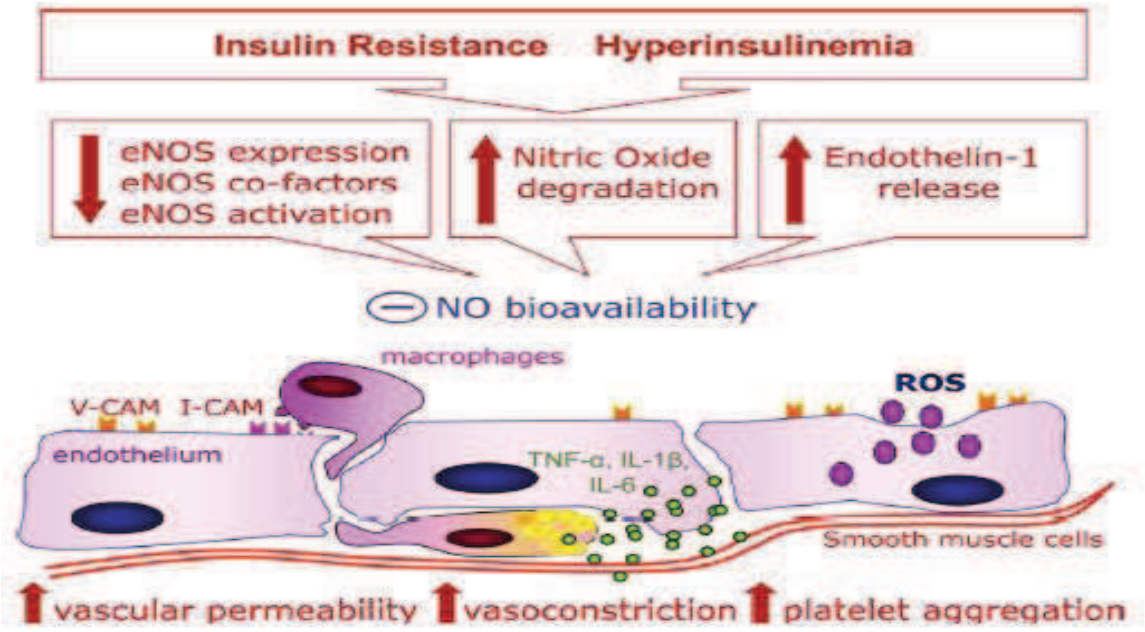

Figure 11. Pathological pathways of endothelial dysfunction associated with insulin resistance and hyperinsulinemia. Endothelial dysfunction characterised by increased expression of adhesion molecules, stimulates macrophage infiltration and release of proinflammatory cytokines (green spheres), induces proliferation of VSMC, and increases platelet aggregation. VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; eNOS, endothelial NO synthase; ROS, reactive oxygen species. (Potenza et al. 2009).

1.3.3 Atherosclerosis

Atherosclerosis is a multifactorial disease that is the major cause of cardiovascular death. It affects arterial blood vessels and is associated with a chronic inflammatory response in the arterial wall (Ross 1993). Atherosclerosis is primarily characterized by elevated circulating and vascular levels of low-density lipoprotein (LDL) particles, which can be associated with an increased interaction of monocytes, macrophages, and platelets with endothelial and smooth muscle cells. Atherosclerosis results from several pathological processes including inflammation in the vessel wall in response to retained LDL that become susceptible to oxidation by ROS, leading to oxidized LDL. LDL oxidation is also involved in the induction of arterial damages mediated by the immune system, through the recruitment of macrophages absorbing the oxidized-LDL, leading to the formation of foam cells. Macrophages are not able to process the oxidized-LDL, and accumulate oxidized particles until they ultimately enter necrosis, depositing a greater amount of oxidized fatty acids and crystalized cholesterol into the arterial wall (Figure 12). The development of this necrotic core in the intima is associated with the migration and proliferation of underlying smooth muscle

cells and fibroblasts, leading to the constitution of a fibrous cap, a hard covering of the necrotic core. The development of both necrotic core and fibrous cap causes the narrowing of the lumen of the artery, leading to a reduction and a perturbation of the blood flow (Libby et al. 2002). Moreover, endothelial dysfunction has been reported to be the result of the accumulation of oxidized lipid derivatives such as lysophosphatidylcholine (LPC), an attenuated eNOS activity, and the stimulation of eicosanoids formation and release by COX, lipoxygenases, and cytochrome P450 (Millanvoye-Van Brussel et al. 2004). In addition, endothelial dysfunction is associated with the initiation and progression of atherosclerosis, and with an increased level of vascular ROS (Miller et al. 1998).

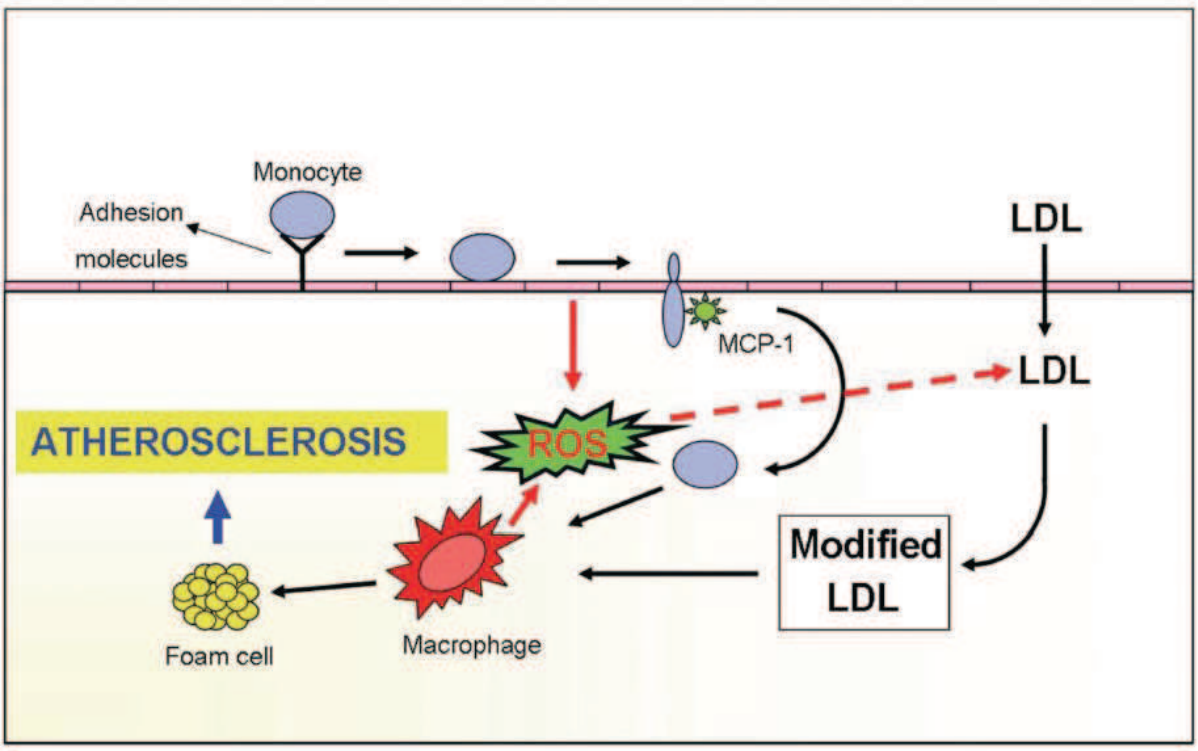

Figure 12. Role of low-density lipoprotein (LDL) in the atherosclerotic process. Oxidative stress increases free radical production. These radicals provoke an oxidative modification from low-density lipoprotein (LDL) to oxidized low-density lipoproteins (ox-LDL). Circulating monocytes migrate to the sub endothelial space in response to ox-LDL and causes endothelial cell injury. The modified LDL is taken up by macrophages which become foam cells, leading to the formation of atherosclerotic plaque. (Bonomini et al. 2008).

The development of the atheroma, constituted of a soft necrotic core under a rigid fibrous cap, can lead to an instable situation prone to rupture or erosion. Such a rupture will expose pro-thrombotic factors that will induce the formation of an intra-arterial thrombus, thus drastically reducing the blood flow. The thrombus causes ischemia in the tissue irrigated

by the artery, thus leading to myocardial infarction in the case of a coronary artery, stroke for a cerebral artery, and localised infarction for other vascular beds (mesenteric, renal, splenic, muscular, etc…) (Weiss and Taylor 2008). The mechanism underlying intra-arterial thrombosis might be attributed to platelets adhesion to sub-endothelial glycoproteins exposed to the bloodstream when the endothelium is injured, including collagen, von Willebrand factor (vWF), fibronectin and vitronectin (Brass 2003; Eisenberg and Ghigliotti 1999; Kroll et al. 1996). Moreover, the activation of platelets plays a critical role in the development of arterial thrombosis due to their adhesion to vascular cells and generation of lipid peroxides, which lead to the inhibition of the endothelium-dependent relaxation through reduced NO bioavailability and decreased prostacyclin production (Eisenberg and Ghigliotti 1999).

Chapter Two:

Omega-3 fatty acids and cardiovascular diseases

2. Nutrition and dietary lipids

Many dietary studies have linked the daily intake of fat, such as polyunsaturated fatty acids, and coronary heart diseases (Fiaccavento et al. 2006). The Mediterranean diet which is characterized by a high consumption of fruit and vegetables and moderate consumption of fish and olive oil as major sources of fat has been shown to reduce the risk of cardiovascular diseases in the general population (Kourlaba and Panagiotakos 2009). Findings from the Seven Countries Studies reported a low cardiovascular mortality in Southern Europe compared to Northern Europe and the US (Renaud et al. 1995). Results from meta-analyses have further suggested beneficial effects of the Mediterranean diet on CVD risk (Sofi et al. 2010; Sofi et al. 2008). The outcome of a meta-analysis including more than 2 million subjects and 50,000 deaths or incident cases from any causes and/or from any cardiovascular diseases, suggest significant protection of the adherence to the Mediterranean diet and the health status. These findings indicated an 8 % reduction of total mortality and 10 % reduction from death and/or incidence of cardio- and cerebrovascular diseases (Sofi et al. 2010).

2.1 Dietary omega-3 fatty acids

The inverse association of cardiovascular risk with intake of omega-3 polyunsaturated fatty acids was suspected early in populations that are known to have a high consumption of fish and fish oil. The beneficial effects of omega-3 fatty acid consumption were first reported in Greenland Eskimos who have a high consumption of marine-derived food including seal, walrus and whale (Simopoulos 2002). Indeed, they have a lower rate of cardiovascular diseases compared to the Danish population, whose diet contains about 22% of saturated fatty acid (Bang et al. 1980; Dyerberg et al. 1975). Omega-3 fatty acids, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been reported to have several beneficial effects on cardiovascular risk factors such as reduced blood pressure, heart rate, triglycerides level, inflammation, and an improved endothelial function and cardiac diastolic level (Abeywardena and Head 2001; Bhatnagar and Durrington 2003; Geelen et al. 2004; Thies et al. 2003). A prospective study has shown that consumption of fish or fish oil at least twice a week is associated with a significant reduction by 50% of the relative risk of fatal coronary heart disease (Kromhout et al. 1985*)*. In 2004, the US Food and Drug Administration (FDA) reviewed the beneficial effects of an increased consumption of omega-3 fatty acids, in particular EPA and DHA from four randomized, controlled, clinical intervention trials. The outcome of the four studies has suggested that an increased intake of 850 mg/d of EPA, 882

mg/d of DHA and alpha-linolenic acid (ALA) is associated with a decreased risk of cardiovascular diseases defined as death, non-fatal myocardial infarction, and non-fatal stroke (Burr et al. 1994; Ng et al. 1999; Singh et al. 1997; von Schacky et al. 1999) in patients who had survived a recent myocardial infraction. Additional randomized trials have provided evidence that replacing unsaturated fatty acids by polyunsaturated fatty acids, particularly EPA and DHA, protects against cardiovascular risk, but not against cardiovascular diseases mortality (Hooper et al. 2001). More recently, epidemiological studies have suggested an association between increased consumption of fish and decreased risk for coronary heart diseases and cancer (Daviglus et al. 1997; Kromhout et al. 1995; Lim et al. 2009), and inflammatory diseases (Simopoulos 2002). In the DART trial (diet and reinfarction trial), subjects recovering from myocardial infarction and advised to consume more fatty fish (two to three times a week) showed a 29% reduction in total mortality after 2 years compared to patients not advised to do so (Burr et al. 1989). These observations provide evidence that an increased consumption of omega-3 fatty acids, including EPA, DHA and α-Linolenic acid (ALA), has a beneficial effect in primary and secondary prevention of cardiovascular diseases (Albert et al. 2002; Alter and Rupp 2011; Marchioli et al. 2002). Moreover, several mechanisms have been suggested to explain the beneficial effects of dietary omega-3 fatty acids on cardiovascular diseases. They include anti-inflammatory and anti-arrhythmic effects, and decreased triglycerides level, blood pressure, thrombosis, and platelet aggregation, as well as plaque stabilization (de Lorgeril et al. 1994; Peter et al. 2013). The beneficial effects of omega-3 fatty acids on the cardiovascular system may also be attributed, at least in part, to their ability to improve the endothelial function (Wang et al. 2012). The omega-3 fatty acids can increase the endothelial release of vasoprotecting factors such as NO and endothelium-dependent hyperpolarization in human atherosclerotic arteries (Tagawa et al. 1999), porcine coronary arteries (Shimokawa et al. 1987), and in animal models of hypercholesterolemia (Komori et al. 1989; Shimokawa and Vanhoutte 1988). Moreover, omega-3 fatty acids have also been suggested to increase the NO formation in endothelial cells due, at least in part, to changes in lipid composition in caveolae micro-domains and also changes in the localisation of caveolin-1 that may eventually lead of activation of eNOS (Li et al. 2007).

2.2 Structure and classification of fatty acids

Lipids are defined as a group of organic compounds including fatty acids (FA), monoacylglycerols (MG), diacylglycerols (DG), triacylglycerols (TG), phospholipids (PL), and eicosanoids, resolvins, sterols, sterol esters, carotenoids, vitamins A, E, fatty alcohols, hydrocarbons and wax esters. Lipids are insoluble in water but soluble in organic compounds. In the body, lipids serve as energy storage in adipose tissue, and play a central role in the production of various compounds involved in signalling pathways, including prostaglandins, thromboxanes and leukotrienes. It has been observed that dietary intake of fats such as polyunsaturated fatty acids affect a wide variety of physiological processes by modulating the lipid metabolism in the cell membrane. Moreover, lipids such as triacylglycerols and phospholipids are important for the regulation of membrane functions and activation of membrane-bound enzymes, receptors, and ion channels (Clandinin et al. 1994). The diverse biological effects of dietary lipids have been reported as deleterious in the case of excessive consumption of cholesterol and saturated fatty acids (SFAs), while polyunsaturated fatty acids are generally regarded as beneficial for health.

Fatty acids are classically classified into three families based on their degree of unsaturation, namely the saturated fatty acids (SFA), the monounsaturated fatty acids (MUFA) and the polyunsaturated fatty acids (PUFA; Table 1).

Table 1. Structure and taxonomy of fatty acids (Simopoulos 1998)

Structure	Taxonomy	Common name
C4:0	Butanoïc acid	Butyric acid
C6:0	Hexanoïc acid	Caproïc acid
C8:0	Octanoïque acid	Caprylic acid
C10:0	Decanoïc acid	Capric acid
C12:0	Didecanoïc acid	Lauric acid
C14:0	Tetradecanoïc acid	Myristic acid
C16:0	Hexadecanoïc acid	Palmitic acid
C16:1 ω-7	*Cis*-9 hexadecaenoïc acid	Palmitoleic acid
C18:0	Octadecanoïc acid	Stearic acid
C18:1 ω-9	*Cis*-9 octadecaenoïc acid	Oleic acid (OA)
C18:2 ω-6	*Cis*-9,12 octadecadienoïc acid	Linoleic acid (LA)
C18:3 ω-6	*Cis*-6,9,12 octadecatrienoïc acid	γ-linolenic acid (GLA)
C18:3 ω-3	*Cis*-9,12,15 octadecatrienoïc acid	α-linolenic acid (ALA)
C20:0	Eicosanoïc acid	Arachidic acid
C20:4 ω-6	*Cis*-5,8,11,14 eicosatetraenoïc acid	Arachidonic acid (AA)
C20:5 ω-3	*Cis*-5,8,11,14,17 eicosapentaenoïc acid	Timnodonic acid (EPA)
C22:0	Docosanoïc acid	Behenic acid
C22:5 ω-3	*Cis*-7,10,13,16,19 docosapentaenoïc acid	Lupanodonic acid (DPA)
C22:6 ω-3	*Cis*-4,7,10,13,16,19 docosahexaenoïc acid	Cervonic acid (DHA)
C24:0	Tetracosanoïc acid	Lignoceric acid

2.2.1 Saturated fatty acids (SFA)

Saturated fatty acids consist of an aliphatic chain (straight hydrocarbon chain) without any double bond. They are classified as short, medium and long chain, depending on the number of atoms of carbon in the chain. At room temperature, they are solid or semi-solid. Saturated fatty acids such as lauric acid (C12:0), myristic acid (C14:0), stearic acid (C18:0), and palmitic acid (C16:0) are present in large quantities in certain food such as whole milk, cocoa butter, palm oil and coconut oil (Figure 13). Many clinical trials have shown the association of an increased consumption of saturated fatty acids with an increased low-density lipoprotein (LDL) cholesterol level in humans and animal models leading to an increasing risk of cardiovascular diseases like coronary heart diseases and cancer (Hu et al. 1997; Siri-Tarino et al. 2010).

2.2.2 Monounsaturated fatty acids (MUFA)

Monounsaturated fatty acids have a single double bond in the aliphatic chain (Figure 13). Monounsaturated fatty acids, such as oleic acid (C18:1n-9), palmitoleic acid (C16:1n-7) and vaccenic acid (C18:1n-11), are found only in plants but in a wide variety of plants including sunflower, corn, soybean, peanut, palm, sesame, and cottonseed, with the highest amount being found in canola acid (rapeseed) oil and olive oil by 75% and 85%, respectively (Schwingshackl and Hoffmann 2014). Clinical studies have shown that the consumption of mono or polyunsaturated fatty acids is associated with a reduced risk of cardiovascular diseases (Skeaff and Miller 2009) and stroke (Samieri et al. 2011), as well as a reduction by 26% of the total mortality in the Spanish branch of the EPIC study (Buckland et al. 2012).

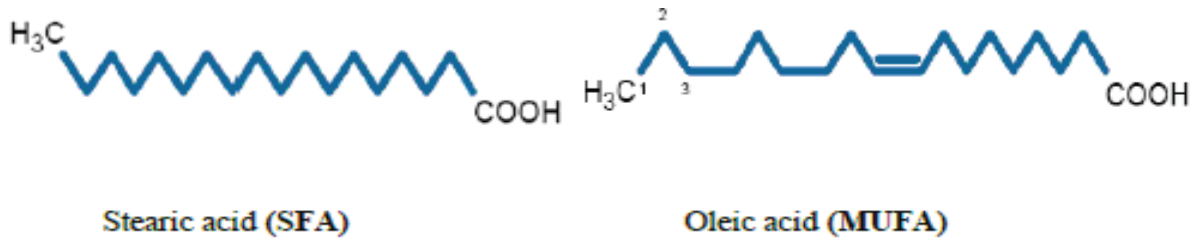

Figure 13 . Saturated fatty acids (SFA), Monounsaturated fatty acids (MUFA).

2.2.3 Polyunsaturated fatty acids (PUFA)

Polyunsaturated fatty acids are fatty acids which are characterized by the presence of two or more double bonds in the aliphatic chain (Figure 14). The number following "Omega-" represents the position of the first double bond, counting from the terminal methyl group on the molecule. Thus, omega-3 fatty acids have their first double bond between the 3rd and the 4th carbon atom from the methyl end of the molecule, whereas in omega-6 fatty acids the double bond is between the 6th and 7th carbon atom (Figure 14).

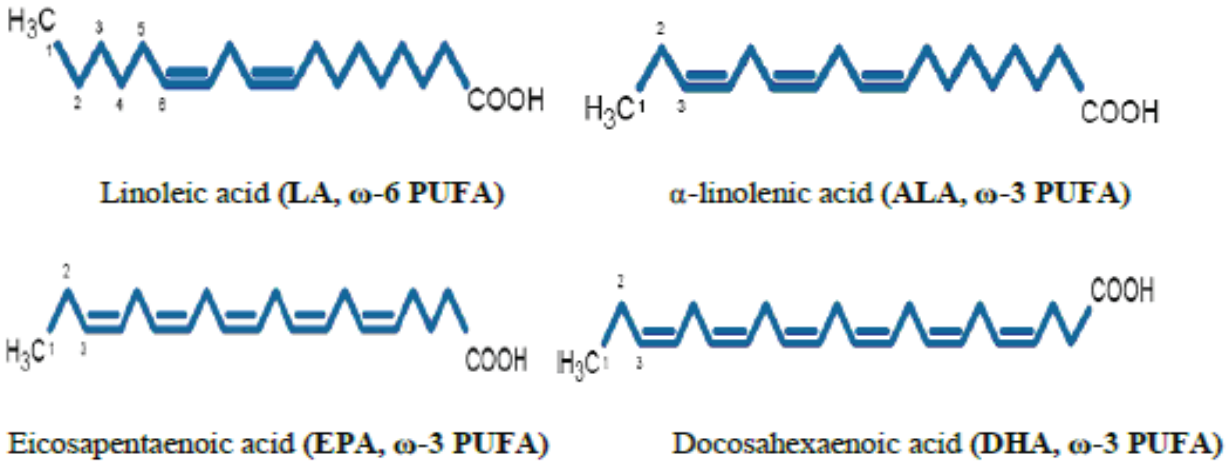

Figure 14. Polyunsaturated fatty acids (PUFA). ALA, α-linolenic acid and; LA, Linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid (Nair et al. 1997)**.**

2.2.4 *Trans*-fatty acids

Trans-fatty acids are monounsaturated fatty acids that can be found in certain food items like meat and dairy products (Figure 15). They are also found in higher concentrations in partially hydrogenated vegetable oil, a process used for the conversion of vegetable oil in the agro-food industry. Several studies suggest that an increase intake of *trans*-fatty acids is associated with increased plasma level of low density lipoprotein (LDL) and total cholesterol, and a reduced levels of high density lipoprotein (Lopez-Garcia et al. 2005; Stampfer et al. 1991), as well as an increased release of eicosanoids (Hu et al. 2001), and promotion of inflammation by different mechanisms including increased production of TNF-α and interleukin-6 in humans. All such effects will lead to an increased risk of cardiovascular diseases, endothelial dysfunction and type 2 diabetes mellitus (Ascherio et al. 1996; Kasim-Karakas et al. 1997; Kromhout et al. 1995; Salmeron et al. 2001; Willett et al. 1993). In

January 2006, the US FDA noted that foods containing *trans*-fatty acids must have a label sharing this information (Food and Drug Administration 2006).

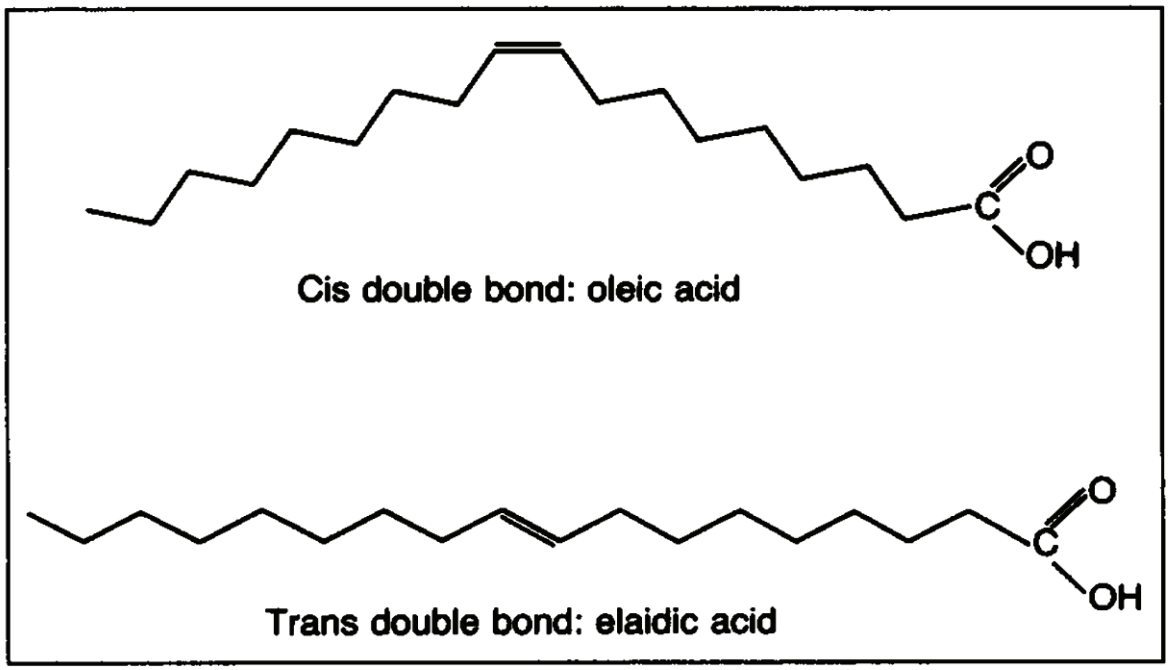

Figure 15 Trans fatty acids structure of oleic acid and elaidic acid (Mensink and Katan 1990).

2.3 Essential fatty acids

The essential fatty acids are long-chain polyunsaturated fatty acids that play an essential role as structural components of all cell membranes as well as important sources of biologically active metabolites. In humans, there are only two essential fatty acids, namely alpha-linolenic acid and linoleic acid, which are required for survival and must be obtained through diet. Indeed, mammals do not have the enzymes necessary to introduce a double bond at the omega-3 or omega-6 positions in aliphatic chains longer than nine carbons. A study from Evans and Burr reported that mammals can use linoleic acid and alpha-linolenic acid to synthesize respectively the omega-6 fatty acids and omega-3 fatty acids (Burr 2000), in particular EPA and DHA through elongation and desaturation mechanism. As the rate of conversion of linoleic acid is limited, most of omega-3 fatty acids are conditional essential fatty acids as the intake from diet represents the main source for these compounds. The alpha-linolenic acid is mostly found in green leafy vegetables, in vegetable oils such as canola and soybean oils, and nuts such as flaxseed and walnuts. A number of studies suggest that an

increased consumption of foods rich in ALA, like flaxseeds that contain about 35 % of ALA, might be associated with a reduced severity of cardiovascular diseases including myocardial infarction, atherosclerosis and hypertension in several animal models (Bierenbaum et al. 1993; Bloedon and Szapary 2004; Craig 1997; Lucas et al. 2002; Prasad 2009). It has also been reported that the intake of ALA is associated with a decreased blood pressure in spontaneously hypertensive rats (Bacova et al. 2013). Moreover, intake of polyunsaturated fatty acids such as ALA has been associated with a decreased platelets clotting activity (Austria et al. 2008; Renaud and de Lorgeril 1989; Renaud et al. 1986). Moreover, dietary intake of omega-3 fatty acids, in particular of EPA and DHA, has been associated with a reduced risk of several chronic diseases including cardiovascular diseases (He et al. 2004; Hu et al. 2002). The main dietary sources of omega-3 fatty acids in humans are the fatty fishes such as herring, mackerel, salmon, albacore tuna, and sardines (Figure 16). The beneficial effect of dietary fish and fish oil is associated with a reduction of cardiovascular diseases risk factors such as a lowering of plasma triglycerides, and blood pressure, and an improvement of the endothelial function (Bays et al. 2011; Gaibazzi and Ziacchi 2004).

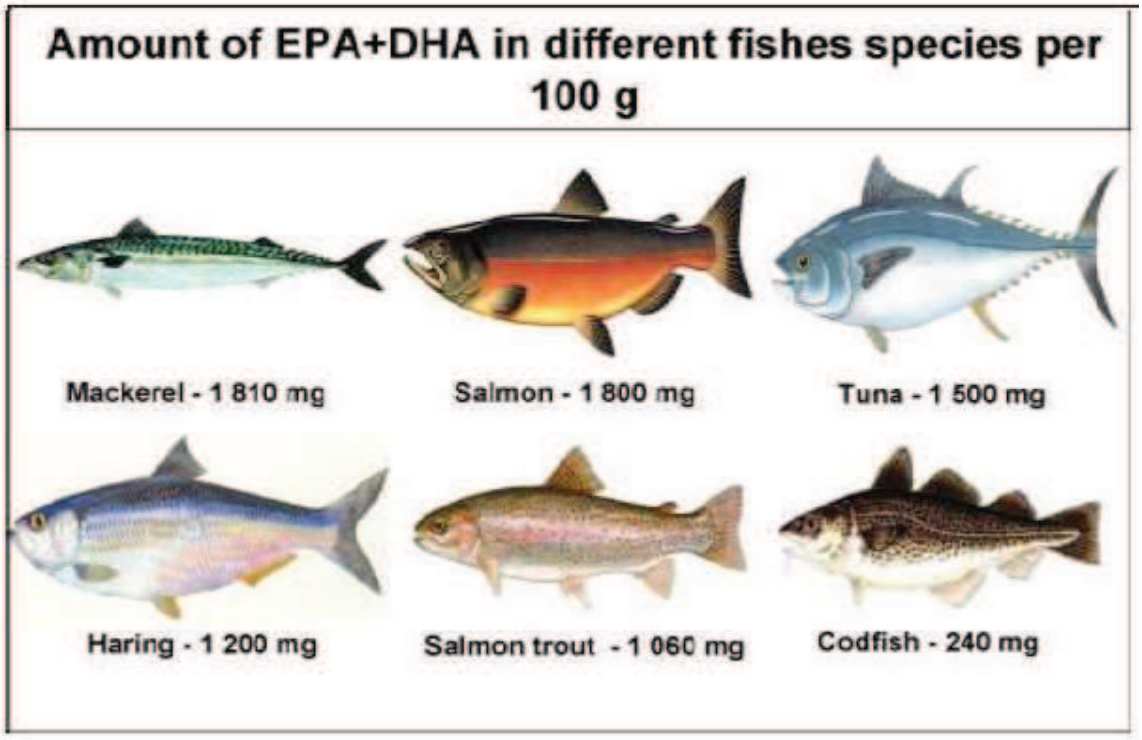

Figure 16. Content of omega-3- PUFA in fishes (Feskens et al. 1993).

2.4 Role of the ratio of omega-6 and omega-3 polyunsaturated fatty acids from our diet

Eicosanoids are 20 carbon molecules derived from omega-3 and omega-6 fatty acids which affect the inflammatory responses and the immune system. Eicosanoids are a family of compounds including prostaglandins, thromboxanes, leukotrienes, lipoxins and eoxins. A balanced intake of both omega-3 and omega-6 fatty acids is important for health because eicosanoids derived from either omega-3 or omega-6 have opposing metabolic properties. Indeed, ALA is the precursor for the 3-series of prostaglandins and thromboxanes, and the 5-series of leukotrienes, which are all associated with anti-inflammatory properties (Figure 17). Conversely, arachidonic acid (AA) is the precursor for the 2-series of prostaglandins and thromboxanes, and the 4-series of leukotrienes, which are associated with pro-inflammatory properties (Figure 17). Thus, increasing the omega-6/omega-3 fatty acids ratio leads to a shift from a physiological state to a pro-thrombotic and pro-aggregatory state associated with increases in blood viscosity and vasospasms (Simopoulos 1997). Several governments and organizations have issued recommendations to decrease the omega-6/omega-3 fatty acids ratio through a decreased omega-6 fatty acids intake and an increased omega-3 fatty acids intake (Kris-Etherton et al. 2000; Mozaffarian 2007). In addition, an increased consumption of omega-6 has also been suggested to be directly involved in inhibition of EPA incorporation

in cell membrane from dietary fish oil in humans subjects (Cleland et al. 1992). Moreover, reduced intake of linoleic acid has been associated with an increased circulating level of omega-3 fatty acids such as EPA and DHA in the human plasma (Stepanova et al. 2007). Similarly, it has been observed that the intake of fish oil containing 1.6 g of EPA and 0.32 g of DHA for 4 weeks is associated with a higher incorporation of EPA into the neutrophil membrane phospholipids in healthy male that are maintained on a low-linoleic acid diet for 3 weeks prior to the supplementation, compared to the group maintained on a high linoleic acid diet (Cleland et al. 1992). Moreover, the intake for 4 weeks by healthy Swedish subjects of a Mediterranean-inspired diet containing 3 times the amount of polyunsaturated fatty acids and twice the amount of omega-3 fatty acids was associated with decreased plasma omega-6 to omega-3 fatty acids ratio, platelets aggregation, and serum VEGF levels in comparison to an ordinary Swedish diet (Simopoulos 2006).

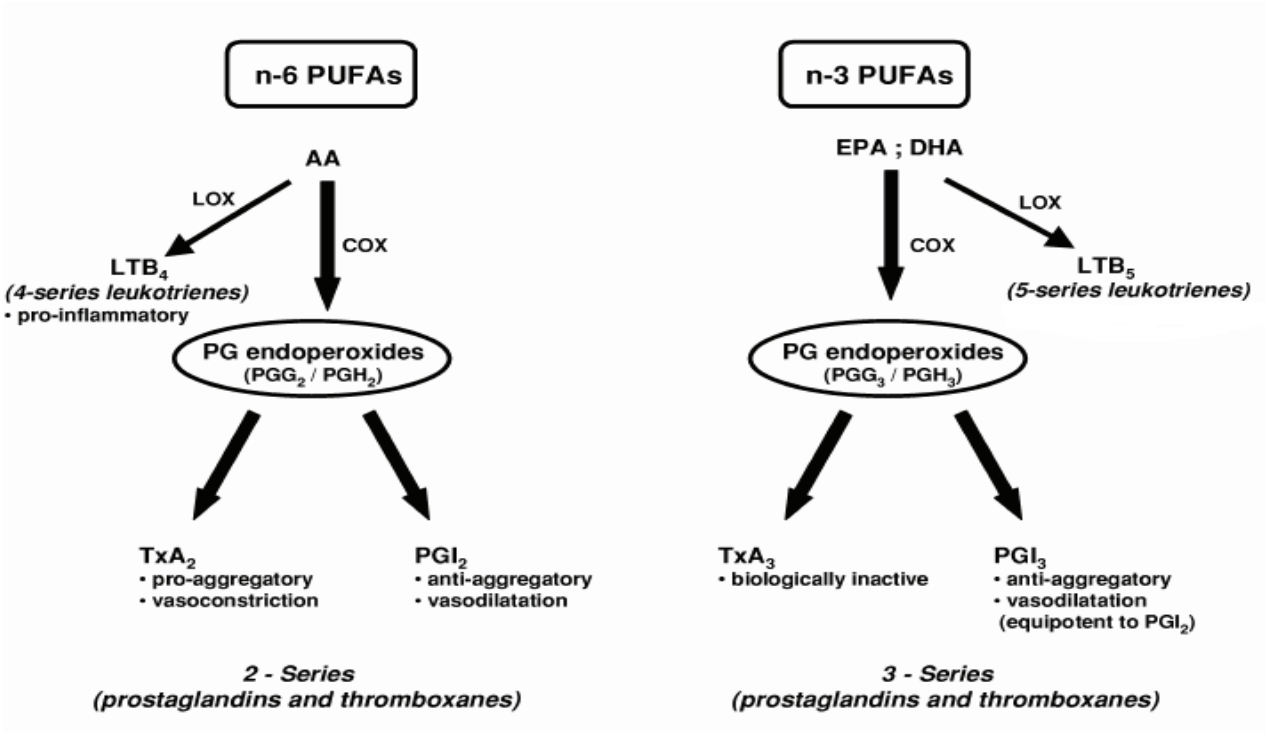

Figure 17. Eicosanoid metabolites derived from omega-3 and omega-6 polyunsaturated fatty acids. COX, cyclooxygenase; LOX, lipoxygenase; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; LT, leukotriens; PG, prostaglandin; TXA_2, thromboxane (Abeywardena and Head 2001).

2.5 Metabolism of polyunsaturated fatty acids

α-linolenic acid (ALA) can be metabolized to some extent by mechanisms including desaturation and elongation to yield EPA, DHA, while linoleic acid (LA) is the metabolic precursor of arachidonic acid (AA). In the omega-6 fatty acids pathway, linoleic acid can be first converted into gamma-linolenic acid (GLA, 18:3, omega-6 fatty acid) by the enzyme Δ6-desaturase before elongation leading to (DGLA) dihomo-GLA (DHGLA, 20:3, omega-6 fatty acids). The dihomo-GLA can be further converted into arachidonic acid (AA, 20:4, omega-6 fatty acids) by the Δ5-desaturase. The omega-3 fatty acids pathway uses the same series of enzymes for converting α-linolenic acid (ALA) into EPA and then into docosapentaenoic acid (DPA) by elongase (Figure 18). The conversion of ALA to EPA and DPA occurs primarily in the liver in the endoplasmic reticulum, whereas the final conversion of DPA to DHA requires a translocation to the peroxisome for a β-oxidation reaction (Arterburn et al. 2006).

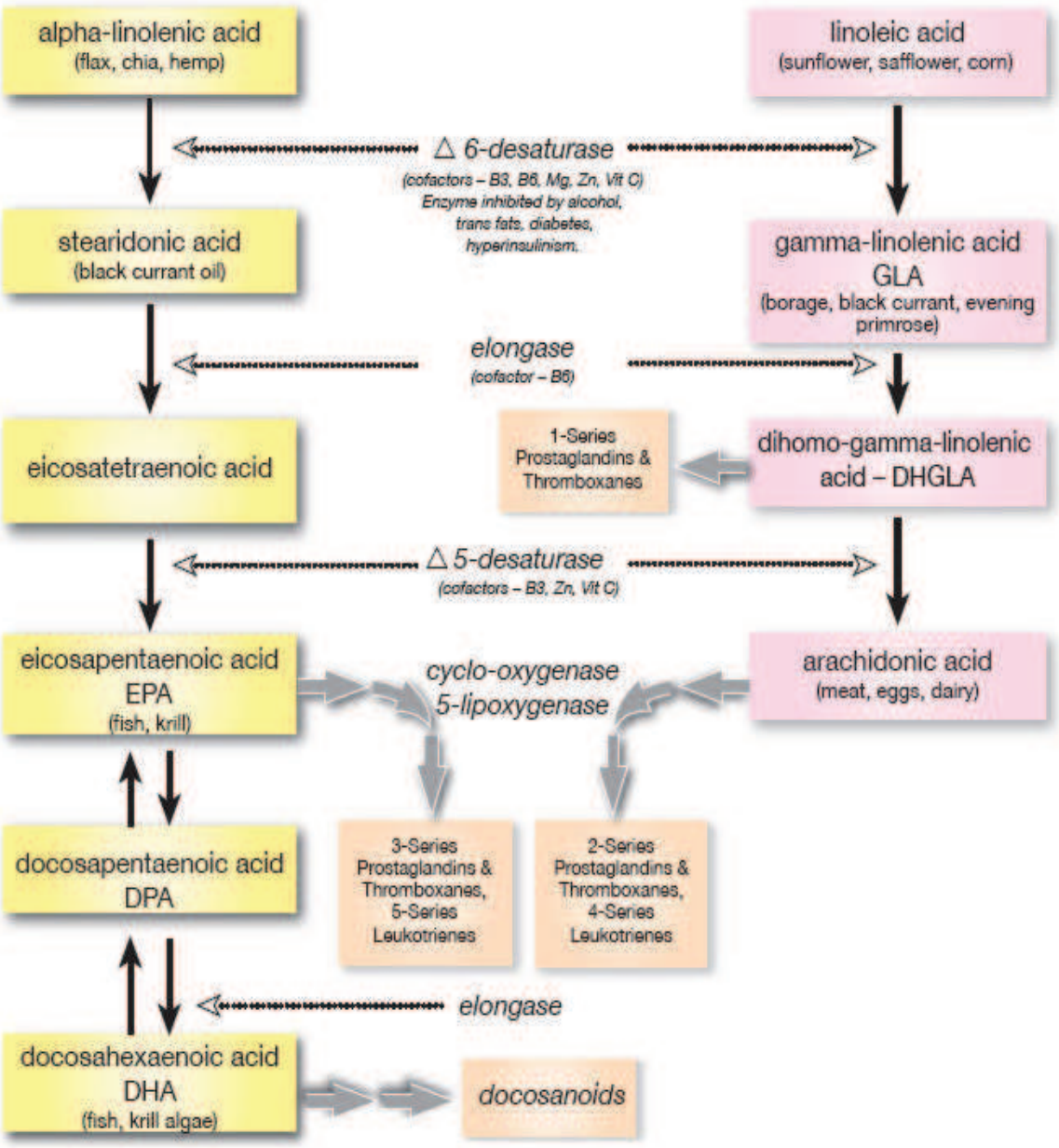

Figure 18. Synthesis pathway of omega-6 and omega-3 fatty acids in mammals (Kidd 2007).

2.6 Beneficial effect of polyunsaturated fatty acids

2.6.1 Biological effects of omega-3 fatty acids in cell membrane

Polyunsaturated fatty acids are the major component of membrane phospholipids, especially in the myocardium, retina, and brain, where they are important for function and growth, and are implicated in many physiological processes (Connor 2000). They are also found in many blood cells including red blood cells and platelets. Dietary consumption of polyunsaturated fatty acids, such as EPA and DHA, has beneficial health effects in many chronic diseases, including heart diseases, diabetes and inflammatory disorders. Many studies have suggested that the beneficial properties of omega-3 fatty acids affect a large range of organs and physiological systems. *In vivo* studies both in humans and animals have shown that dietary intake of omega-3 fatty acids increases the plasma content of fatty acids such as DHA and EPA (Christensen et al. 2001; Harris et al. 2008; Holub 1989; McLennan 2001), reduces the content of saturated fatty acids in membranes particularly in lipid rafts and calveolae (Garattini 2007). Moreover, omega-3 fatty acids can alter the basic properties of cell membrane including fluidity, elastic compressibility, ion permeability, and membrane remodelling (Stillwell and Wassall 2003). Recently, it has been also shown that omega-3 fatty acids, particularly EPA, can induce a change of the lipid environment in caveolae and thus induce the translocation of caveolin-1 outside of caveolae, thereby leading to the subsequent activation of eNOS (Li et al. 2007).

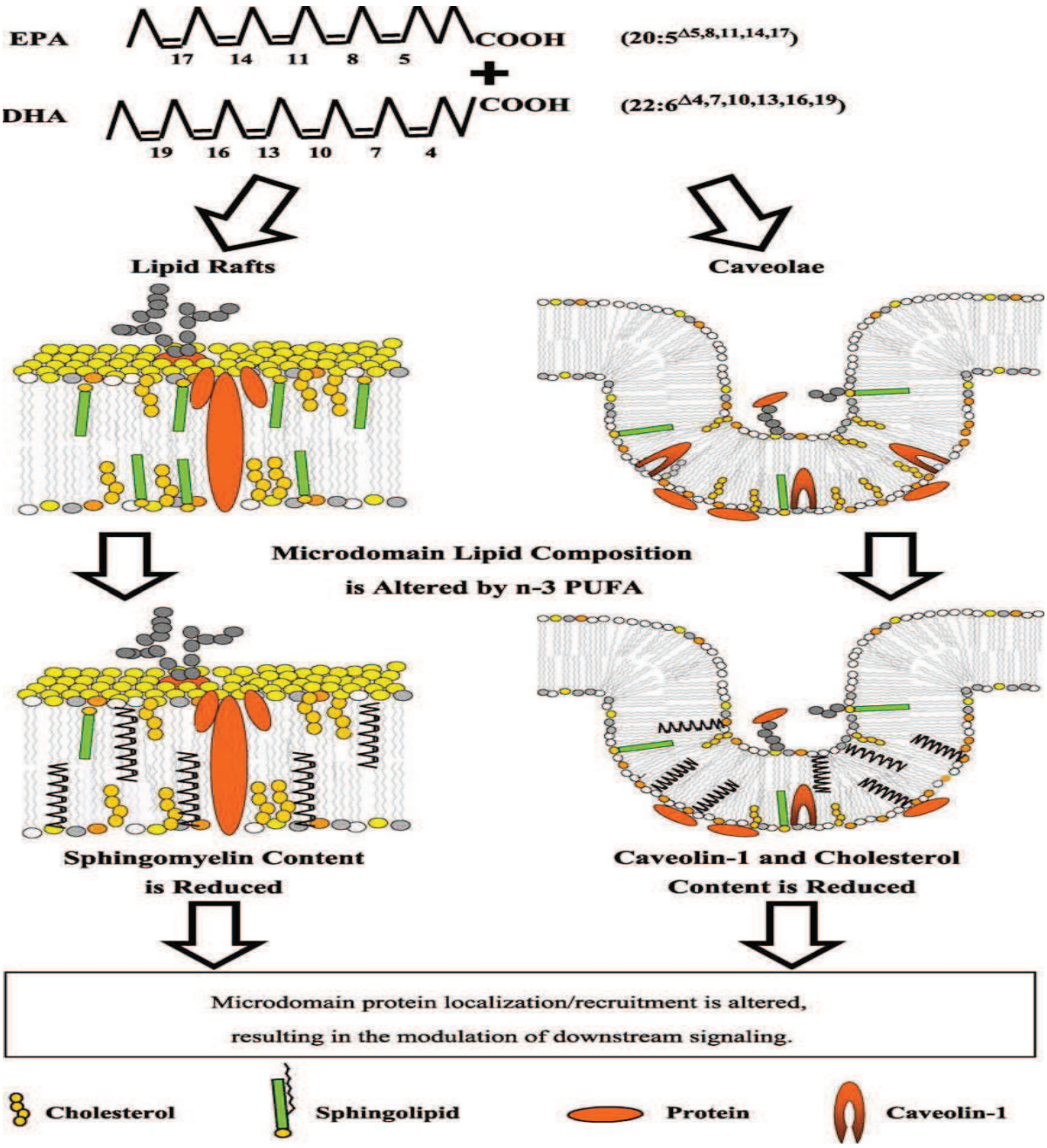

Figure 19. Incorporation of the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into lipid rafts and caveolae. Modulation of the microdomain lipid composition, resulting in the modulation of downstream cellular signaling events (Ma et al. 2004).

2.6.2 Lowering triglyceride levels

Several epidemiological studies have reported the positive association between an increased level of triglycerides in plasma and an increased development of cardiovascular diseases (Harris 1997; Sacks and Katan 2002). Recent studies suggest that omega-3 fatty acids play an important role in the regulation and reduction of triglycerides concentration via inhibition of the hepatic secretion of VLDL and triglycerides synthesis (Harris and Bulchandani 2006; Sampath and Ntambi 2005). Moreover, a study has shown that consumption of 3.4 g/day of EPA and DHA reduces the plasma triglycerides level by 17 % (Eritsland et al. 1989). Moreover the beneficial effect of omega-3 fatty acids on the plasma TG level has been reflected to inhibition an increased intracellular degradation of apolipoprotein B-100.

2.6.3 Anti-inflammatory effects of omega-3 fatty acids

Studies on the effects of lipids derived from polyunsaturated fatty acids in human have attracted much attention in recent years. Both omega-6 and omega-3 fatty acids have been associated with the activation and resolution of inflammatory responses, respectively. Arachidonic acid (AA) derived from omega-6 fatty acids is the most important precursor which is converted by different enzymes, such as COX and lipoxygenases (LOX), to different eicosanoid families including the prostaglandins, thromboxanes, leukotrienes, lipoxins, hydroxy and hydroperoxy-eicosatetraenoic acids (Calder 2015). Arachidonic acid metabolites are implicated in various pathological processes such as platelet aggregation, haemorrhage, and vasoconstriction (Rizos et al. 1988). Conversely, dietary omega-3 fatty acids, and particularly EPA, are the main sources of the 3-series of prostaglandins (PGs) and thromboxanes, and 5-series leukotrienes (LTs), all having anti-inflammatory and vasorelaxant effects. In addition, anti-inflammatory DHA-derived metabolites have been identified as d-series resolvins and protectins (Hong et al. 2003). Cells culture and animal feeding studies have shown that the EPA- and DHA-derived resolvins and related compounds, such as resolvin E1, resolvin D1 and protectin D1, have anti-inflammatory and pro-resolution effects (Calder 2010). These effects are mediated, at least in part, through down-regulation of TNF expression, which plays a major role in inflammatory gene expression (Serhan et al. 2008). Moreover, studies have suggested that omega-3 fatty acids may down-regulate the nuclear factor (NF)-kB pathway by the activation of peroxisome proliferators-activated receptors (Adkins and Kelley 2010; de Winther et al. 2005).

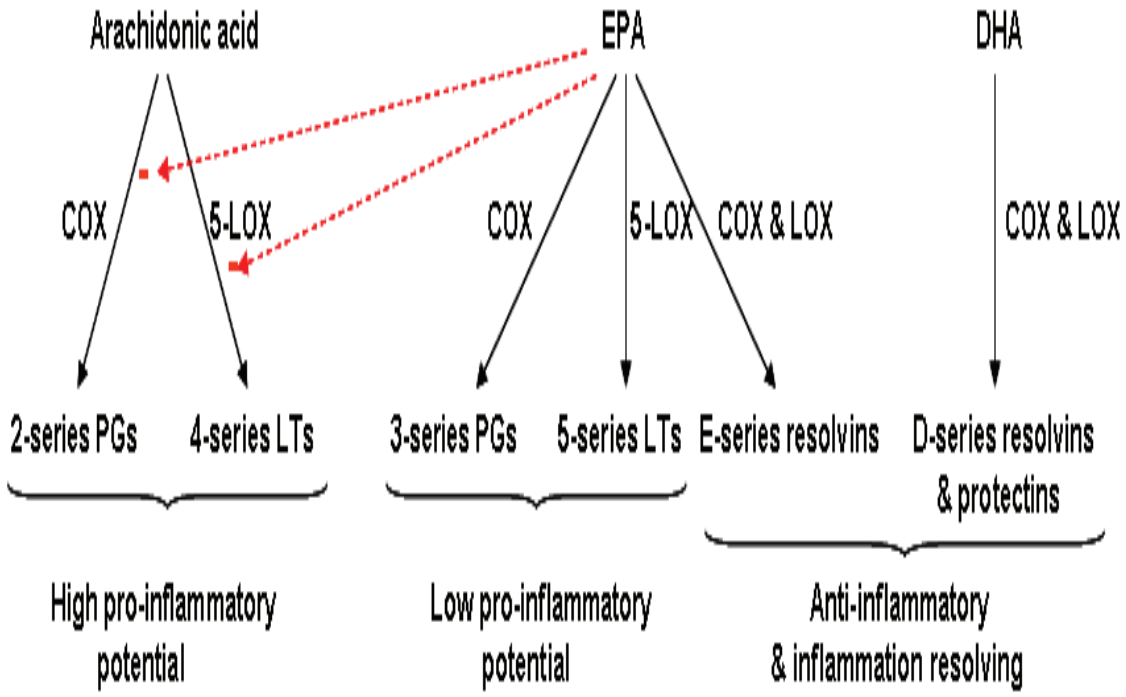

Figure 20. Synthesis and actions of lipid mediators produced from arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). COX, cyclooxygenase; LOX, Lipoxygenase; LT, Leukotriene; PG, prostaglandin; 5-LOX, Lipoxygenases; LTs, Leukotrienes (Calder 2010).

2.7 Cardiovascular protection

Cardiovascular diseases are the major causes of mortality worldwide and are associated, at least in part, with an increased intake of dietary saturated fats leading to an increased plasma level of LDLs, VLDLs, cholesterol and triglycerides. Since the first studies that reported that the traditional diet of Greenland Eskimos is associated with a decreased risk of cardiovascular diseases, several clinical studies have shown the beneficial effects of the consumption of fish and fish oil rich in omega-3 fatty acids, particularly EPA and DHA, in preventing cardiovascular diseases (Kromhout et al. 2010; Shaikh et al. 2014; Svensson et al. 2006). Furthermore, several studies have reported that fish oil could exert a beneficial role in primary and secondary prevention of cardiovascular diseases such as myocardial infarction, sudden cardiac death, coronary heart disease, arterial fibrillation and heart failure (Kromhout et al. 1985; Liou et al. 2008; Sheard 1998; von Schacky et al. 1999; Yokoyama et al. 2007). Similarly, the GISSI-P (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico - Prevenzionne) study indicated that dietary supplementation with 850 mg/d of EPA and 882 mg/d of DHA as ethyl esters was associated with a reduction of major cardiovascular events (20% for total deaths, 30% for cardiovascular deaths, and 45% for sudden deaths) in patients surviving a recent myocardial infarction over 3.5 years (von

Schacky et al. 1999). In the Zutphen study a cohort of 1373 males were examined repeatedly after long-term consumption of polyunsaturated fatty acids. This study showed that the strength of the association between long-term fish consumption and CHD death decreased with increasing age. Moreover, fatty-fish consumption lowered sudden coronary death risk while there was no clear dose-response relationship between EPA+DHA intake and sudden coronary death (Streppel et al. 2008). Further studies reported that the beneficial effects of polyunsaturated fatty acids on the cardiovascular system have been attributed to an inhibition of platelets aggregation and improvement of lipoproteins metabolism including a decreased plasma triglycerides level, decreased inflammation, lowered blood pressure and improved endothelial function (Calder 2001; Connor and Connor 1997; Geleijnse et al. 2002; Goodfellow et al. 2000; Harris 1997; Hornstra 2001; Knapp 1997; Mori et al. 2000; Sacks and Katan 2002; Simopoulos 1991).

2.7.1 Hypertension

Hypertension is a complex and common disease with a complex aetiology that could involve several different mechanisms (Williams et al. 1990). Many studies have indicated that the dietary consumption of fish, fish oil, and omega-3 fatty acids is associated with a decreased blood pressure in hypertensive subjects. Indeed, the dietary supplementation with fish and fish oil (15 g of omega-3 fatty acids) for four weeks is associated with a reduced blood pressure in mild hypertensive patients (reduction of systolic blood pressure by 6.5 mmHg and of diastolic blood pressure by about 4.4 mmHg) (Knapp and FitzGerald 1989). Moreover, a meta-analysis study has indicated that the supplementation with fish oil is associated with a reduction in the blood pressure values (0.4-0.7 mmHg) in men (Appel et al. 1993; Morris et al. 1993). The beneficial effect on blood pressure is, at least in part, due to an improvement of the endothelium function through an increased NO production within the vascular wall (Omura et al. 2001). Human clinical trials suggested that fish oil intake attenuated the vasoconstriction induced by adrenaline and angiotensin II in human peripheral arteries through an improved endothelial function (Chin et al. 1993; Mori et al. 2000).

2.7.2 Metabolic syndrome

Obesity and diabetes mellitus are increasingly common diseases in the world. Indeed, in 2013 about 282 million persons were diagnosed with type 2 diabetes (T2DM) associated with metabolic disorders such as obesity (Alberti and Zimmet 2014). T2DM is characterized as an independent risk factor of cardiovascular diseases, hypertension, and hyperlipidaemia.

In overweight and mildly hyperlipidemic men, intake of 4 g of marine oil containing EPA and DHA is associated with an increased endothelium-dependent vasorelaxation and decreased blood pressure and heart rate (Stamler et al. 1993). Similarly, intake of EPA and DHA in T2DM patients was associated with increased fasting glucose, C-peptide levels, and insulin sensitivity (Mori et al. 2000; Woodman et al. 2002). In addition, consumption of 3.6 g of purified EPA and DHA decreased the level of urinary F2-isoprostanes, a marker of oxidative stress, in overweight and mildly hyperlipidemic subjects, possibly due to the anti-inflammatory properties of omega-3 fatty acids (Gopaul et al. 1995). Since the level of urinary F2-isoprostanes is also elevated in T2DM patients, omega-3 fatty acids may also exert a beneficial anti-inflammatory effect in these subjects (Davi et al. 1999). Moreover, omega-3 fatty acids intake is associated with an improved endothelial function via the interruption of the insulin-dependent modulation of the PI3-kinase pathway leading to increased endothelial formation of the vasoprotective NO (Fulton et al. 1999; Michell et al. 1999).

2.7.3 Improvement of the endothelial function

As described previously, the endothelium plays a pivotal role in the maintenance of vascular homeostasis, in part, by the formation and release of NO. The endothelium-derived NO exerts multiple vascular effects including the relaxation of the vascular smooth muscle, maintenance of the vascular smooth muscle in a quiescent state, the inhibition of platelet adhesion and aggregation, of leucocytes adhesion, and of pro-atherosclerotic and pro-thrombotic responses, in part, by inhibiting the expression of adhesion molecules, monocyte chemoattractant protein-1, and tissue factor (Schini-Kerth et al. 2010). The intake of omega-3 fatty acids, including EPA and DHA, has been associated with an improvement of the endothelial function and an increased NO bioavailability subsequent of an increased endothelial formation (Boulanger et al. 1990; Chin 1994; Chin and Dart 1995; Chu et al. 1992; Yin et al. 1991). Moreover, supplementation with omega-3 fatty acids (0.8 g/kg/d) in ovariectomized rats is associated with reduced blood pressure, arterial stiffening and vascular oxidative stress, and increased endothelial function due to increased eNOS expression and activity (Gortan Cappellari et al. 2013; Losurdo et al. 2014). In patients with T2DM, the dietary supplementation with 2 g of purified EPA/DHA daily for 6 weeks is associated with a reduction of the postprandial impaired macro- and micro-vascular functions (Stirban et al. 2010). Similarly, the daily intake of 5 g EPA plus DHA for 3 weeks is associated with an increased vasodilatory response to acetylcholine in the coronary artery of heart transplanted patients (Fleischhauer et al. 1993). Moreover, the daily intake of 1.8 g of EPA for 6 weeks by

eight patients with documented coronary artery diseases is associated with an improvement of both the NO-dependent and the NO-independent endothelium-dependent forearm vasodilatation (Tagawa et al. 1999).

The beneficial effect of omega-3 fatty acids on the endothelial function has been attributed mainly to their ability to increase the endothelium-dependent formation of NO through various mechanisms. Indeed, in primary cultures of human coronary artery endothelial cells, DHA increased the expression and phosphorylation of eNOS as well as the phosphorylation of the Akt (Stebbins et al. 2008).

Moreover, several studies demonstrated that EPA exerted protective actions against the palmitic acid induced endothelial dysfunction through the activation of the AMPK, which the phosphorylation of the Akt/eNOS signalling pathway (Lee et al. 2014). On the other side, in cultured endothelial cells, DHA is able to increase the activity of SIRT1 that will catalyse the deacetylaction of lysine 496 and 506 in the calmodulin-binding domain of eNOS, leading to an increase endothelial formation of NO (Jung et al. 2013), whereas EPA, but not oleic acid, increased the insulin-stimulated NO production without modulating the insulin-induced Akt-dependent phosphorylation of eNOS (Lynn et al. 2004). Similarly, the omega-3 fatty acids induced changes in the membrane organization in caveolae that are accompanied by alterations of caveolin-1 and eNOS interaction, leading an increased phosphorylation and activation of eNOS (Li et al. 2007).

AIM OF THE STUDY

The endothelium has an important role in the regulation of the vascular homeostasis, by inducing various vasodilatating mechanisms, which can also inhibit platelet aggregation and reduce the endothelial expression of adhesion molecules, and thus reduce the risk of cardio vascular diseases. Several studies and clinical trials have shown that an endothelial dysfunction has a major role in many cardiovascular diseases and endothelial dysfunction is an early indicator of atherosclerosis. Endothelial dysfunction also occurs as a result of reduced NO bioavailability and increased vascular oxidative stress.

The studies also demonstrate that intake of fish or fish oil rich in polyunsaturated fatty acids, particularly EPA and DHA, improves endothelial function associated with reduced cardiovascular diseases. The improved endothelial function involves an increase NO bioavailability. Such effects have been observed in rat aortic rings and coronary artery rings (Engler and Engler 2000; Omura et al. 2001). Furthermore, other studies have been shown that EPA and DHA profoundly alter the lipid composition of caveolae/lipid rafts and are incorporated into the cell membrane, thereby modifying the location and function of proteins in caveolae. This is associated with selective displacement of caveolae-1 and eNOS from caveolae lading enhanced eNOS activation.

The main goal with this thesis was to determine the role of different ratios of omega-3 fatty acids EPA:DHA 1:1, 3:1, 6:1, 1:3, 1:6 and 1:9 to improve endothelium-dependent relaxation by stimulating the endothelial formation of NO and EDH.

More specifically, the aims were:

- The role of EPA:DHA ratio and amount to induce endothelium-dependent relaxation in rings of porcine coronary and human mammary artery.
- To characterize the mechanism underlying EPA:DHA-induced endothelium dependent–relaxation mediated by NO and EDH in coronary artery and human mammary artery rings.
- To characterize the mechanism underlying the ability of omega-3 fatty acids EPA: DHA 6:1 formulation to stimulate the redox-sensitive Src/PI3-kinase/Akt pathway leading to the phosphorylation of eNOS at Ser1177.
- The ability of omega-3 fatty acids (EPA, DHA and different ratios of EPA:DHA) to cause activation of eNOS in cultured endothelial cells and to determine the underlying signal transduction pathway.

- Evaluate the ability of EPA:DHA to reduce contractile responses in porcine coronary artery and human mammary artery rings.

RESULTS

Article I

Redox-Sensitive Induction of Src/PI3-kinase/Akt and MAPKs Pathways Activate eNOS in Response to EPA:DHA 6:1.

Article I

Redox-Sensitive Induction of Src/PI3-kinase/Akt and MAPKs Pathways Activate eNOS in Response to EPA:DHA 6:1.

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In 1953, Hugh Sinclair published the first report indicating that the Inuit diet, which is rich in seal, fish and fish oil, was associated with lower incidence of cardiovascular diseases (Sinclair 1953). He later attributed the beneficial effects of the diet to its high content in long chain omega-3 fatty acids (Sinclair 1956). The beneficial cardiovascular effects of omega-3 fatty acids, and especially EPA and DHA, have since been reported in several number of clinical studies, randomized controlled trials and meta-analyses (Casula et al. 2013; Mariani et al. 2013; Rizos et al. 2012; Shaikh et al. 2014). Several mechanisms have been suggested to explain the reduction of cardiovascular events associated with the dietary intake of omega-3 fatty acids including the reduction of cardiovascular risk factors such as hypertension, dyslipidemia, inflammation and platelets aggregation (Bays et al. 2011; Geleijnse et al. 2002; He et al. 2009; Mozaffarian et al. 2005; Theobald et al. 2007). Another beneficial effect attributed to omega-3 fatty acids is the improvement of the endothelial function (Abeywardena and Head 2001; He et al. 2009). Indeed, several studies have reported that both EPA and DHA can induce potent endothelium-dependent relaxations due, at least in part, to the activation of eNOS and the subsequent formation of NO (Komori et al. 1989; Shimokawa and Vanhoutte 1988; Tagawa et al. 2002).

However, the beneficial cardiovascular effects of the dietary supplementation in omega-3 fatty acids has been challenged by a meta-analysis by Hooper et al. in 2006 that concluded that there was no clear benefit of omega-3 fatty acids on cardiovascular events (Hooper et al. 2006). Similarly, several randomized double-blind clinical studies did not show a reduction of cardiovascular events after omega-3 fatty acids supplementation (Galan et al. 2010; Kromhout et al. 2010).

The discrepancy of these findings has been attributed to several factors including the heterogeneous population studied and parameters determined. In addition, the conflicting results could also be attributed to the vast range of omega-3 fatty acids sources, formulations and doses used in the different studies. Indeed, the supplementation in omega-3 fatty acids has been provided in form of capsules, oils, emulsions, margarines, fatty fish, whereas the control groups were often undefined (Hooper et al. 2006). Moreover, the daily intake dose of omega-3 fatty acids was within a large range going from 0.4 up to 7.0 g containing EPA, DHA and DPA in very different amounts (Hooper et al. 2006). Similarly, a meta-analysis by Wang et al. evaluating the effects of omega-3 fatty acids on the endothelial function included a large range of omega-3 fatty acids in the 16 randomized clinical studies (Wang et al. 2012). Indeed, the daily dose of omega-3 fatty acids ranged from 0.45 to 4.5 g per day in form of fish oil, walnuts and capsules containing various formulations of EPA and/or DHA (Wang et al. 2012).

Thus, the aim of our study was to evaluate the role of omega-3 fatty acids formulations on the ability to activate the endothelial function, and in particular to promote the endothelial promotion of NO. Firstly, to identify an optimized formulation of omega-3 fatty acids, we have evaluated the ability of EPA, DHA and formulations with different EPA: DHA ratios ranging from 1:9 to 9:1 to induce potent endothelium-dependent relaxations in porcine coronary artery rings. The influence of the purity was also evaluated by comparing an optimized formulation with a formulation containing the same EPA:DHA ratio, but only with a 50% content in omega-3 fatty acids. Finally, the cellular and molecular mechanisms underlying the omega-3 fatty acid induced relaxation was investigated.

Results and discussion

The main finding of our study indicated that omega-3 fatty acids formulations are able to induce potent endothelium-dependent relaxations in porcine coronary artery rings. This findings is in line with several previously published studies that have shown that omega-3 fatty acids and fish oils are able to induce endothelium-dependent relaxations in various types of isolated arteries including the coronary artery, the pulmonary artery and the cerebral artery (Engler et al. 2000; Omura et al. 2001; Shimokawa et al. 1988b; Shimokawa and Vanhoutte 1988; Vanhoutte 1989).

The present findings show that while EPA and DHA are both able to induce endothelium-dependent relaxations in porcine coronary artery rings, the combination of the two omega-3 fatty acids in formulations EPA:DHA 6:1 and 9:1 was associated with an increased potency to induce relaxation. The enrichment of the formulation with EPA was associated with a dose-dependent increase in the relaxation, with the maximal relaxation obtained with the EPA:DHA 6:1 and 9:1 formulations. The enrichment of the formulations in DHA was only associated with a weak increase in relaxations. The optimized formulation of EPA:DHA was then defined as the EPA:DHA 6:1 ratio, which was subsequently used for further characterization. The role of the purity was assessed by comparing the optimized EPA:DHA 6:1 formulation which is highly purified with more than 90% of EPA and DHA, to a formulation containing EPA:DHA 6:1 with a reduced purity of about 50%. The results have indicated that a reduction of the content in omega-3 fatty acids by 50% was associated with significantly less relaxation. Altogether, these findings indicate that the potency of omega-3 fatty acids formulations to induce endothelium-dependent relaxations is affected by both the EPA:DHA ratio and the purity.

Furthermore, we determined the mechanism underlying the relaxation induced by the optimized formulation. The findings show that the optimized formulation induce an endothelium-dependent relaxation that is reduced by the inhibition of eNOS and abolished by the inhibition of eNOS plus that of EDH-mediated relaxations. These observations indicate that the optimized formulation of omega-3 fatty acids induces endothelium-dependent relaxation mainly due to the formation of NO, and also, to some extent, to the EDH-mediated component of the relaxation. Moreover, the induction of the NO-mediated relaxation was reduced by inhibitors of ether oxidative stress, Src kinase, PI3-kinase, p38 MAPK, MEK, or JNK. These findings suggest that the optimized formulation induced relaxations in porcine coronary artery rings through a redox-sensitive activation of the Src/PI3-kinase/Akt and

MAPKs pathways leading to the activation of eNOS. Such a mechanism is similar to that involved in the endothelium-dependent relaxation induced by polyphenol-rich products, another type of natural compounds. Indeed, a redox-sensitive activation of the Src/PI3-kinase/Akt leading to the activation of eNOS and subsequent formation of NO was reported in response to grape-derived products and isolated phenolic compounds (Auger et al. 2010a; Auger et al. 2010b; Kim et al. 2007; Schini-Kerth et al. 2010).

In conclusion, the present study has shown that the ability of omega-3 fatty acids products to induce endothelium-dependent relaxations is dependent on the ratio and the amount of the EPA:DHA formulation. Furthermore, the optimized EPA:DHA 6:1 formulation induces endothelium-dependent relaxations via the stimulation of the endothelial formation of NO, and to a lesser extent to the activation of the EDH pathway. The NO-mediated relaxation involves an intracellular oxidative stress triggering the activation by phosphorylation of the Src/PI3-kinase/Akt and MAPKs pathways, ultimately leading to increase eNOS activity.

Article II

The omega-3 EPA:DHA 6:1 formulation prevents the platelets-induced arterial contraction by abolishing the serotonin-mediated contraction in porcine coronary and human internal mammary artery rings.

Article II

The omega-3 EPA:DHA 6:1 formulation prevents the platelets-induced arterial contraction by abolishing the serotonin-mediated contraction in porcine coronary and human internal mammary artery rings

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In preparation

Our previous work has indicated that various formulations of omega-3 fatty acids products induce potent relaxations in porcine coronary artery rings depending on the EPA:DHA ratio and degree of purity. Indeed, the EPA:DHA 6:1 ratio with a high purity rate was amongst the most potent product tested. Moreover, the relaxation induced in porcine coronary artery rings by omega-3 fatty acids products is an endothelium-dependent event mediated mainly by the activation of eNOS but also, to a lesser extent, to the EDH component. The EPA:DHA 6:1 ratio has been shown to induce the endothelium-dependent NO-mediated relaxation through redox activation of Src/PI3-kinase/Akt and MAPKs with the subsequent activation of eNOS. While these findings indicate that omega-3 fatty acids products activate the endothelial function in healthy porcine coronary artery rings, the relevance of the beneficial effects of omega-3 products in pathophysiological conditions remains to be determined.

Indeed, in pathophysiological conditions such as atherosclerosis, hypertension, metabolic syndrome or diabetes, blood vessels often present an endothelial dysfunction which is associated a reduced formation of endothelium-derived relaxing factors and increased

formation of contracting factors and expression of pro-atherothrombotic factors. Activated platelets are known to induce potent vasoconstriction through the formation and release of several contracting factors including thromboxane A_2, serotonin and eicosanoids. Moreover, the platelet-induced contractions are more pronounced in blood vessels with an endothelial dysfunction compared with healthy blood vessels.

Thus, the aim of the present study was to evaluate the potency of omega-3 fatty acids products to prevent the contraction induced by platelets in porcine coronary artery rings and, if so, to investigate the underlying mechanism. For this purpose, contractions were induced by human washed platelets in porcine artery rings, and the inhibitory effect of omega-3 fatty acids products was evaluated. The mechanisms involved in the platelet-induced contraction and in the preventive effect of omega-3 fatty acids products were determined. To determine the potential clinical relevance, the beneficial effect of omega-3 fatty acids products as well as the underlying mechanisms were studied in human internal mammary artery rings.

Results and discussion

The present study indicates that human washed platelets can induce dose-dependent contractions in porcine coronary artery rings. The platelet-induced contraction is significantly inhibited in the presence of the EPA:DHA 6:1 formulation, whereas the EPA:DHA 1:1 formulation had little effect. Moreover, the EPA:DHA 6:1 formulation dose-dependently prevented the serotonin (5-HT)-induced contraction, whereas it was without effect on the thromboxane A_2 analogue (U46619)-induced contraction. The latter, associated to the fact that the platelet-induced contraction was markedly reduced by ketanserin, a 5-HT_{2A} receptor antagonist, strongly suggests that human washed platelets induced the contraction in porcine coronary artery rings mainly through serotonin released from activated platelets. The EPA:DHA 6:1 at 0.4 % (v/v) was able to prevent both the platelet-induced and the 5HT-induced contractions only in the presence of a functional endothelium, whereas it was without effect in endothelium-denuded rings or in the presence of an eNOS inhibitor. These findings suggest that the preventive effect of EPA:DHA 6:1 on platelet-induced serotonin-mediated contraction is mainly due to its ability to induce the endothelium-dependent formation of NO. This hypothesis is reinforced by the fact that a low concentration SNP, a NO donor, significantly inhibited the contraction induced by 5-HT, but not that by U46619. In addition, the EPA:DHA 6:1 formulation at 0.4 % (v/v) also significantly prevented the 5-HT-induced contraction in human mammary artery rings through NO-mediated mechanisms.

In conclusion, the present findings show that the optimized EPA:DHA 6:1 formulation is able to prevent the 5-HT-mediated platelet-induced contraction in porcine coronary and human internal mammary artery rings through an endothelium-dependent NO-mediated mechanism. These results suggest that optimized omega-3 products may exert potent beneficial effect on vascular health by activating the endothelial function leading to an increased formation of NO to prevent the deleterious vascular effects of activated platelets.

GENERAL DISCUSSION AND PERSPECTIVES

The endothelium plays a pivotal role in the regulation of vascular homeostasis mainly through the formation and release of vasodilatating and vasocontracting factors. Mumerous studies have shown an association between endothelial dysfunction, an imbalance of the formation of relaxing and contracting factors released by the endothelium, and the development of cardiovascular diseases in the presence of risk factors such as hypertension, diabetes mellitus, atherosclerosis, thrombosis and platelet aggregation (Anderson 1999; Forstermann et al. 1994; Hadi and Suwaidi 2007; Sase and Michel 1995). The protective effects of the endothelium towards the vascular function has been attributed, at least in part, to its ability to produce vasoprotective dilating factors which are mainly NO, EDH, and PGI_2. The impairment of the endothelial function is generally associated with decreased NO bioavailability in many cardiovascular diseases and risk factors such as hypertension, diabetes, and atherosclerosis (Davignon and Ganz 2004; Forstermann et al. 1994).

The beneficial effect of intake of omega-3 fatty acids on the risk of cardiovascular diseases has been reported in numerous studies (Forstermann et al. 1994; Lopez-Garcia et al. 2004; Nettleton et al. 2010; Nettleton et al. 2008; Nettleton et al. 2007). Indeed, the initial studies reported that the high intake of fatty fish and fish oil by the Greenland Eskimos compared to Danes was associated with a reduced mortality from cardiovascular diseases (Dyerberg and Bang 1979; Dyerberg et al. 1978; Forstermann et al. 1994). Several further studies have since reported the beneficial effects of the consumption of fatty acids such as EPA and DHA in various diseases such as hypertension, diabetes, and atherosclerosis (Albert et al. 1998; Burr et al. 1989; de Lorgeril et al. 1994; Forstermann et al. 1994; Marchioli et al. 2002; Mozaffarian et al. 2004; Siscovick et al. 1995).
However, recent meta-analyses of clinical studies have suggested a limited beneficial effect of omega-3 fatty acids supplementation due to conflicting results amongst various studies (Delgado-Lista et al. 2012; Enns et al. 2014; Forstermann et al. 1994; Kromhout et al. 2012). These conflicting results are due, at least in part, to the heterogeneity of omega-3 sources, formulations and doses used in the different studies.

The aim of the present work was firstly to evaluate the role of the formulation of omega-3 fatty acids on the induction of endothelium-dependent relaxations, the contribution of both NO- and EDH-mediated relaxations, and to determine the underlying mechanisms. The second aim of the study was to evaluate the beneficial vascular effect of omega-3 fatty

acids on the platelet-induced coronary artery contraction, and to characterize the role serotonin and vasoconstricting prostanoids.

Therefore, we have evaluated various ratios and different levels of purity of EPA and DHA formulations based on their potency to induce potent endothelium-dependent relaxations in porcine coronary artery rings. Indeed, our findings show that the induction of endothelium-dependent relaxation, and especially the activation of the NO formation, is dependent on both the purity and the ratio of the EPA:DHA formulation. While isolated EPA and DHA are able to induce endothelium-dependent relaxations in porcine coronary artery rings, EPA induced significantly greater relaxations than DHA at the concentration of 0.4% (v/v). Similarly, the evaluation of various ratio of EPA:DHA indicates that the relaxing effect is maximized in presence of an EPA:DHA ratio of 6 or 9 to 1, whereas formulation with lower content of EPA induced significantly weaker relaxations. Moreover, an EPA:DHA 6:1 formulation with a EPA:DHA content reduced by 50% also induced significantly weaker relaxations than the optimized EPA:DHA 6:1 formulation with a high EPA:DHA purity. These findings thus suggest that the beneficial vascular effects of omega-3 formulations are dependent of the content in term of both total omega-3 contents and EPA:DHA ratio. These parameters should be taken into account when comparing results from both preclinical and clinical studies using different products rich in omega-3 fatty acids. Moreover, the present findings may also help to understand the discrepancies found in several clinical studies assessing the cardiovascular effects of various omega-3 formulations ranging from fish oil to isolated purified EPA or DHA used at different doses.

Indeed, in meta-analyses, the clinical studies selected have used various approaches for omega-3 fatty acids supplementation, including dietary advice to eat 2-3 fish meals/week, consumption of fish oil or supplementation with omega-3 fatty acids formulations (Delgado-Lista et al. 2012; Enns et al. 2014; Forstermann et al. 1994; Kromhout et al. 2012). Moreover, the doses used in the clinical trials are very heterogeneous, with reported omega-3 formulations ranging from fish oil to purified EPA or DHA, with doses from 0.18 up to 10 g/day (Delgado-Lista et al. 2012; Enns et al. 2014; Forstermann et al. 1994).

Moreover, the various clinical studies published in the literature have focused on different types of patients and measured various clinical parameters. Indeed, the randomized clinical trials published over the years have assessed the effect of omega-3 fatty acids supplementation in patients with documented angina (Burr et al. 1989; Forstermann et al. 1994), arrhythmia (SOFA), acute myocardial infarction (GISSI-P, DART, OMEGA, Alpha Omega), chronic heart failure (GISSI-HP), hypercholesterolemia (JELIS), and evaluated

different primary and secondary outcomes (DiNicolantonio et al. 2014; Forstermann et al. 1994). In addition, the discrepancy of the randomized clinical trials could also be due, at least in part, to the fact that the studied population had different medications such as statins, and dietary exposition to omega-3 fatty acids (Delgado-Lista et al. 2012; DiNicolantonio et al. 2014; Forstermann et al. 1994).

The characterization of the relaxation induced by the optimized EPA:DHA 6:1 formulation shows that it is able to induce endothelium-dependent relaxation involving mainly the NO- and EDH-mediated components of the relaxation. Moreover, the relaxation induced by the optimized EPA:DHA 6:1 formulation was significantly reduced in the presence of inhibitors of Src kinase, PI3-kinase, p38 MAPK, MEK, or JNK as well as by intracellular ROS chelators. Altogether, the results indicate that the EPA:DHA 6:1 formulation induces an intracellular activation of the redox-sensitive PI3-kinase/Akt and MAPKs pathways leading to the activation of eNOS.

The redox-sensitive activation of the PI3-kinase/Akt/eNOS pathway by EPA:DHA is similar to the pathway activated by other natural products such as the polyphenolic compounds. Indeed, polyphenol-rich products including red wine extract (Forstermann et al. 1994; Ndiaye et al. 2005; Ndiaye et al. 2004), grape skin extract (Madeira et al. 2009), grape juice (Anselm et al. 2007), EGCg (Auger et al. 2010a; Kim et al. 2007) and plant extracts (Rattmann et al. 2012; Tokoudagba et al. 2010) induced a redox-sensitive endothelium-dependent relaxations. The intracellular formation of ROS leading to the activation of eNOS induced by polyphenols is dependent of the structure of the compounds (Auger et al. 2010a; Auger et al. 2010b). Indeed, the presence of hydroxyl groups on the phenolic structure is critical for the formation of ROS most likely through an auto-oxidation process (Auger et al. 2010b; Kurita et al. 2013). However, omega-3 fatty acids do not have the hydroxyl moieties needed to undergo auto-oxidation, and thus should induce the intracellular formation of ROS mostly likely through another mechanism, unless they are converted to hydroxylated metabolites. Indeed, the metabolomics analysis of the eicosanoids founds in human plasma after omega-3 fatty acids supplementation indicates that the levels of CYP450 epoxy metabolites and corresponding dihydroxy metabolites from EPA and DHA were significantly increased (Zhang et al. 2015). The possibility that these metabolites could be responsible for the endothelial formation of ROS remains to be demonstrated. Another possibility is that the omega-3 fatty acids incorporation into the cell membranes induces the activation of ROS producing enzymes. Indeed, upon its incorporation in cell membranes, DHA could induce the activation of various

membrane proteins such as protein kinase C, cytochrome P450, several transporters and insulin receptor (Murphy 1990; Slater et al. 1994; Stillwell and Wassall 2003). However, the possibility that such mechanisms contribute to the redox-sensitive endothelium-dependent formation of NO remains to be clarified.

The aim of our second study was to evaluate the potency of the optimized EPA:DHA 6:1 formulation to prevent the platelet-induced vasoconstriction. Indeed, in pathological coronary arteries, platelets can be locally activated and induce vasoconstriction. Similarly, platelets aggregation is associated with contraction of vascular smooth muscles in human and porcine coronary artery rings (Chand and Altura 1980; Freeman et al. 1981; Joiner et al. 1975). In arteries with an altered endothelium, such as coronary arteries with atherosclerotic plaques, local activation of platelets and subsequent contraction could lead to ischemia and possibly also vasospasm.

The present findings indicate that in porcine coronary artery rings, human washed platelets induced dose-dependent contractions that were significantly inhibited by the 5-HT_{2A} receptor antagonist ketanserin and not affected by indomethacin, suggesting that the platelet-induced contraction is mainly due to the release of serotonin from activated platelets. Moreover, the short-term incubation of the rings with the EPA:DHA 6:1 optimized formulation, but not with the EPA:DHA 1:1 formulation, resulted in the abolition of the platelet-induced contraction. The fact that the prevention of the platelets-induced contraction by EPA:DHA 6:1 0.4% (v/v) was not observed in endothelium-denuded rings and in the presence of an eNOS inhibitor suggests that the EPA:DHA 6:1 exerts its inhibitory effects through an increased endothelial formation of NO.

Previous *in vivo* studies have indicated that in dog, the platelets-induced contraction of the large epicardial coronary artery is due both to thromboxane A_2 and serotonin (Golino et al. 1989). Thus, we have assessed the potency of the omega-3 fatty acids formulation to prevent the contractions induced by both agents. The EPA:DHA 6:1 formulation dose-dependently prevented the 5-HT-induced contraction, whereas it was without effect on that induced by the thromboxane A_2 analogue U46619.

As vascular beds from different species can display different type of reactivity, the extrapolation of the mechanisms observed in the porcine coronary artery to the human artery needs to be validated. Similar results were observed in human internal mammary artery rings, where the EPA:DHA 6:1 formulation at 0.4 % (v/v) significantly prevented the 5-HT-induced

contraction and it also induced vasorelaxation through NO-mediated mechanisms. Indeed, 5-HT has been reported as a possible spasmogenic agent in human internal mammary artery (Rosenfeldt et al. 1999), and in particular in arteries with an endothelial dysfunction (He 1999). In our setting, we were able to observe 5-HT-induced contractions in human internal mammary artery rings, possibly due to the fact that these arteries obtained from patients undergoing coronary artery bypass grafting most likely have an established endothelial dysfunction.

Altogether, these findings indicate that the optimized EPA:DHA 6:1 formulation is able to prevent the 5-HT-mediated platelet-induced contraction in porcine coronary and human internal mammary artery rings through an endothelium-dependent NO-mediated mechanism.

The main limitation of the present findings is the fact that they were obtained *ex vivo* using artery rings suspended in organ baths. Moreover, we have assessed only the short term effects of the optimized EPA:DHA 6:1 formulation, whereas the beneficial cardiovascular effects of omega-3 fatty acids supplementation reported by clinical trials is most likely due to the chronic impregnation of tissues leading to long-term effects. The present work requires further studies to better characterize the beneficial endothelial effects of omega-3 fatty acids. Firstly, the mechanism involved in the intracellular formation of ROS in response to the EPA:DHA 6:1 formulation needs to be clarified. Similarly, the long-term effects of the EPA:DHA 6:1 formulation on the endothelium should be evaluated, and in particular its potential ability to induce an up-regulation of eNOS expression leading to an improved basal NO formation in the endothelium, which can be responsible for an improved endothelial function and a decreased risk of developing cardiovascular diseases.

Secondly, the present work on the endothelial effects of EPA:DHA 6:1 formulation needs to be confirmed using *in vivo* models of endothelial dysfunction. The chronic oral intake of omega-3 has been associated with increased levels of omega-3 fatty acids in plasma, but also of various metabolites which can exert different effects on the endothelium. Thus, the ability of the EPA:DHA 6:1 formulation to prevent the endothelial dysfunction and/or to improve the endothelial function needs to be evaluated in *in vivo* models of endothelial dysfunction such as the angiotensin II-induced hypertension in rats. Moreover, such studies will also allow the identification of cellular and molecular mechanisms involved in the beneficial effects of omega-3 fatty acids on the endothelium.

Moreover, these *in vivo* studies could lead to the identification of pertinent biological markers of the EPA:DHA effects on the endothelial function. Such markers would allow a better

evaluation of the beneficial effects of the EPA:DHA 6:1 formulation during future randomized clinical trials in patients with cardiovascular diseases or risk factors associated with endothelial dysfunction.

In conclusion, the present work has assessed the potency of EPA:DHA formulations to protect the endothelial function *ex vivo*. The ability of EPA:DHA formulation to induce endothelium-dependent relaxations is dependent on both the ratio and the purity of the formulation, with the maximal effect obtained with a highly purified EPA:DHA 6:1 ratio. The endothelium-dependent relaxation induced by the optimized EPA:DHA 6:1 formulation is mainly mediated by a redox-sensitive activation of PI3-kinase/Akt and/or MAPKs pathways leading to the activation of eNOS by phosphorylation. Moreover, the optimized EPA:DHA 6:1 formulation is able to effectively inhibit the platelet-induced serotonin-mediated contraction of porcine coronary artery rings, but not the thromboxane A_2-mediated contraction. The inhibition by the optimized EPA:DHA 6:1 formulation of the 5-HT-induced contraction in both porcine coronary artery and human internal mammary artery rings is mainly due to an increased endothelial formation of NO. Taken together, the present findings indicate that the optimized EPA:DHA 6:1 formulation is able to exert potent beneficial cardiovascular effects through the activation of the endothelial function leading to an increased formation of NO.

Resume

L'endothélium joue un rôle clé dans la régulation de l'homéostasie vasculaire principalement via la formation et la libération de facteurs vasodilatateurs ou vasoconstricteurs. Plusieurs études ont montré une association entre la présence d'une dysfonction endothéliale, caractérisée par un déséquilibre dans la formation endothéliale des facteurs vasorelaxants et des facteurs vasoconstricteurs, et le développement des maladies cardiovasculaires comme l'hypertension et l'athérosclérose (Anderson 1999; Forstermann et al. 1994; Hadi and Suwaidi 2007; Sase and Michel 1995). Les effets protecteurs de l'endothélium vis-à-vis de la fonction vasculaire ont été en partie attribués à sa capacité à produire plusieurs facteurs vasoprotecteurs qui sont principalement le NO, l'EDH, et le PGI_2. La détérioration de la fonction endothéliale est généralement associée à une diminution de la biodisponibilité du NO dans de nombreuses maladies chroniques comme l'hypertension, le diabète ou l'athérosclérose (Davignon and Ganz 2004; Forstermann et al. 1994).

De nombreuses études ont rapporté les effets bénéfiques de la consommation d'acides gras de type oméga-3 sur les risques de développer une maladie cardiovasculaire (Forstermann et al. 1994; Lopez-Garcia et al. 2004; Nettleton et al. 2010; Nettleton et al. 2008; Nettleton et al. 2007). En effet, les premières études ont montré qu'en comparaison de la population danoise, la consommation élevée de poissons gras et d'huile de poissons par les esquimaux du Groenland était associée avec une réduction de la mortalité cardiovasculaire (Dyerberg and Bang 1979; Dyerberg et al. 1978; Forstermann et al. 1994). Par la suite, plusieurs études ont montré les effets bénéfiques de la consommation d'acides gras oméga-3 comme l'EPA et le DHA dans de nombreuses maladies comme l'hypertension, le diabète et l'athérosclérose (Albert et al. 1998; Burr et al. 1989; de Lorgeril et al. 1994; Forstermann et al. 1994; Marchioli et al. 2002).

Cependant, les récentes méta-analyses d'essais cliniques suggèrent un effet bénéfique limité de l'apport d'oméga-3 du fait de résultats contradictoires rapportés par plusieurs études (Delgado-Lista et al. 2012; Enns et al. 2014; Forstermann et al. 1994; Kromhout et al. 2012). Ces résultats contradictoires pourraient en partie s'expliquer par la grande variabilité de sources d'oméga-3, des formulations et des dosages utilisés dans les différentes études.

L'objectif du présent travail de thèse a été dans un premier temps d'évaluer le rôle de la formulation en oméga-3 sur l'induction des relaxations dépendantes de l'endothélium, ainsi que la contribution respectives des composantes NO et EDH de la relaxation, et également de

déterminer les mécanismes sous-jacents. Le second objectif a été d'évaluer l'effet vasculaire bénéfique des oméga-3 sur la contraction des artères coronaires induite par les plaquettes et de caractériser l'implication de la sérotonine et des prostanoïdes vasoconstricteurs.

Pour cela, nous avons évalué la capacité de formulations présentant différents ratios et degrés de pureté en EPA et DHA à induire des relaxations dépendantes de l'endothélium dans des anneaux d'artères coronaires de porc. Ainsi, nos résultats montrent que l'induction de la fonction endothéliale, et en particulier l'activation de la formation endothéliale de NO, est dépendante à la fois du ratio EPA:DHA et de la pureté des formulations en oméga-3. L'EPA ou le DHA isolés sont capables d'induire des relaxations dépendantes de l'endothélium dans des anneaux d'artère coronaire de porc, la relaxation en réponse à l'EPA à une concentration de 0,4% (v/v) étant significativement plus importante que celle du DHA. De même, l'étude des différents ratios EPA:DHA indique que l'effet relaxant est maximal pour un rapport EPA:DHA de 6 :1 ou 9 :1, tandis que les formulations avec des ratios EPA:DHA inférieurs induisent des relaxations plus faibles. De plus, une formulation EPA:DHA 6 :1 avec une pureté relative en omega-3 de 50 % induit une relaxation significativement plus faible que celle obtenue avec une formulation EPA:DHA de grande pureté (> 90 %). Ces résultats suggèrent que les effets bénéfiques des formulations en oméga-3 vis-à-vis du système vasculaire sont dépendants à la fois de la composition en oméga-3 totaux et du rapport EPA:DHA. Ces paramètres devraient donc être pris en compte pour l'interprétation et la comparaison des résultats des essais précliniques et cliniques utilisant différents produits riches en oméga-3. De plus, ces travaux peuvent aussi aider à comprendre les différences trouvés dans les résultats de méta-analyses évaluant les effets cardiovasculaires de plusieurs formulations allant de l'huile de poissons à des omégas-3 (EPA et/ou DHA) extraits et purifiés à différents ratios et doses.

En effet, dans plusieurs méta-analyses, les essais cliniques sélectionnés rapportent l'utilisation de plusieurs approches pour l'apport en acides gras de type oméga-3 incluant des conseils nutritionnels (comme manger du poisson 2 à 3 fois par semaine), une consommation d'huile de poissons ou une supplémentation avec diverses formulations d'oméga-3 (Delgado-Lista et al. 2012; Enns et al. 2014; Forstermann et al. 1994; Kromhout et al. 2012). De plus, les doses d'oméga-3 utilisées dans les essais cliniques sont très hétérogènes avec des teneurs allant de 0,18 à 10 g par jour sous forme d'huiles de poissons ou de formulations d'EPA et/ou de DHA purifiés (Delgado-Lista et al. 2012; Enns et al. 2014; Forstermann et al. 1994; Kromhout et al. 2012).

En outre, les divers essais cliniques publiés dans la littérature montrent une grande variabilité dans les paramètres d'inclusion des patients et des volontaires, ainsi que dans les événements étudiés et les paramètres cliniques déterminés. En effet, les essais cliniques randomisés ont évalué l'effet d'un apport en acides gras de type oméga-3 chez des patients ayant soit une angine de poitrine (Burr et al. 1989; Forstermann et al. 1994), un infarctus du myocarde (GISSI-P, DART, OMEGA, Alpha Omega), une insuffisance cardiaque (GISSI-HP), ou une hypercholestérolémie (JELIS), avec une grande variété d'événements étudiés en prévention primaire ou secondaire (DiNicolantonio et al. 2014; Forstermann et al. 1994).
Enfin, les divergences observées dans les résultats des essais cliniques randomisés pourraient également être attribuées, du moins en partie, au fait que les populations incluses dans les essais présentaient des différences dans leurs prises en charge thérapeutique (par exemple prescription de statines) et dans leurs consommations alimentaires en acides gras de type oméga-3 (Delgado-Lista et al. 2012; DiNicolantonio et al. 2014; Forstermann et al. 1994).

La caractérisation de la relaxation indique que la formulation optimisée EPA:DHA 6:1 induit une relaxation dépendante de l'endothélium impliquant principalement les composantes NO et EDH de la relaxation. De plus, la relaxation induite par la formulation optimisée EPA:DHA 6:1 est réduite significativement en présence des inhibiteurs de la Src kinase, PI3-kinase, p38 MAPK, MEK, ou JNK, aussi bien que par les chélateurs de ROS intracellulaires. Ces résultats indiquent que formulation EPA:DHA 6:1 induit une activation redox-sensible des voies PI3-kinase/Akt and MAPKs conduisant à une activation de la eNOS.
Cette activation redox-sensible de la voie PI3-kinase/Akt/eNOS par la formulation optimisée EPA:DHA 6:1 est similaire à celle induite par d'autres produits naturels comme les composés polyphénoliques. En effet, les produits riches en polyphénols comme un extrait de vin rouge (Forstermann et al. 1994; Ndiaye et al. 2005; Ndiaye et al. 2004), de pellicule de raisin (Madeira et al. 2009), le jus de raisin Concord (Anselm et al. 2007), l'EGCg (Auger et al. 2010a; Kim et al. 2007) ou encore des extraits de plantes (Rattmann et al. 2012; Tokoudagba et al. 2010), induisent une activation redox-sensible des relaxations dépendantes de l'endothélium. La formation intracellulaire de ROS conduisant à l'activation de la eNOS en réponse aux polyphénols est dépendante de la structure de ces composés (Auger et al. 2010a; Auger et al. 2010b). En effet, la présence de groupements hydroxyles dans la structure phénolique est cruciale pour la formation de ROS, probablement par un processus d'auto-oxydation (Auger et al. 2010b; Kurita et al. 2013). Cependant, les acides gras de type oméga-3 n'ont pas de fonctions hydroxyles pouvant conduire à une auto-oxydation, et par conséquent

induisent vraisemblablement la formation intracellulaire de ROS par d'autres mécanismes, à moins qu'ils ne soient transformés en métabolites hydroxylés. En effet, des analyses métabolomiques des eicosanoïdes trouvés dans le plasma humain indiquent que les concentrations des métabolites époxy d'EPA et de DHA provenant du CYP450 et des métabolites dihydroxy correspondants sont significativement augmentées après ingestion d'acides gras oméga-3 (Zhang et al. 2015). La possibilité que ces métabolites soient responsables de la formation endothéliale de ROS reste cependant à démontrer. Alternativement, l'incorporation des acides gras oméga-3 dans les membranes cellulaires pourrait induire l'activation des enzymes pro-oxydantes menant à la formation de ROS. En effet, après incorporation dans les membranes cellulaires, le DHA a été impliqué dans l'activation de nombreuses protéines membranaires comme la protéine kinase C, le cytochrome P450, plusieurs transporteurs et les récepteurs de l'insuline (Murphy 1990; Slater et al. 1994; Stillwell and Wassall 2003).

Le but de la seconde étude a été d'évaluer la capacité de la formulation optimisée EPA:DHA 6:1 à prévenir la vasoconstriction induite par les plaquettes. Dans les artères coronaires pathologiques, les plaquettes peuvent être activées localement et induire une vasoconstriction. En effet, l'agrégation plaquettaire est associée avec la contraction du muscle lisse vasculaire chez l'homme et dans l'artère coronaire de porc (Chand and Altura 1980; Freeman et al. 1981; Joiner et al. 1975). Dans les artères présentant une altération de l'endothélium, comme les artères coronaires ayant des plaques d'athérosclérose, l'activation locale des plaquettes et la contraction consécutive peuvent conduire à l'ischémie, voire à un possible vasospasme.

Nos résultats indiquent que dans des anneaux d'artères coronaires de porc, les plaquettes humaines lavées induisent des contractions dépendantes de la dose qui sont significativement inhibées en présence de kétansérine, un antagoniste du récepteur de la sérotonine 5-HT_{2A}, ce qui suggère que la contraction induite par les plaquettes est principalement due à une libération de sérotonine secondaire à l'activation plaquettaire. De plus, l'incubation à court terme des anneaux avec la formulation optimisée EPA:DHA 6:1 conduit à la suppression de la contraction induite par les plaquettes, alors que la formulation EPA:DHA 1:1 est sans effet. Le fait que la prévention de la contraction par la formulation optimisée EPA:DHA 6:1 à 0,4% (v/v) n'est pas observée en l'absence d'endothélium fonctionnel ou en présence d'un inhibiteur de la eNOS suggère que la formulation optimisée EPA:DHA 6:1 exerce ses effets inhibiteurs par une augmentation de la formation endothéliale de NO.

De précédentes études réalisées *in vivo* ont montré que la contraction induite par les plaquettes dans des artères coronaires canines est due à la fois à la libération plaquettaire de

thromboxane A_2 et de 5-HT (Golino et al. 1989). Ainsi, nous avons évalué le potentiel de la formulation en acides gras oméga-3 à empêcher les contractions induites par ces deux vasoconstricteurs. La formulation EPA:DHA 6:1 supprime de façon dépendante de la dose la contraction induite par la 5-HT, tandis qu'elle est sans effet sur celle induite par un analogue du thromboxane A_2 (U46619).

Comme les réponses des lits vasculaires peuvent varier selon l'espèce considérée, l'extrapolation des mécanismes observés pour l'artère coronaire de porc nécessite d'être validée dans une artère humaine. Ainsi, nous avons pu observer des résultats similaires dans des anneaux d'artères mammaires internes humaines, la formulation EPA:DHA 6:1 à 0,4% (v/v) inhibant significativement la contraction induite par la 5-HT et provoquant une vasorelaxation par des mécanismes dépendants du NO endothélial. Toutefois, la 5-HT est décrite comme un agent pouvant induire des spasmes dans l'artère mammaire interne humaine (Rosenfeldt et al. 1999) et en particulier dans les artères ayant une dysfonction endothéliale (He 1999). Lors de notre étude, nous avons pu observer la contraction induite par la 5-HT dans les anneaux d'artères mammaires internes humaines, ce qui peut être dû au fait que ces artères proviennent de patients ayant subis un pontage aorto-coronarien présentant probablement aussi une dysfonction endothéliale avérée.

Dans l'ensemble, ces résultats indiquent que la formulation optimisée EPA:DHA 6:1 est capable de supprimer les contractions induites par la 5-HT plaquettaire dans la coronaire de porc et l'artère mammaire interne humaine via des mécanismes dépendants de l'endothélium et impliquant le NO.

Une principale limitation de nos résultats vient du fait qu'ils sont obtenus *ex vivo* en utilisant des anneaux d'artères suspendus dans des cuves à organes isolés. De plus, nous avons évalué seulement les effets à court terme induits par la formulation optimisée EPA:DHA 6:1, tandis que les effets bénéfiques d'un apport en acides gras de type oméga-3 sur le système cardiovasculaire décrits dans les essais cliniques sont peut-être dû à une modulation à long-terme induite par l'imprégnation chronique des tissus. Le travail présent demande donc des recherches complémentaires pour mieux caractériser l'effet bénéfique des acides gras de type oméga-3 sur l'endothélium. D'abord, les mécanismes impliqués dans la formation intracellulaire de ROS en réponse à la formulation EPA:DHA 6:1 nécessitent d'être clarifiés. De même, les effets à long terme de la formulation EPA:DHA 6:1 doivent être évalués sur l'endothélium, et en particulier la possibilité d'induire une expression de la eNOS conduisant à une augmentation de la formation basale de NO dans l'endothélium, ce qui peut à terme

conduire à l'amélioration de la fonction endothéliale et à une diminution du risque de développer une maladie cardiovasculaire.

De plus, le travail actuel sur les effets endothéliaux de la formulation EPA:DHA 6:1 doivent être confirmés sur des modèles de dysfonction endothéliale *in vivo*. La prise orale chronique d'oméga-3 a été associée à une augmentation plasmatique du taux d'acides gras de type oméga-3, ainsi qu'à la présence de nombreux métabolites circulants pouvant exercer des effets sur l'endothélium. Ainsi, la capacité de la formulation EPA:DHA 6:1 à empêcher la dysfonction endothéliale et/ou améliorer la fonction endothéliale doit être évaluée sur des modèles *in vivo* de dysfonction endothéliale comme l'hypertension induite par l'angiotensine-II chez le rat. De plus, de telles études pourraient également permettre d'identifier les mécanismes cellulaires et moléculaires impliqués dans les effets bénéfiques des acides gras oméga-3 sur l'endothélium.

En outre, ces études *in vivo* pourront conduire à l'identification de nouveaux marqueurs biologiques pertinents des effets de la formulation EPA:DHA 6:1 sur la fonction endothéliale. Ces marqueurs pourront *in fine* permettre une meilleure évaluation des effets bénéfiques de la formulation EPA:DHA 6:1 lors de futurs essais cliniques randomisés chez des patients ayant des maladies cardiovasculaires ou des facteurs à risques associés à une dysfonction endothéliale.

En conclusion, le travail actuel a évalué l'activité de formulations EPA:DHA à protéger la fonction endothéliale *ex vivo*. La capacité de la formulation EPA:DHA à induire des relaxations dépendantes de l'endothélium est liée à la fois à la teneur et à la pureté de la formulation, avec un effet maximal obtenu avec un rapport EPA:DHA de 6:1 hautement purifiés. La relaxation dépendante de l'endothélium induite par la formulation optimisée EPA:DHA 6:1 est principalement due à une activation redox-sensible des voies PI3-kinase/Akt et/ou MAPKs conduisant à une phosphorylation activatrice de la eNOS. De plus, la formulation optimisée EPA:DHA 6:1 est capable d'inhiber fortement la contraction induite par la 5-HT plaquettaire dans les anneaux d'artères coronaires de porc, mais n'a pas d'effet sur la contraction induite par un analogue du thromboxane A_2. L'inhibition de la formulation optimisée EPA:DHA 6:1 sur la contraction induite par la 5-HT dans les artères coronaires de porc et sur les anneaux d'artères mammaires internes humaines est principalement due à une augmentation de la formation endothéliale de NO. Dans l'ensemble, ces résultats indiquent que la formulation optimisée EPA:DHA 6:1 est capable d'exercer de puissants effets bénéfiques sur le système cardiovasculaire via l'activation de la fonction endothéliale

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Protection vasculaire par la nouvelle formation d'omega3 : Rôle de la NO synthase endothéliale

Résume

Le but du présent travail est d'évaluer le potentiel d'oméga-3 à induire la fonction endothélial, et à inhiber la contraction des artères coronaires en réponse aux plaquettes activées.

L'induction des relaxations dépendantes de l'endothélium est affecté à la fois par le ratio et la pureté des formulations, avec un effet maximal obtenu pour la formulation EPA:DHA 6:1 à haut degré de pureté via une activation redox-sensible des voies de signalisation PI3-kinase/Akt et/ou MAPKs menant à l'activation de eNOS. La formulation EPA:DHA 6:1 inhibe les contractions induites par les plaquettes impliquant la sérotonine dans les artères coronaires de porc, et les contractions à la sérotonine dans les artères coronaires porcines et mammaires internes humaines, principalement par une augmentation de la formation endothéliale de NO. Ces résultats indiquent que la formulation optimisée EPA:DHA 6:1 exerce un effet bénéfique sur le système cardiovasculaire via l'activation de la fonction endothéliale.

Abstract

The aim of the present work was to evaluate both the potency of omega-3 formulations to induce the endothelial function and the effect of omega-3 on the platelet-induced coronary artery contraction.

The endothelium-dependent relaxations induced by EPA:DHA formulations is dependent on both the ratio and the purity of the formulation, with the maximal effect obtained with a highly purified EPA:DHA 6:1 ratio through a redox-sensitive activation of PI3-kinase/Akt and/or MAPKs pathways and subsequent activation of eNOS. The EPA:DHA 6:1 formulation inhibits the platelet-induced serotonin-mediated contraction of porcine coronary rings and the 5HT-induced contraction in both porcine coronary artery and human internal mammary artery rings, mainly due to an increased endothelial formation of NO. Taken together, the present findings indicate that the optimized EPA:DHA 6:1 formulation is able to exert potent beneficial cardiovascular effects through the activation of the endothelial function.