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THE ENDOTHELIAL DYSFUNCTION IN PORTAL HYPERTENSION: ROLE OF THE OXIDATIVE STRESS AND ANGIOTENSIN SYSTEM

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TO ASLAN, YASSER, TAQWA AND MY BEST BELOVER; My WIFE

I DEDICATE THIS WORK

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Publications

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3. Oswald-Mammosser Monique, **Rashid Sherzad K.**, Boehm Nelly, Agin Arnaud, Geny Bernard, Schini-Kerth Valérie, Charloux Anne. Effect of Fulvestrant,an estrogen receptor antagonist, on the cirrhotic rat lung, *soumis pour publication*
4. Amissi Said, Boisramé-Helms Julie, Burban Mélanie, **Rashid Sherzad K.**, Cyril Auger, Florence Toti, Ferhat Meziani, Valérie B. Schini-Kerth. Lipid emulsions used in parenteral nutrition induce endothelial dysfunction in porcine coronary artery rings: Role of oxidative stress and cyclooxygenase-derived vasoconstrictors, *En preparation*
5. Mahmoud Alhosin, Eric Anselm, **Sherzad K. Rashid**, Jong Hun Kim, Socorro Vanesca Frota Madeira, Christian Bronner, and Valérie B. Schini-Kerth. Redox-Sensitive Up-Regulation of eNOS by Purple Grape Juice in Endothelial Cells: Role of PI3-Kinase/Akt, p38MAPK, JNK, FoxO1 and FoxO3a.PLOS ONE 2013;8(3):e57883. doi: 10.1371/journal.pone.0057883. Epub 2013 Mar 22.
6. Faraj Zgheel, Mahmoud Alhosin, **Sherzad K. Rashid**, Cyril Auger and 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, *soumis pour publication*.
7. Mahmoud Alhosin, Israa Dandache, Agnès Lelay, **Sherzad K. Rashid**, Luc-Matthieu Fornecker, Laurent Mauvieux, Raoul Herbrecht and Valérie B. Schini-Kerth. Bilberry

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3. **Sherzad K. Rashid**, Noureddine Idris-Khodja, Cyril Auger , Mahmoud Alhosin, Nelly Boehm, Monique Oswald-Mammosser and Valérie B. Schini-Kerth. Oral intake of blackcurrant juice prevents endothelial dysfunction in the mesenteric artery of cirrhotic rats with portal hypertension.MOVD 2013, 11th International Symposium on Mechanisms of Vasodilatation, P2/2, 4-6 October 2013 University hospital Zurich.

4. Faraj Zgheel, Mahmoud Alhosin, **Sherzad K. Rashid**, Cyril Auger and Valérie B. Schini-Kerth. The NO-mediated relaxation induced by highly purified EPA: DHA 6:1 product involves a copper-dependent redox-sensitive activation of the PI3-Kinase/Akt pathway leading to eNOS activation.11th International Symposium on Mechanisms of Vasodilatation, P1/2, 4-6 October 2013 University hospital Zurich.

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ABBRAVATIONS

AA : arachidonic acids

AC : adenylyl cyclise

ACE : angiotensin converting enzyme

ACh : acetylcholine

ACE : angiotensin-converting enzyme

ADP : adenosine diphosphate

ATI : angiotensin I

AT II : angiotensin II

ATP : adenosine triphosphate

BH4 : tetrahydrobiopterin

cAMP : cyclic adenosine-3',5'-monophosphate

CBDL : common bile duct ligation

CO : carbon monooxide

COX : cyclooxygenase

EC : endothelial cell

EDCF : endothelium-derived contracting factor

EDH : endothelial derived hyperpolarizing

EDHF : endothelium-derived hyperpolarizing factor

EDRF : endothelium-derived relaxing factor

EETs : epoxyeicosatrienoic acids

ER : estrogen receptor

ET-1 : endothelin-1

GPx : glutathione peroxidase

H_2O_2 : hydrogen peroxide

HCS : hyperdynamic circulatory state

HO-1 : hemeoxygense-1

HPS : hepatopulmonary syndrome

IK_{ca} channels : potassium-dependant channels of intermediate-conductance

iNOS : inducible NO synthase

K_{ca} : Ca^{2+}- sensitive K^+ channels

L-NA : N-nitro-L-arginine

L-NAME : L-nitro-L-arginine methylester

LPS : lipopolysaccaride

MCP-1 : monocyte chemoattractant protein-1

MGJ : myoendothelial gap junction

MnTMPyP : Mn(III) tetrakis(1-methyl-4pyridyl)porphyrin, superoxyde dismutase mimetic

MMPs : matrix metalloproteinases

NO : nitric oxide

NOS : nitric oxide synthase

nNOS : neuronal nitric oxide synthase

O_2^- : superoxide anions

OH^{-} : hydroxyl groups

PDGF : platelet-derived growth factor

PEG-SOD : polyethylene glycol-superoxide dismutase

PGE_2 : prostaglandins E_2

PGH_2 : prostaglandins H_2

PGI_2 : prostacyclin

PI3K : Phosphoinositide 3-kinase

PIMs : pulmonary intravascular macrophage

PKA : protein kinase A

PKC : protein kinase C

PKG : protein kinase G

RAAS : rennin-angiotensin-aldosterone-system

ROS : reactive oxygen species

SID : selective intestinal decontamination

SK_{ca} channels : K_{ca} channels of small-conductance

sGC : soluble guanylyl cyclase

SMC : smooth muscle cell

SOD : superoxide dismutase

TGF-B1 : transforming growth factor-B1

TNF-α : tumor necrosis factor-alpha

TXA_2 : thrombxane A_2

VEGF-A : vacular endothelial growth factor A

VEGFR : vacular endothelial growth factor receptor

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ABSTRACT

Chronic liver diseases are characterized by a progressive vasodilation that particularly affects splanchnic and pulmonary vascular beds. The vasodilation is due to portal hypertension with or without cirrhosis.

Vasodilation in the lungs can cause a shunt which may lead to hypoxemia. These abnormalities are known as the hepatopulmonary syndrome (HPS): arterial de-oxygenation due to pulmonary vascular dilatation in the context of liver disease. It is important to diagnose HPS because it is an independent prognostic factor of survival and because it also affects postoperative mortality in liver transplantation. Till now the only available treatment for HPS is liver transplantation which leads to a regression and often a disappearance of the HPS. Nevertheless, having an efficient medical treatment is necessary for two reasons. First, given that there is no close correlation between the occurrence of hypoxemia and the severity of liver disease, there are cases where severe hypoxemia requires liver transplantation although liver disease is only mild and would otherwise not be an indication for transplantation. Improving hypoxemia would therefore allow delaying transplantation in such cases. Second, since HPS is associated with an increased transplantation morbidity and mortality, improving hypoxemia could ameliorate survival in patients with a HPS during and after transplantation.

The mechanisms leading to vasodilation due to portal hypertension are not fully understood. Till now, nitric oxide (NO) has been thought to play a major role. It is a well known vasodilator the production of which has been shown to be increased in the rat model of biliary cirrhosis. Moreover, in animal studies chronic treatment with N^G-nitro-L-arginine methyl ester (L-NAME), a non-specific NO synthase inhibitor attenuated or prevented the occurrence of HPS. However clinical trials of treatment with inhaled L-NAME led to contradictory results. This can be explained by the fact that other factors play a role. It has been suggested that the renin-angiotensin-aldosterone system associated to oxidative stress may be important. Oxidative stress is linked in part to bacterial translocation that is present in a significant proportion of patients with advanced cirrhosis. Finally, other factors have been implicated: the carbon monoxide which is a vasodilator produced by the heme-oxygenase and it has also been suggested that estrogens (high levels in some patients) could lead to pulmonary vascular dilatation.

The aims of our work were thus to estimate the effects of a probiotic which could act on bacterial translocation, the effects of polyphenols on oxidative stress and the possible improvement of HPS by the estrogen receptor antagonist fulvestrant in a rat model of biliary cirrhosis. The first two studies have been devoted to the effects of a probiotic and a polyphenol rich compound on the splanchnic vessels and the last study to the effects of fulvestrant on the lung. Biliary cirrhosis with portal hypertension has been induced by bile duct ligation in wistar rats under general anesthesia. This leads to a hyperdynamic circulatory state and a hepatopulmonary syndrome. The rats were examined 4 weeks after surgery.

It was shown in an experimental model of partial portal vein ligation in the rat that the initial event because of portal hypertension is an up-regulation in the expression of VEGF (vascular endothelial growth factor) and eNOS (endothelial nitric oxide synthase) in the intestinal microcirculation. Thereafter, a systemic vasodilation occurs, and in particular in the lung leading to a HPS.

The effects of the probiotic and polyphenols were mainly studied on the mesenteric artery and the aorta:

- Vascular reactivity studies were performed in isolated organ chambers in the presence of indomethacin to prevent the formation of vasoactive prostanoids. The NO component of relaxation was studied in the presence of charybdotoxin and apamin (inhibitors of the EDHF response). The EDHF (endothelial derived hyperpolarizing factor) component was studied in the presence of N^G-nitro-L-arginine.

- The formation of reactive oxygen species (ROS) was estimated using the fluorescent probe dihydroethidine before the determination of different sources of ROS.

- Immunofluorescence and western blot analyses were used to quantify the expression of different components of the vascular reactivity and of the renin-angiotensin-aldosterone system (RAAS).

- Inflammatory cytokines levels were measured in the plasma

The effects of fulvestrant were studied on the lungs:

- Immunohistochemistry on lung sections allowed to quantify the intravascular macrophages and the small vessels diameter.

- Western blot analyses was used to quantify the expression of eNOS, iNOS, nitrotyrosine and p-VASP, and VEGF and p-Akt.

- Nitrites and hormones levels were measured in the plasma and COHb was measured in arterial blood samples.

The first study showed that treatment with probiotics improved the NO- and EDHF-dependent components of relaxation in the mesenteric vascular bed. This was accompanied by a decreased production of ROS and expression of RAAS factors and a decrease in pro-inflammatory cytokines in the plasma. Bacterial translocation and oxidative stress leading to the production of pro-inflammatory cytokines and to the stimulation of local RAAS is probably an important factor in the development of endothelial dysfunction in cirrhotic rats. The administration of probiotics has improved these abnormalities.

The second study showed that the administration of polyphenols improves endothelial dysfunction in cirrhotic rats: improved EDHF-mediated relaxations, decreased expression of eNOS, ROS production, and some components of the RAAS and decreased levels of proinflammatory cytokines. Oxidative stress, part of which may be due to bacterial translocation, but other factors are also involved, is probably a key factor in the endothelial dysfunction in cirrhotic rats. The relation between the RAAS and oxidative stress is again highlighted in this study which confirms our previous studies where treatment with losartan (angiotensin type 1 receptor antagonist) improved endothelial dysfunction in rats with biliary cirrhosis.

In the third study, treatment with fulvestrant (an estrogen receptor antagonist) decreased the expression of eNOS and nitrotyrosines production in the lung. However, the expression of p-VASP which is a marker of the action of NO was reduced in cirrhotic rat lungs and fulvestrant had no effect on this marker. The hypothesis is that despite the increase in eNOS expression, the produced NO is captured by ROS which would prevent its activity. This could also explain the impaired NO component of relaxation in the two earlier studies. Fulvestrant had no effect neither on the expression of VEGF, nor on the number of macrophages in the pulmonary vessels, nor on the diameter of small pulmonary vessels. In conclusion, either fuvestrant is not effective in cirrhotic rats or the relative increase in estradiol in male rat has no role in the occurrence of a HPS

The important role of oxidative stress in the development of endothelial dysfunction in rats with biliary cirrhosis is emphasized in our studies. Oxidative stress is associated with the stimulation of the RAAS and bacterial translocation. Treatment with antioxidants (polyphenols) and probiotics have clearly demonstrated their beneficial effects on the endothelial dysfunction in our cirrhotic rats. The role of NO as a pure vasodilator is perhaps not essential which may explain the disappointing clinical results published by some authors.

Résumé

Les atteintes hépatiques chroniques se caractérisent par une vasodilatation progressive qui touche en particulier les lits vasculaires splanchnique et pulmonaire. La vasodilatation est due à l'hypertension portale que celle-ci soit la conséquence d'une cirrhose hépatique ou qu'elle soit primitive.

Au niveau pulmonaire la vasodilatation peut entraîner un shunt pouvant conduire à une hypoxémie. On parle alors de syndrome hépato-pulmonaire (SHP) dont la définition est une triade : défaut d'oxygénation artériel dû à des dilatations vasculaires pulmonaires dans un contexte de maladie hépatique. Il est important d'en faire le diagnostic car c'est un facteur pronostique indépendant de survie qui conditionne aussi la mortalité post-opératoire au cours de la greffe hépatique. Pour le moment seule la transplantation hépatique permet une régression ou une disparition du SHP mais un traitement médical efficace est nécessaire pour deux raisons. D'une part étant donné qu'il n'existe pas de corrélation étroite entre la survenue d'une hypoxémie et la sévérité de l'atteinte hépatique, il y a des cas où l'hypoxémie profonde nécessite la transplantation hépatique alors que d'un point de vue hépatique elle n'est pas encore justifiée. Améliorer l'hypoxémie pourrait donc surseoir à la greffe. D'autre part la survenue d'un SHP sévère entraîne un risque opératoire important et un traitement médical atténuant la complication pulmonaire devrait pouvoir faciliter la prise en charge pré- et post-opératoire de ces patients.

Les mécanismes conduisant à la vasodilatation due à l'hypertension portale ne sont pas complètement élucidés. La molécule qui a été incriminé le plus souvent est le NO. C'est un vasodilatateur bien connu et dont on a montré la surproduction dans un modèle de rat de cirrhose biliaire accompagnée d'un SHP. En expérimentation animale un traitement chronique par le N^G-nitro-L-arginine methyl ester (L-NAME), inhibiteur non spécifique des NO

synthases atténuait ou empêchait la survenue du SHP. Néanmoins des essais cliniques de traitement par inhalation de L-NAME donnent des résultats contradictoires. Ceci peut s'expliquer par le fait que d'autres facteurs jouent un rôle. Il en est ainsi du système-rénine-angiotensine-aldostérone probablement par le biais d'un stress oxydant. Ce dernier a aussi été lié à la translocation bactérienne qui est présente chez une proportion importante de patients présentant une cirrhose évoluée. Enfin d'autres facteurs ont été incriminés : le monoxide de carbone qui est un vasodilatateur produit par la hème-oxygénase et il a aussi été suggéré que les estrogènes (taux élevés chez certains patients) pouvaient entraîner une dilatation vasculaire pulmonaire.

Le but des différents travaux était donc d'estimer l'action de probiotiques agissant sur la translocation bactérienne, de polyphénols agissant sur la composante stress oxydant et d'un antagoniste des récepteurs de l'estrogène dans un modèle de rat ayant une cirrhose biliaire. L'analyse des effets a été essentiellement réalisée dans la circulation splanchnique et dans une moindre mesure (étude fulvestrant) au niveau pulmonaire.

Premier chapitre

Un rappel général sur les cellules endothéliales et notamment leur action sur le tonus vasculaire par le biais des différents facteurs vasoconstricteurs et vasodilatateurs produits par l'endothélium est fait dans un premier chapitre. Ainsi ont été revus comme vasodilatateurs le monoxyde d'azote (NO), le facteur hyperpolarisant dérivé de l'endothelium (endothelium-derived-hyperpolarization-factor, EDHF), et la prostacycline (PGI2). Parmi les facteurs vasoconstricteurs ont été présentés : le thromboxane A_2 (TXA_2), et la prostaglandine H_2 (PGH_2), les espèces réactives de l'oxygène (ROS), l'endotheline 1 (ET1) and l'angiotensine II.

Deuxième chapitre

Le deuxième chapitre est dédié à la dysfonction endothéliale dans l'hypertension portale.

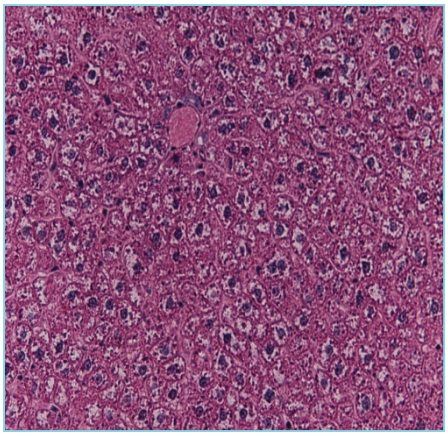
D'après des études antérieures réalisées sur un modèle de ligature partielle de la veine porte chez le rat, les premières anomalies survenant au niveau de la microcirculation intestinale est une augmentation de l'expression du facteur de croissance vasculaire (vascular–endothelial-growth factor, VEGF) et de la NO synthase endothéliale (eNOS). Survient par la suite une vasodilatation généralisée avec syndrome hyperdynamique imputable à des mécanismes encore non complètement élucidés. Plusieurs facteurs sont très probablement impliqués : NO, le monoxyde de carbone (CO, possiblement en relation étroite avec le système NO), VEGF et peut-être la prostacycline (peu étudiée à vrai dire). Il est aussi insisté dans ce chapitre sur le rôle très probable de la translocation bactérienne (BT) par le biais de cytokines et des ROS en partie étroitement liés à l'activation du système rénine-angiotensine (SRA). Enfin, ces dernières années a été souligné le rôle très probable d'une angiogénèse anormale qui survient tant au niveau de la circulation splanchnique qu'au niveau pulmonaire.

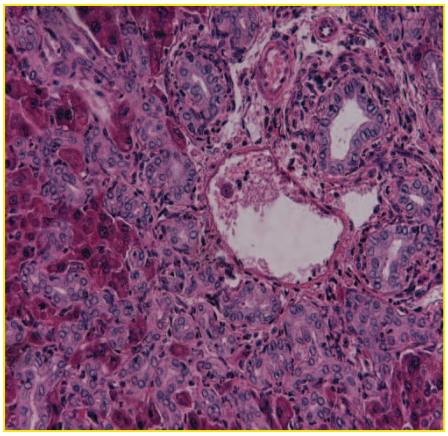
Troisième chapitre

Les résultats observés au cours d'études antérieures justifient les choix faits en termes d'expérimentations réalisées pour cette thèse : action sur la translocation bactérienne par l'utilisation de probiotiques, action sur le stress oxydant en évaluant un traitement par polyphénols. La troisième étude a permis d'estimer la possibilité d'agir sur la composante NO par le biais de l'administration de fulvestrant. Ce dernier est un antagoniste des récepteurs de l'estrogène dont le taux est souvent augmenté dans la cirrhose et qui stimule l'expression de eNOS.

Le modèle de cirrhose biliaire a été bien validé et consiste à ligaturer la voie biliaire du rat sous anesthésie générale. Ceci induit une cirrhose biliaire avec hypertension portale, un état circulatoire hyperkinétique et un syndrome hépato-pulmonaire. Les rats ont été étudiés 4 semaines après l'intervention.

La figure ci-dessous montre à gauche un foie chez un rat normal et à droite le foie d'un rat présentant une cirrhose biliaire. Dans ce dernier cas il existe une nette prolifération des canaux biliaires.





Foie normal Cirhhose biliaire

Dans les études concernant l'action des probiotiques et polyphénols ont été essentiellement étudiés les effets au niveau de l'artère mésentérique et de l'aorte :

- réactivité vasculaire dans des cuves à organe isolé en présence d'indométacine pour prévenir la formation de prostanoïdes vasoactifs. La composante NO de la relaxation est étudiée en présence de charybdotoxine et d'apamine (inhibiteurs de la réponse EDHF-dépendante). La composante EDHF (endothelial derived hyperpolarizing factor) est étudiée en présence de N^G-nitro-L-arginine, inhibiteur de la réponse NO-dépendante.

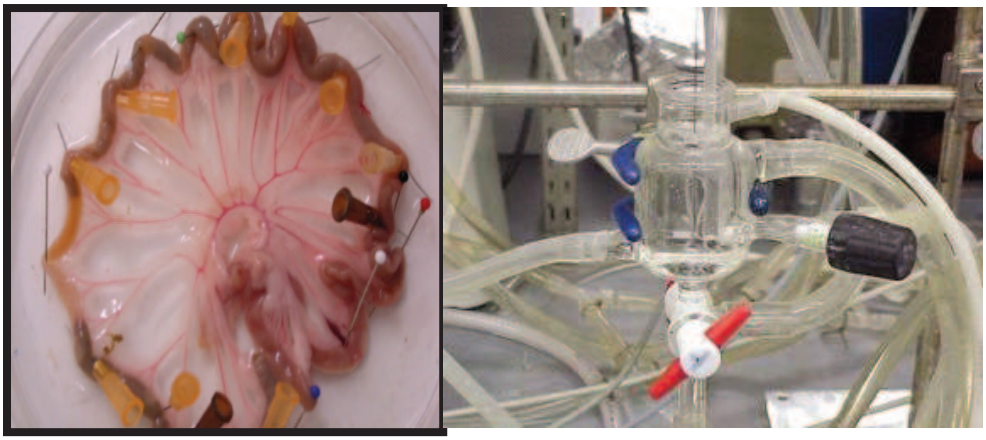
- formation d'espèces réactives de l'oxygène (ROS) déterminée à l'aide d'une sonde fluorescente, la dihydroéthidine et détermination des différentes sources de ROS

- quantifications de l'expression de différents composants de la réactivité vasculaire et du système rénine-angiotensine-aldostérone (SRAA) par immunofluorescence et en western blot

- dosages plasmatiques de cytokines inflammatoires

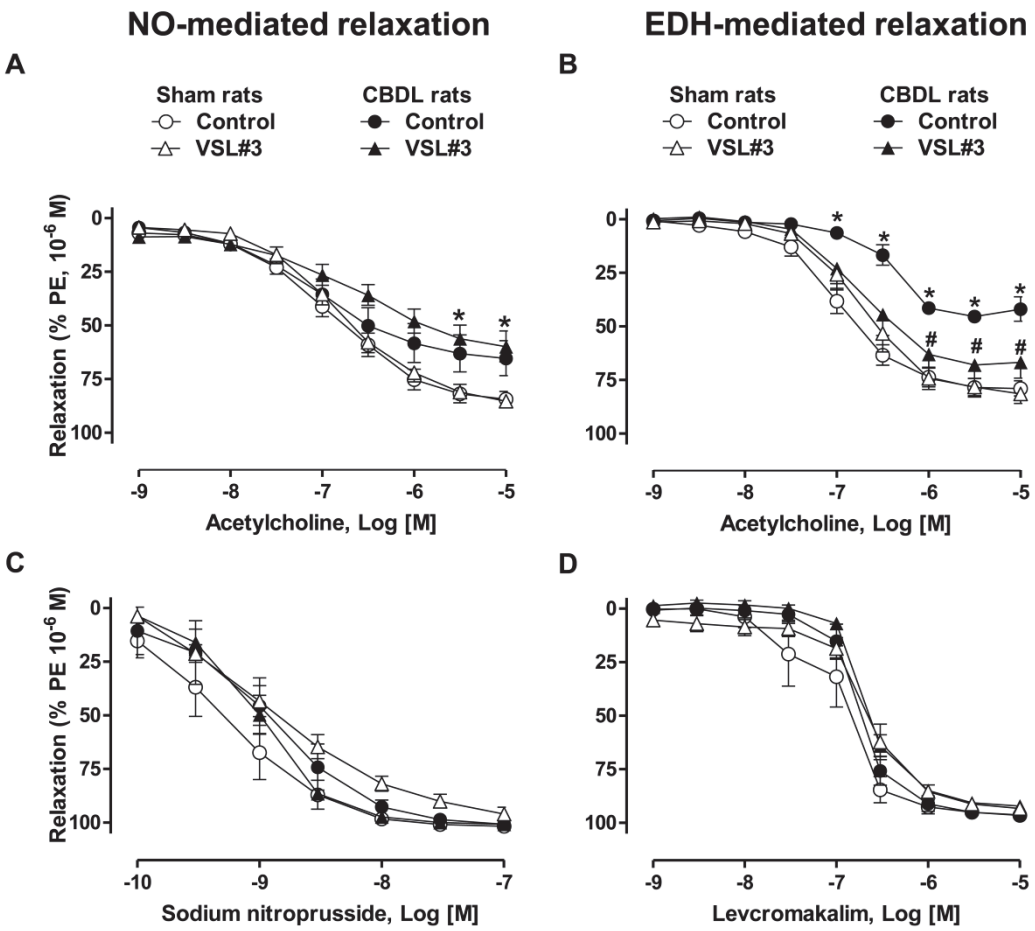
Quant à l'action du fulvestrant (antagoniste des récepteurs de l'estrogène) il a été étudié au niveau du poumon :

- étude des macrophages intravasculaires pulmonaires et des petits vaisseaux (immunohistochimie sur des coupes du poumon)
- western blot sur tissu pulmonaire pour la quantification de l'expression de eNOS, iNOS, nitrotyrosine, VEGF et des marqueurs de l'action de NO (p-VASP) et de VEGF (p-Akt), récepteurs-α des estrogènes
- dosages plasmatiques de nitrites, HbCO et du taux d'hormones.

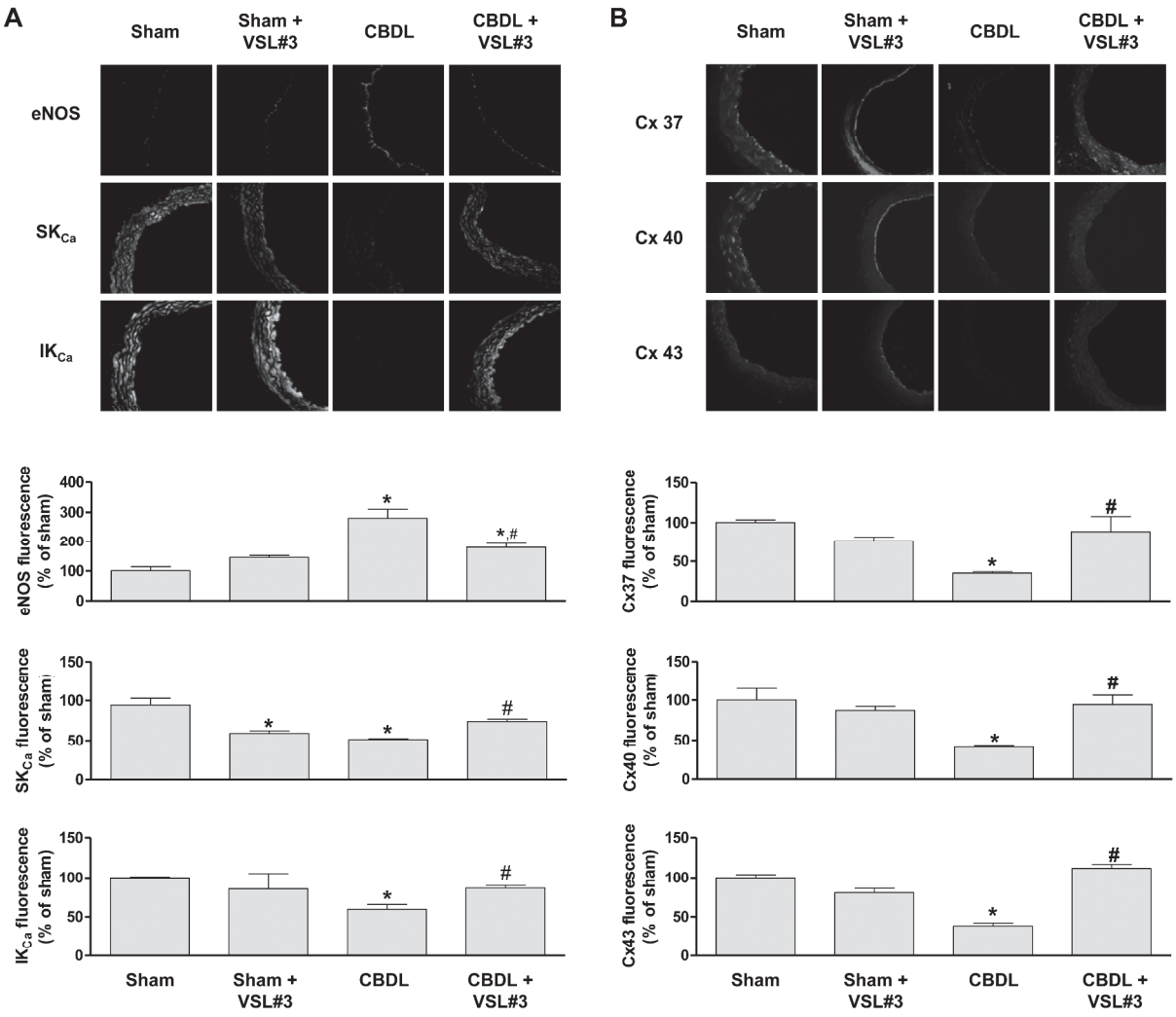


Etude de la réactivité vasculaire

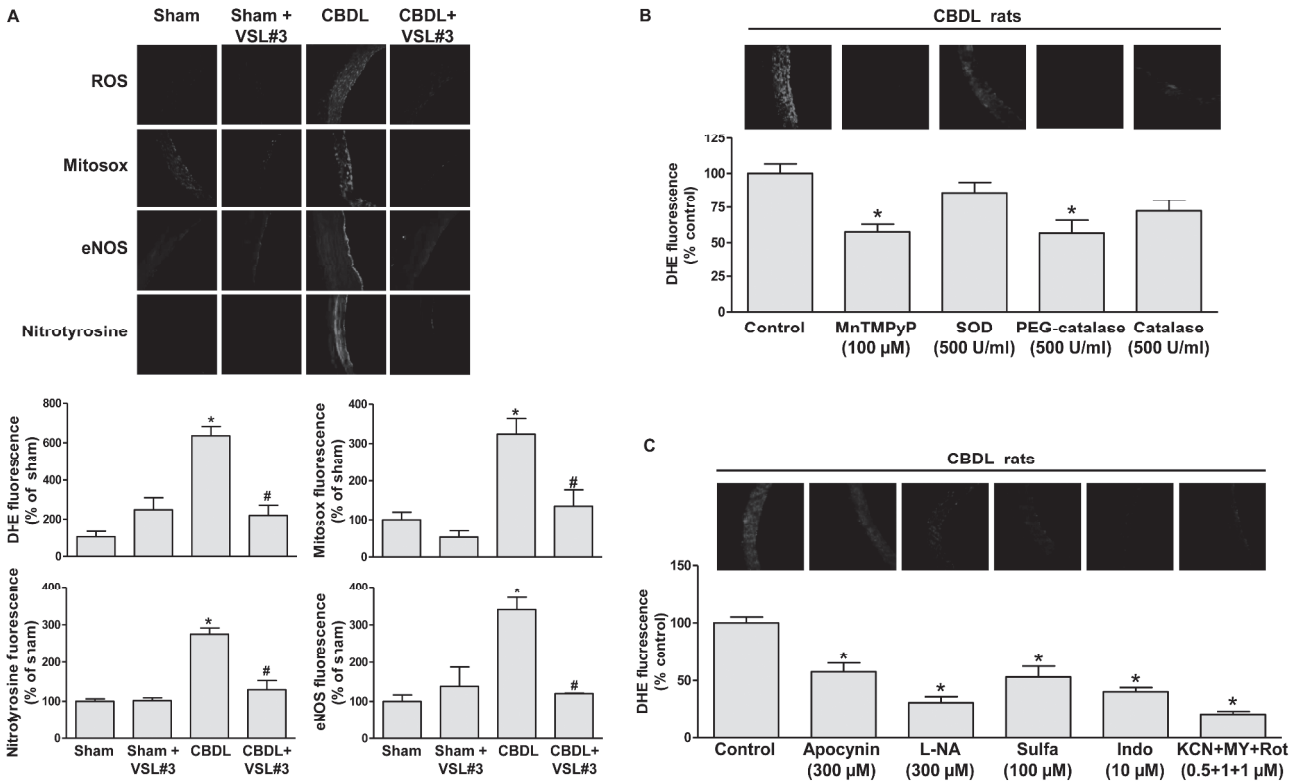
La première étude a montré qu'un traitement par le probiotique VSL#3 améliorait la composante EDHF- dépendante de la relaxation au niveau mésentérique. Ceci était accompagné de la diminution de production des ROS et des expressions facteurs du SRA ainsi qu'une diminution des cytokines pro-inflammatoires dans le plasma. Le stress oxydant dû à la translocation bactérienne et conduisant à la production de cytokines pro-inflammatoires et à une stimulation du SRA local est probablement un facteur important dans la survenue de la dysfonction endothéliale chez le rat cirrhotique. L'administration de probiotiques a amélioré ces anomalies.



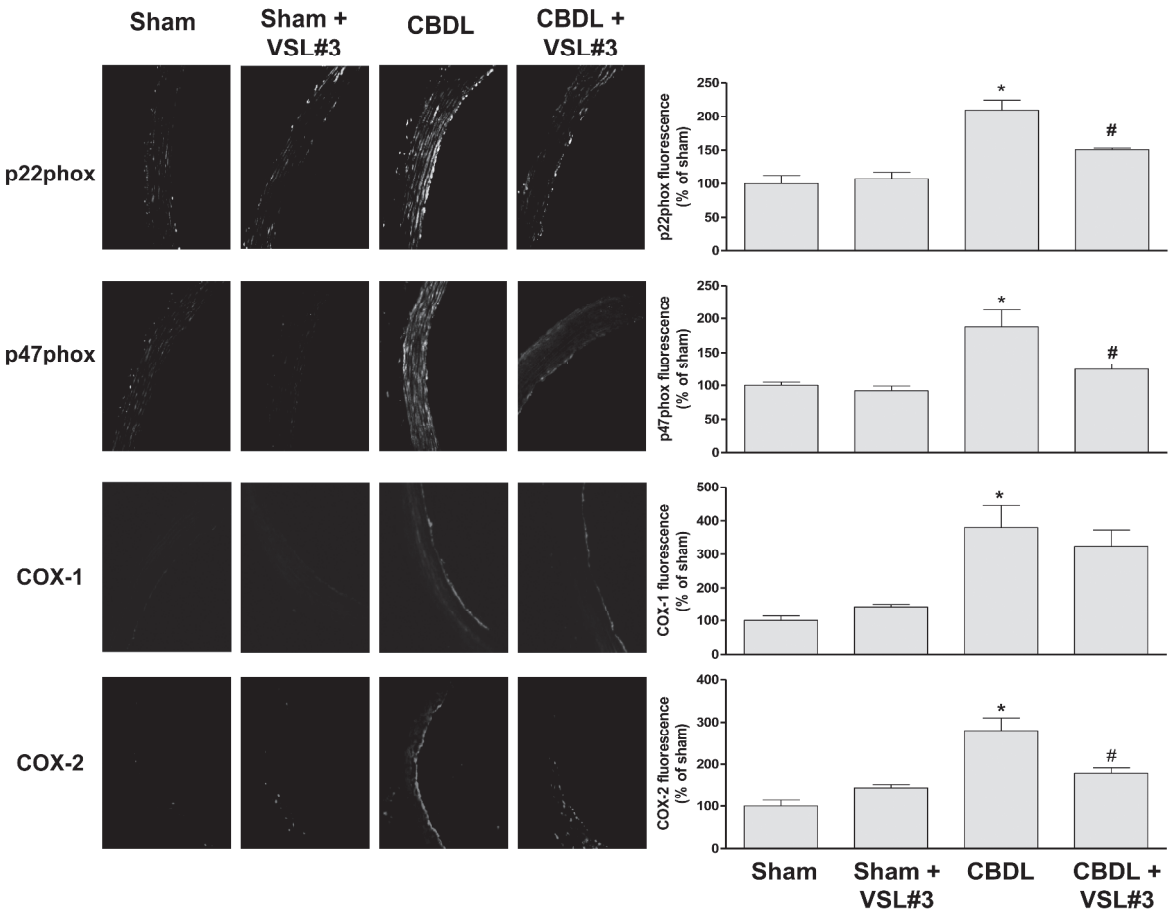
Le probiotique VSL#3 améliore la fonction EDHF-dépendante de la relaxation de l'artère mésentérique chez le rat cirrhotique ; A) composante NO en présence d'indométacine (10 µM) et d'apamine plus charybdotoxine (100 nM chacun), B) composante EDH en présence d'indométacine et de N^G-nitro-L-arginine (300 µM) C) réponse au nitroprussiate de sodium (donneur exogène de NO), D) réponse au levcromakalim (ouvreur de canaux potassiques) ; CBDL : rats cirrhotiques ; **P*<0.05 CBDL vs sham, et $^{\#}$*P*<0.05 CBDL+VSL#3 vs CBDL.



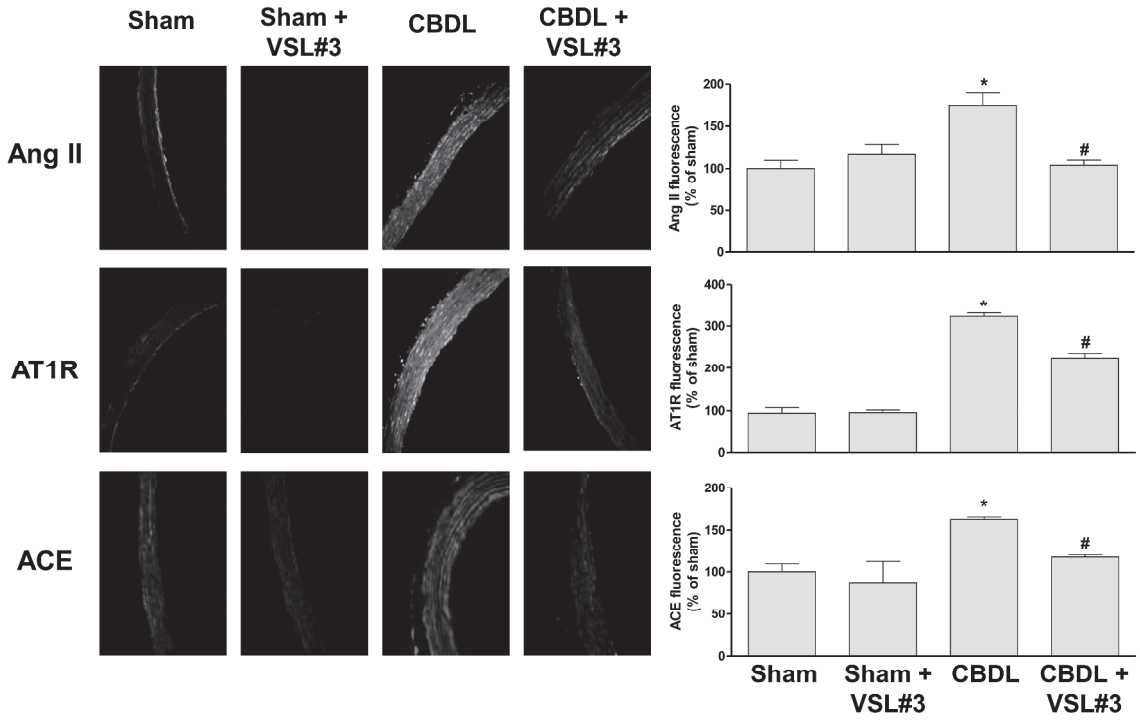
Immmuno-fluorescence au niveau de l'artère mésentérique : VSL#3 diminue l'expression de eNOS et augmente celle des canaux potassiques SK_{Ca} et IK_{Ca} (A) et des connexines (B) impliquées dans la réponse EDHF-dépendante. Panneau du haut : exemple d'immunofluorescence, panneau du bas : données cumulées pour 4-5 rats ; $^{*}P<0.05$ CBDL vs sham, et $^{\#}P<0.05$ CBDL+VSL#3 vs CBDL.



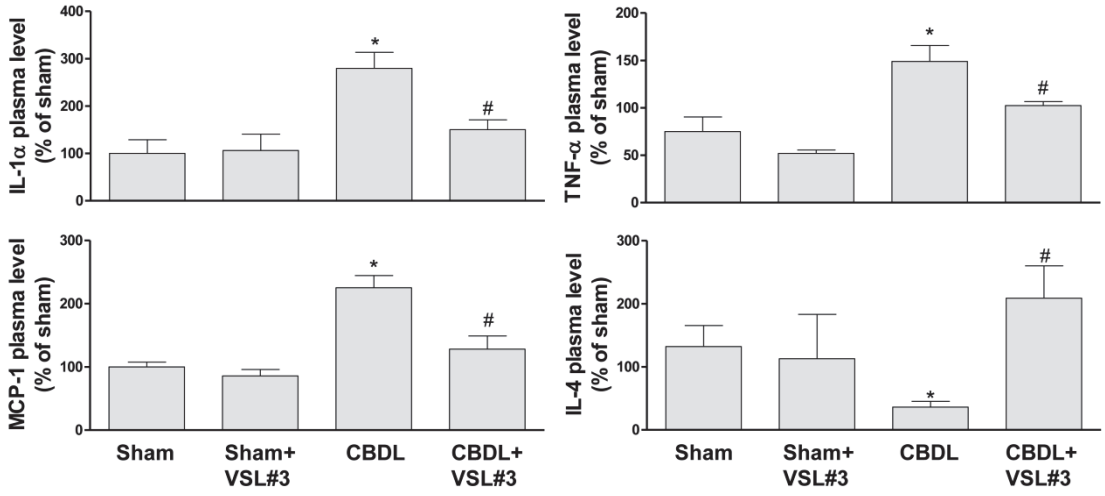
Le traitement par VSL#3 diminue le stress oxydant et l'expression de eNOS dans la paroi de l'aorte des rats cirrhotiques (A) ; rôle d'anions superoxydes intracellulaires et du superoxyde d'hydrogène, anneaux aortiques exposés au MnTMPyP (membrane permeant superoxide dismutase mimetic), à la superoxyde dismutase (SOD), PEG-catalase (membrane permeant catalase) et à la catalase. (B) ; sources cellulaires des ROS, anneaux aortiques exposés à l'antioxydant et inhibiteur de la NADPH oxydase apocynine, au L-NA inhibiteur de la NOS, au sulfaphenazol inhibiteur du cytochrome P450, à l'indométacine inhibiteur de la cyclooxygénase et à la combinaison KCN, myxothiazol et roténone (KCN+MY+Rot), inhibitrice de la respiration mitochondriale, 30 minutes avant le marquage DHE (C) ; A) **P*<0.05 CBDL vs sham, et $^{\#}$*P*<0.05 CBDL+VSL#3 vs CBDL; B et C) *P<0.05 pour CBDL avec et sans inhibiteurs.



Le traitement par VSL#3 prévient la surexpression des sous-unités p22phox et p47phox de la NADPH oxydase et de COX-2 mais pas de COX-1 dans la paroi aortique. Panneau de gauche : exemple d'immunofluorescence, panneau de droite : données cumulées pour 4-5 rats ; **P*<0.05 CBDL vs sham, et $^{\#}$*P*<0.05 CBDL+VSL# 3 vs CBDL.



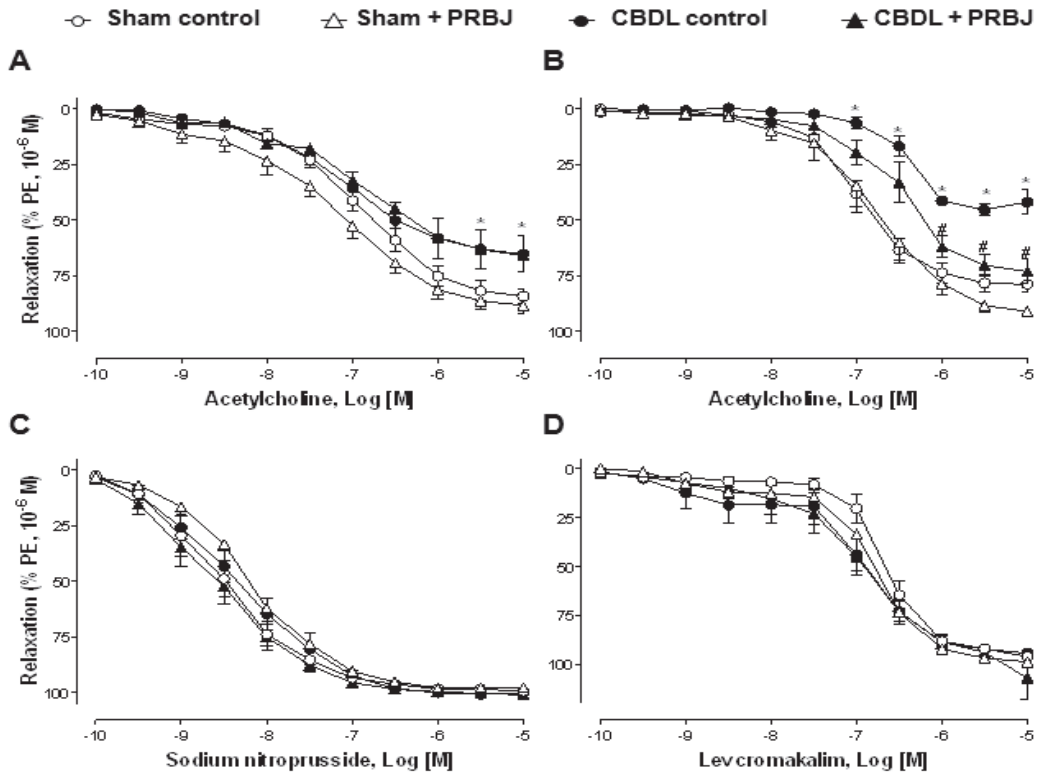
Action du VSL#3 sur les composantes du système RAS (Ang II : angiotensine II, AT1R : récepteur 1 de l'angiotensine, ACE : enzyme de conversion de l'angiotensine) au niveau de la paroi aortique; Panneau de gauche : exemple d'immunofluorescence, panneau de droite : données cumulées pour 4 rats différents; $^{*}P<0.05$ CBDL vs sham, et $^{\#}P<0.05$ CBDL+VSL#3 vs CBDL.



Diminution des cytokines pro-inflammatoires IL-1α, MCP-1 et TNF-α et augmentation de la cytokine anti-inflammatoire IL-4 par le VSL#3 ; $^{*}P<0.05$ CBDL vs sham, et $^{\#}P< 0.05$ CBDL+VSL#3 vs CBDL.

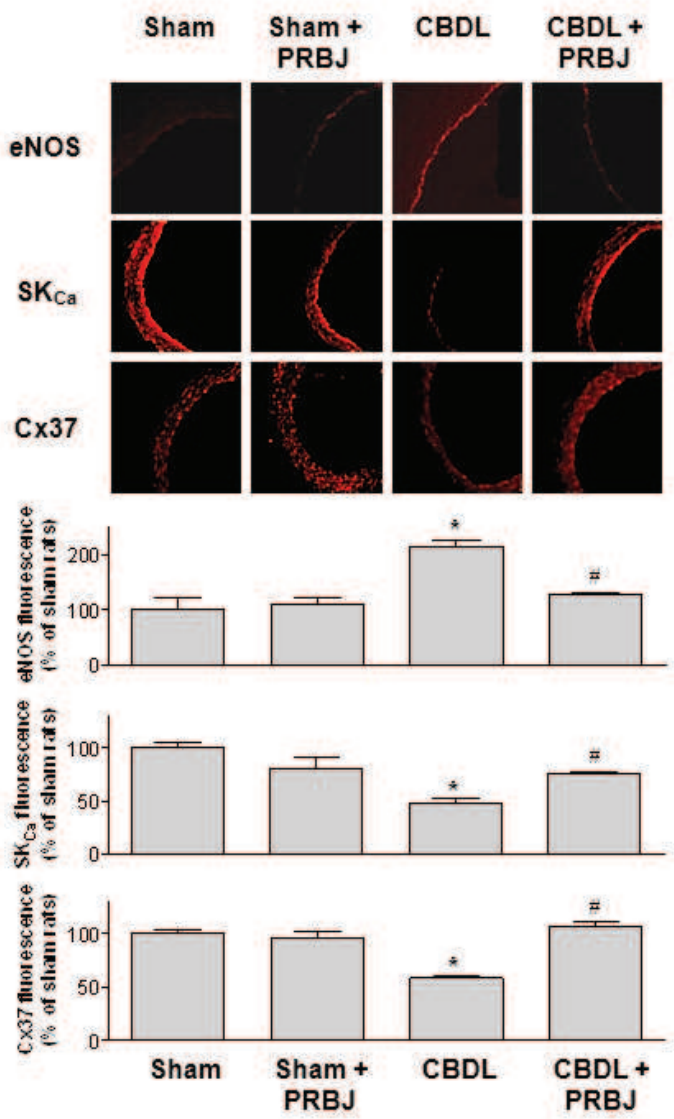
La deuxième étude a montré que l'administration de polyphénols (jus de cassis riche en polyphénols, PRBJ) améliorait la dysfonction endothéliale chez le rat cirrhotique : amélioration de la relaxation EDHF-dépendante, diminution de l'expression de eNOS, des ROS, du SRA et des cytokines pro-inflammatoires. Le stress oxydant, dont une part peut être due à la translocation bactérienne mais d'autres facteurs sont également en jeu, est probablement un facteur clé dans la dysfonction endothéliale chez le rat cirrhotique. Le rôle du SRA lié au stress oxydant est à nouveau mis en exergue dans cette étude ce qui confirme une de nos études antérieures où un traitement par losartan (antagoniste des récepteurs 1 de l'angiotensine) améliorait la dysfonction endothéliale chez le rat présentant une cirrhose biliaire.

Figure 1



Le traitement par PRBJ améliore la fonction EDHF-dépendante de la relaxation de l'artère mésentérique chez le rat cirrhotique ; A) composante NO en présence d'indométacine (10 µM) et d'apamine plus charybdotoxine (100 nM chacun), B) composante EDHF en présence d'indométacine et de N^G-nitro-L-arginine (300 µM) C) réponse au nitroprussiate de sodium (donneur exogène de NO), D) réponse au levcromakalim (ouvreur de canaux potassiques) ; CBDL : rats cirrhotiques ; **P*<0.05 CBDL vs sham, et $^{\#}$*P*<0.05 CBDL+PRBJ vs CBDL

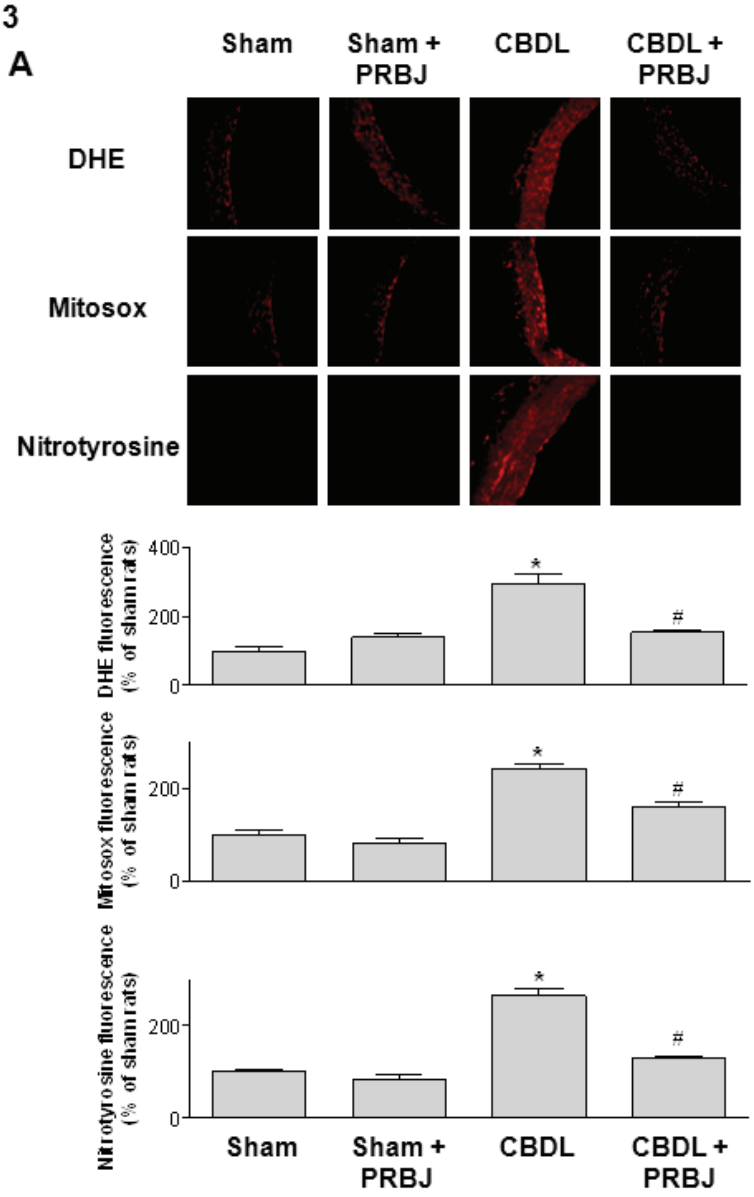
Figure 2

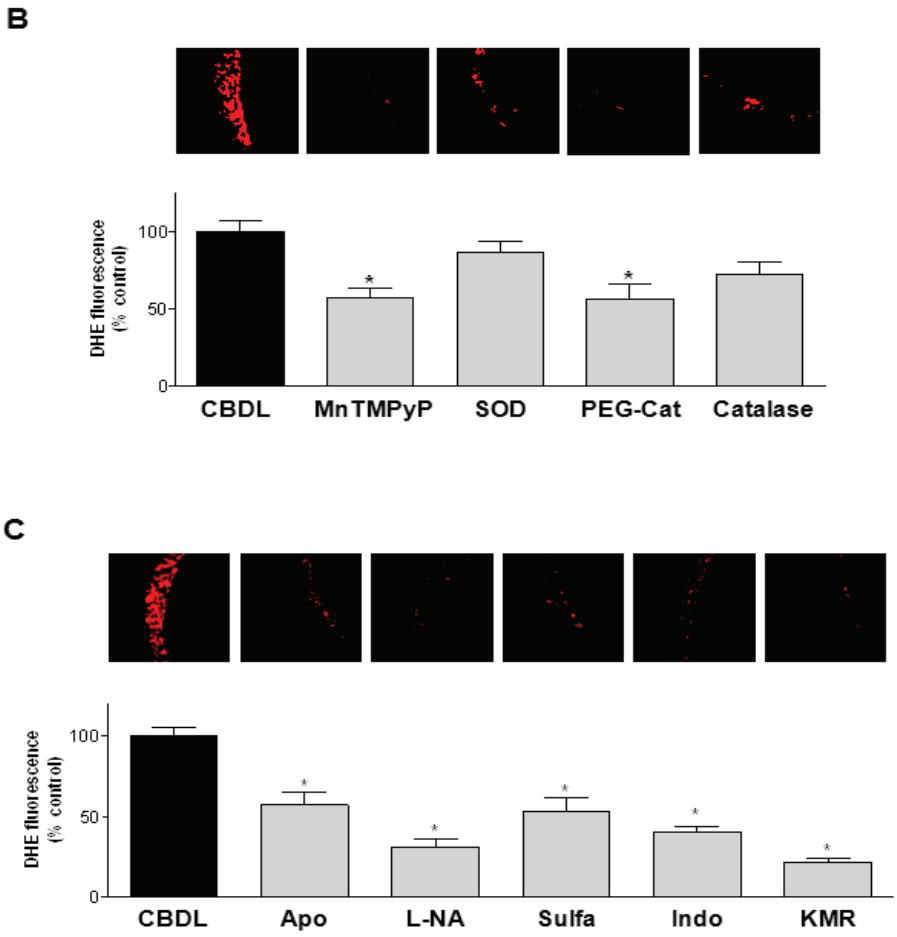


Immmuno-fluorescence au niveau de l'artère mésentérique : PRBJ diminue l'expression de eNOS et augmente celle des canaux potassiques SK_{Ca} et de la connexine 37 impliqués dans la réponse EDHF-dépendante. Panneau du haut : exemple d'immunofluorescence, panneau du

bas : données cumulées pour 4-5 rats ; **P*<0.05 for CBDL vs sham, et $^{\#}$*P*<0.05 CBDL+PRBJ vs CBDL

Figure 3

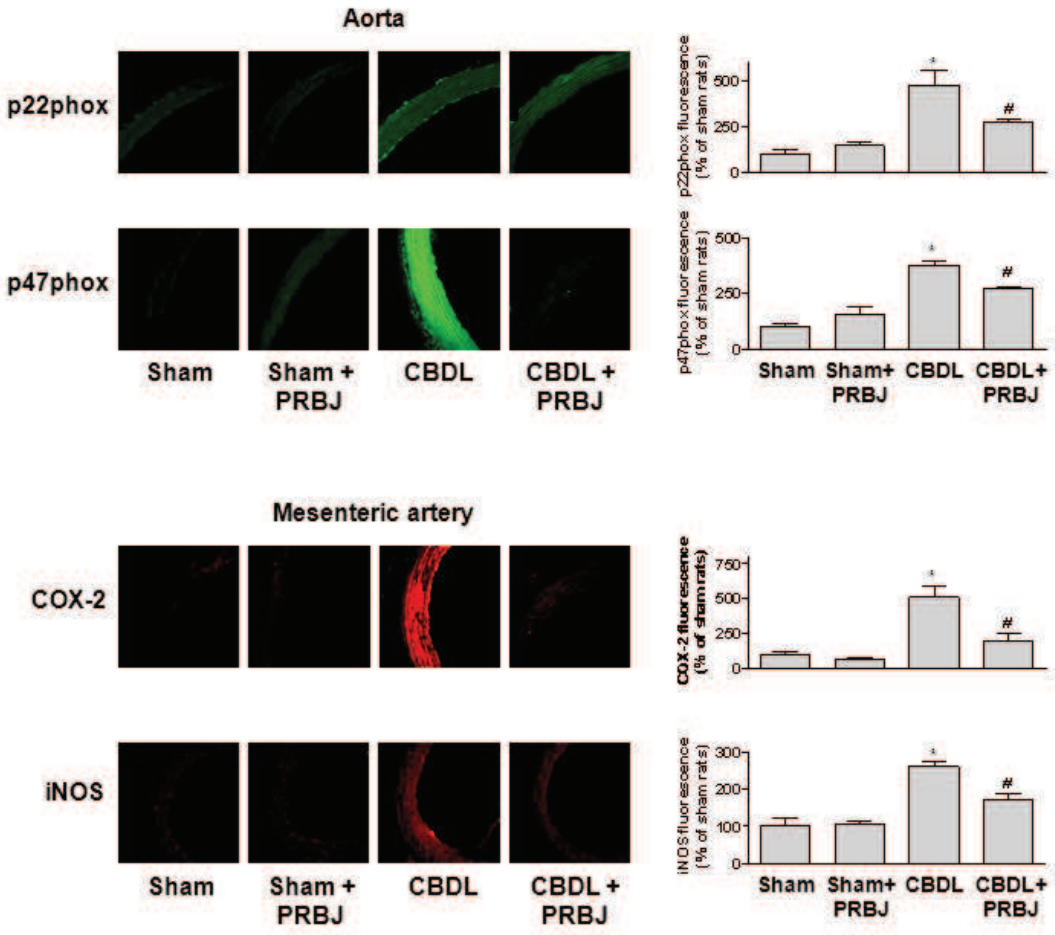




Le traitement par PRBJ diminue le stress oxydant et l'expression de eNOS dans la paroi de l'aorte des rats cirrhotiques (A) ; rôle d'anions superoxydes intracellulaires et du superoxyde d'hydrogène, anneaux aortiques exposés au MnTMPyP (membrane permeant superoxide dismutase mimetic), à la superoxyde dismutase (SOD), PEG-catalase (membrane permeant catalase) et à la catalase (B) ; sources cellulaires des ROS, anneaux aortiques exposés à

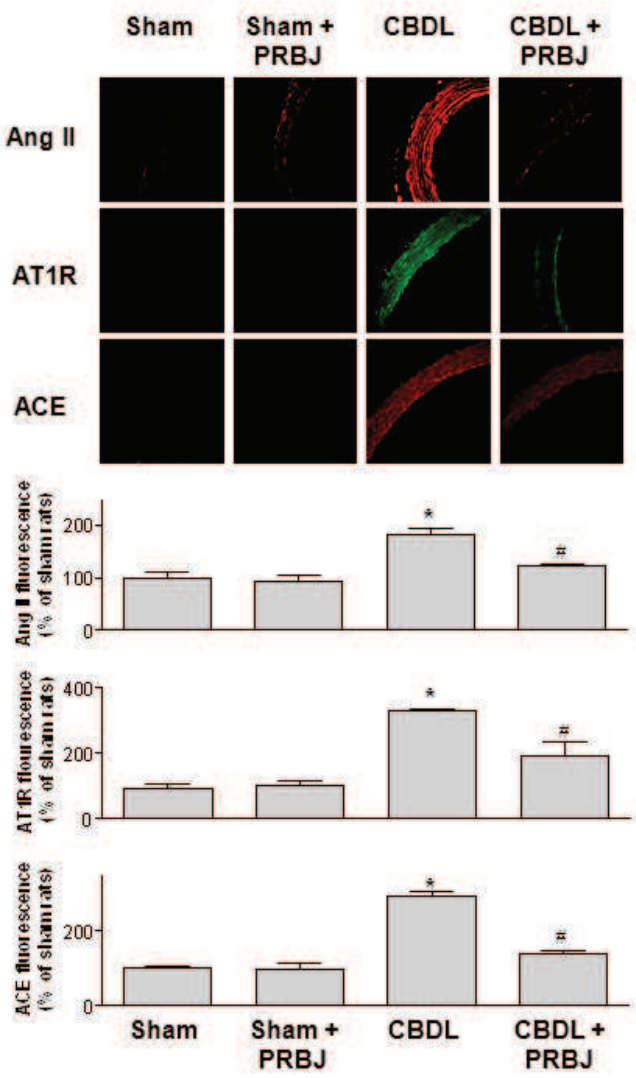
l'antioxydant et inhibiteur de la NADPH oxydase apocynine, au L-NA inhibiteur de la NOS, au sulfaphenazol inhibiteur du cytochrome P450, à l'indométacine inhibiteur de la cyclooxygénase et à la combinaison KCN, myxothiazol et roténone (KCN+MY+Rot), inhibiteur de la respiration mitochondriale, avant le marquage DHE (C). Panneaux du haut : exemple d'immunofluorescence, panneaux du bas : données cumulées pour 4 rats A) $*P<0.05$ CBDL vs sham, et $^{\#}P<0.05$ CBDL+VSL#3 vs CBDL; B and C) *P<0.05 for CBDL avec et sans inhibiteurs.

Figure 4



Le traitement par PRBJ prévient la surexpression des sous-unités p22phox et p47phox de la NADPH oxydase, de COX-2 et de iNOS dans la paroi aortique. Panneau de gauche : exemple d'immunofluorescence, panneau de droite : données cumulées pour 4 rats différents ;*P<0.05 CBDL vs sham, et $^{\#}P$<0.05 CBDL+PRBJ vs CBDL.

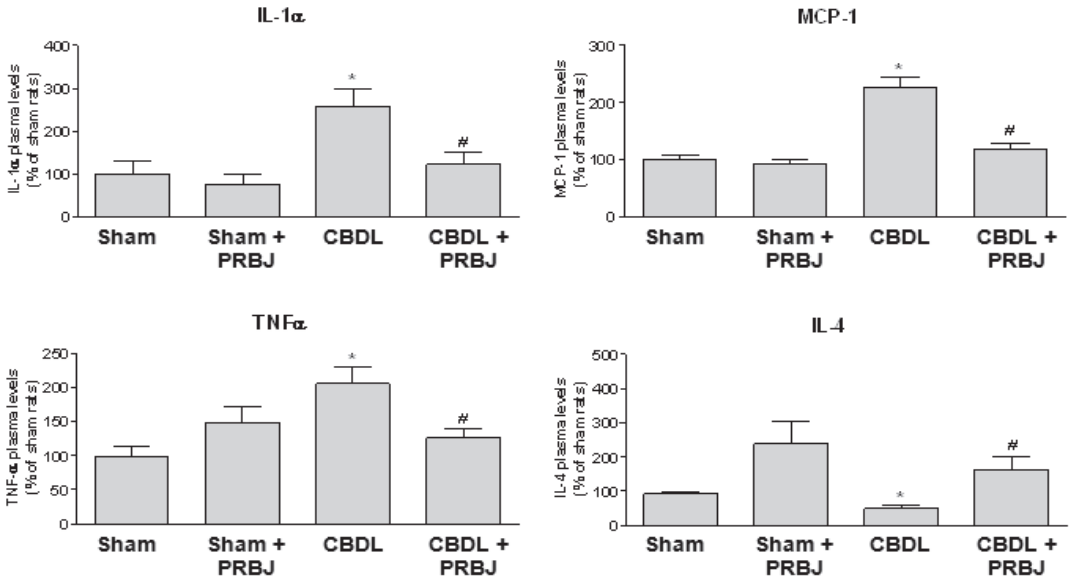
Figure 5



Action du PRBJ sur les composantes du système RAS (Ang II : angiotensine II, AT1R : récepteur 1 de l'angiotensine, ACE : enzyme de conversion de l'angiotensine) au niveau de la paroi aortique; Panneau du haut : exemple d'immunofluorescence, panneau du bas : données

cumulées pour 4 rats différents ; *P<0.05 CBDL vs sham, et $^{\#}P$<0.05 CBDL+PRBJ vs CBDL.

Figure 6



Diminution des cytokines pro-inflammatoires IL-1α, MCP-1 et TNF-α et augmentation de la cytokine anti-inflammatoire IL-4 par le PRBJ ; $*P<0.05$ CBDL vs sham, et $^{\#}P<$ 0.05 CBDL+PRBJ vs CBDL.

Dans la troisième étude, un traitement par fulvestrant (antagoniste des récepteurs de l'estrogène) diminuait l'expression de eNOS et des nitrotyrosines au niveau du poumon. Néanmoins le taux de p-VASP qui est un marqueur de l'action de NO était diminué dans le poumon du rat cirrhotique et le fulvestrant n'avait aucun effet sur ce marqueur. L'hypothèse est que malgré l'augmentation de l'expression de eNOS, le NO produit est capté par les ROS ce qui empêcherait son activité. Ceci pourrait d'ailleurs aussi expliquer la diminution de la composante NO de la relaxation dans les 2 études précédentes. Le fulvestrant n'a aucun effet non plus sur l'expression de VEGF ni sur la quantification des macrophages dans les vaisseaux pulmonaires, ni sur le diamètre des petits vaisseaux pulmonaires. En conclusion, soit le fuvestrant n'est pas efficace, soit l'hyperoestrogénie relative chez nos rats cirrhotiques mâles n'a pas de rôle à jouer dans le survenue du SHP.

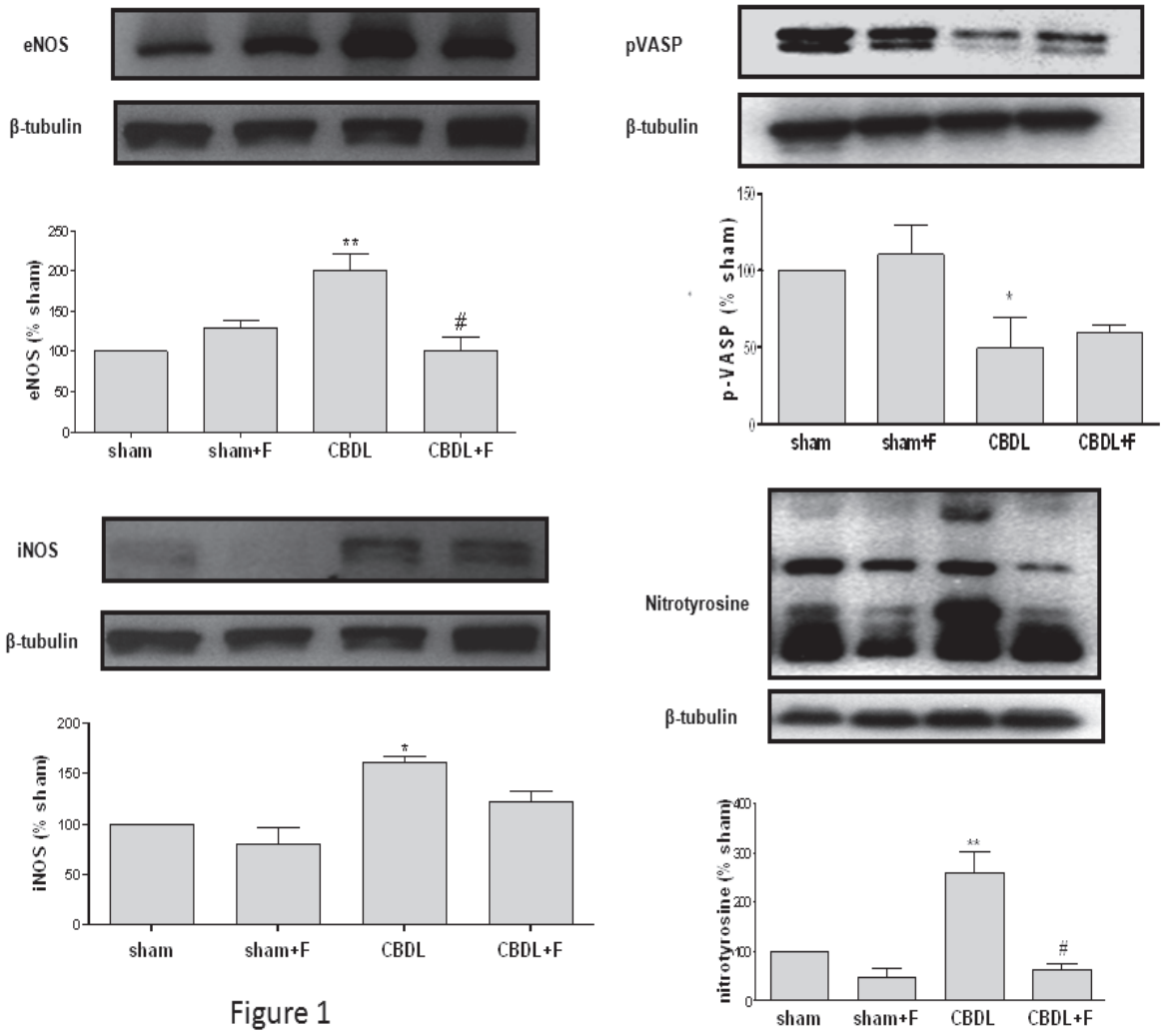


Figure 1

Résultats des western blots pour eNOS (NO synthase endothéliale), iNOS (NO synthase inductiblel), nitrotyrosine, p-VASP (phosphorylated vasodilator-stimulated phosphoprotein) dans les poumons.

Le fulvestrant diminue l'expression de eNOS et de la nitrotyrosine ; de manière surprenante, p-VASP qui est un marqueur de l'action de NO, est diminué dans le poumon du rat cirrhotique et le fulvestrant n'a aucun effet sur ce marqueur

* : P<0.05 pour CBDL vs sham rats; ** : P<0.01 pour CDL vs sham; # : P<0.01 pour CBDL vs CBDL+F

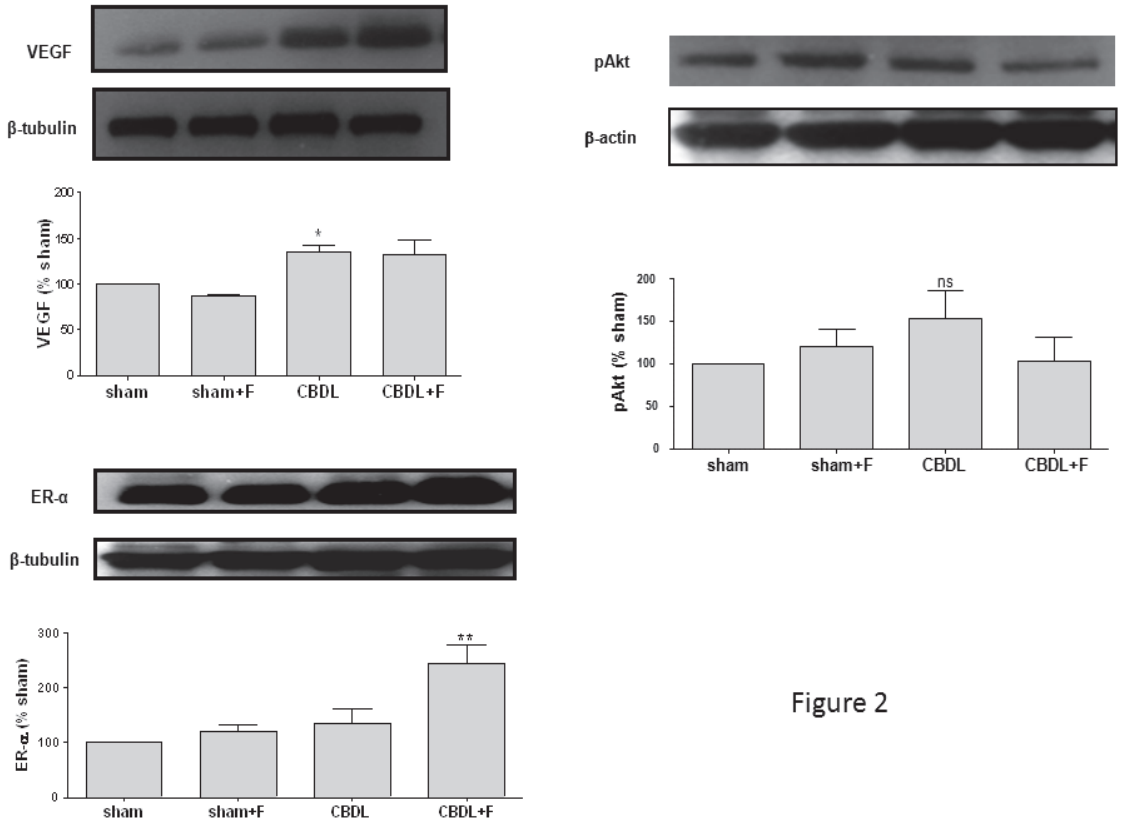


Figure 2

Résultats des western blots pour VEGF (vascular endothelial growth factor), ER-α (récepteur –alpha des estrogènes), p-Akt (phosphorylated serine/threonine kinase Akt) dans les poumons. Le fulvestrant augmente l'expression de ER-α mais n'a aucun effet sur VEGF ni p-Akt.* : P<0.05 pour CBDL vs sham; ** : P<0.01 pour CBDL vs CBDL+F

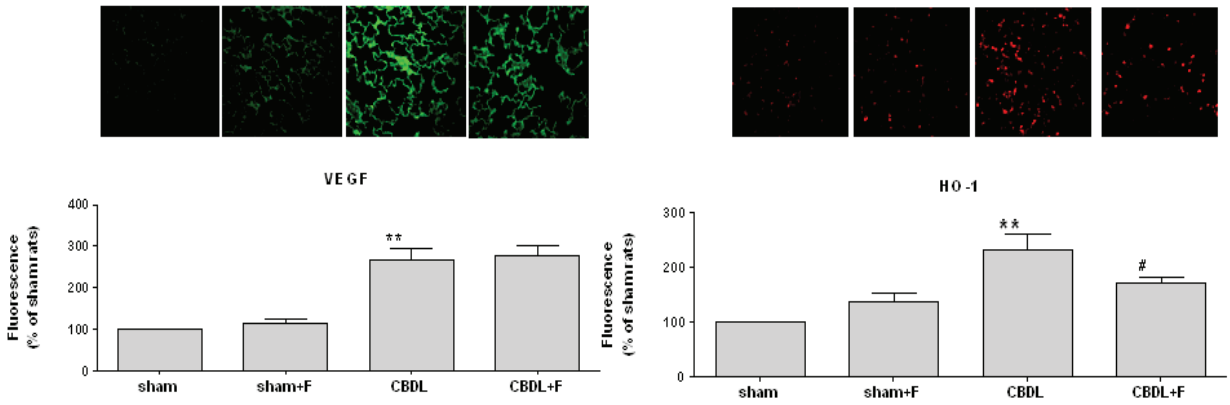
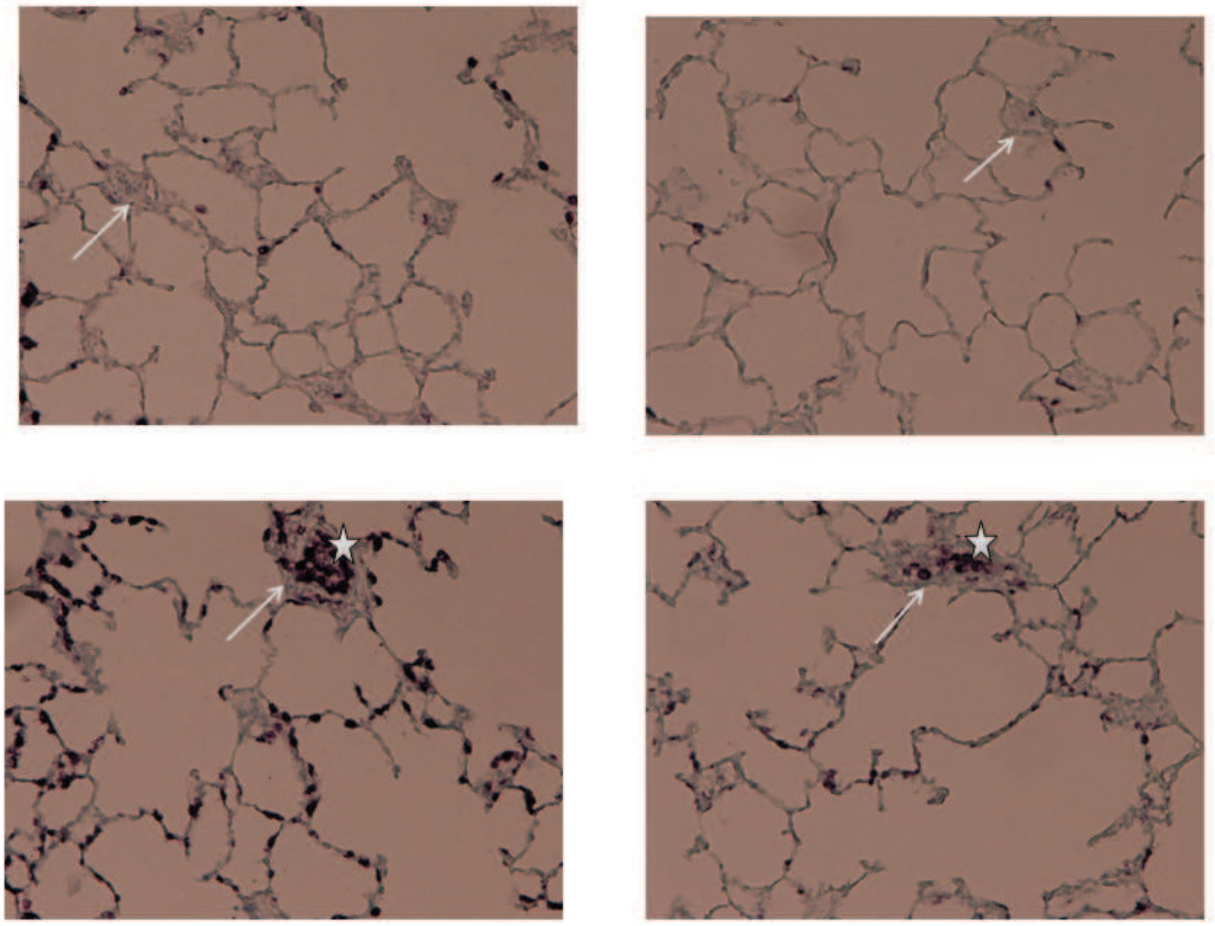


Figure 3

Marquage en immunofluorescence pour VEGF (vascular endothelial growth factor) et la hème-oxygénase-1 dans les poumons. Les deux facteurs sont élevés mais seule HO-1 est diminuée avec le traitement par fulvestrant (possible effet de la diminution de NO, qui peut stimuler HO-1, par le fulvestrant).

** : P<0.05 pour CBDL vs sham; # : P<0.05 pour CBDL vs CBDL+F



Immunohistochimie d'échantillons pulmonaires. On observe de nombreux macrophages intravasculaires (étoiles) et une augmentation du diamètre de petits vaisseaux (flèches) dans le poumon de rats cirrhotiques (panneaux du bas, 2 rats différents) en comparaison avec des rats normaux (panneaux du haut).

Conclusions

L'ensemble de nos études soulignent le rôle important du stress oxydant qui est lié à la stimulation du SRA dans la survenue de la dysfonction endothéliale chez le rat présentant une cirrhose biliaire. L'action sur des facteurs pouvant induire le stress oxydant comme la

translocation bactérienne et le traitement par des antioxydants (polyphénols) a clairement montré des actions bénéfiques chez nos rats cirrhotiques. Le rôle du NO, en tous cas en tant que vasodilatateur pur, n'est peut-être pas primordial ce qui peut expliquer les résultats décevants en clinique publiés par certains auteurs et ce qui peut également expliquer en partie les résultats de notre troisième étude.

CHAPTER I

INTRODUCTION

Chapter 1

Introduction:

1.1. Vascular endothelium

The vascular endothelium plays a major role in controlling the vascular tone through different mechanisms. The vessel's wall consists of three individualized layers (Figure 1) from the lumen to the periphery: the tunica intima (comprising the endothelium with a basement membrane and the internal elastic lamina), the media (smooth muscle cells and collagen fibers limited by the external elastic lamina) and the adventitia (where are nerve endings, vasa vasorum, fibroblast and macrophages in connective tissue) (Mulvany 1990). The importance of each layer depends on the size of the arteries: more elastic fibers in the media in large conducting arteries, more smooth muscles in muscular arteries, less smooth muscle cells with only an internal elastic lamina in arterioles and only endothelium and basement membrane and connective tissue in capillaries). The endothelium covers the whole entire wall of all vessels from the heart to small vessels. Hence the endothelium has many function, this enables it to be called the chief regulator of body homeostasis. Due to their position and their large surface area, endothelial cells (ECs) assume a large variety of functions: synthesis and secretion of various molecules, including vasodilator and vasoconstrictor factors, control of smooth muscle cell (SMC) proliferation, exchanges of molecules between the plasma and the interstitial fluid; they have also a role in the balance between pro- and anticoagulant factors and in immunity.

1.2. The major endothelial functions

Endothelial cells respond to physical and chemical stimuli such as pressure, shear stress, and pH (Feletou and Vanhoutte 2006; Moncada and Higgs 2006) and respond also to other stimuli like microparticles and pro-inflammatory mediators. In response to stimuli the endothelium has the capacity to regulate local vascular homeostasis by maintaining the balance between vasodilation and vasoconstriction, by modulating the proliferation and migration of smooth muscle cells and by acting on thrombosis and fibrinolysis by the release of various factors (Davignon and Ganz 2004). In case of endothelial dysfunction, imbalance of these different mechanisms occurs and may lead to serious cardiovascular diseases. One of the endothelial dysfunction may be due to imbalance between vasodilators and vasoconstrictors. The principal vasoconstrictors are: thromboxane A_2, prostaglandin H_2

(PGH_2), endothelin-1, angiotensin II and the superoxide anions, (Furchgott and Vanhoutte 1989; Touyz, Yao et al. 2004). Vasodilator factors are : nitric oxide (NO), the prostacyclin or prostaglandin I_2 (PGI_2) and the endothelium derived hyperpolarizing (EDH) (Feletou and Vanhoutte 2006; Mombouli and Vanhoutte 1999; Moncada and Higgs 2006) (Table1).

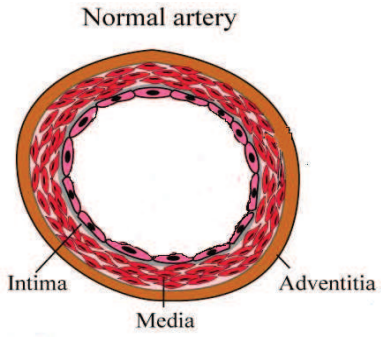


Figure 1. The three layers of normal artery

Vasodilation	Vasocontraction
Nitric oxide (NO	*Reactive oxygen species (ROS)*
Hyperpolarizing factor	*Angiotensin II (Ang II)*
Prostacyclin (PGI2)	*Thromboxane A2 (TXA2)*
	Prostaglandin H2 (PGH2)
	Endothelin

Table 1. Endothelium-derived vasoactive factors. (Calles-Escandon and Cipolla, 2001)

1.2.1. Endothelium-derived vasorelaxing factors

1.2.2. Nitric oxide (NO)

NO is an important cellular signaling molecule involved in different physiological and pathological processes. NO was first known as a ligand capable of activating the soluble guanylyl cyclase and responsible for vascular smooth muscle cells relaxation (Katsuki, Arnold et al. 1977). Soon after, Furchgott and Zawadzki (1980) discovered that the endothelium is responsible for vascular smooth muscle relaxation through production of an endothelium-derived relaxing factor, EDRF. Finally, NO was identified as being the EDRF

described earlier (Palmer, Ferrige et al. 1987; Palmer, Ashton et al. 1988; Palmer and Moncada 1989; Moncada 1997). The endothelium constitutively expresses a NO synthase (endothelial NO synthase, eNOS) which is one of the three isoforms of NOS (Mombouli and Vanhoutte 1999; Stuehr and Griffith 1992) (Table 2). Under normal conditions, inactive eNOS is bound to the protein caveolin and is located in microdomains in the cell membrane called caveolae (Bucci, Gratton et al. 2000). When intracellular Ca^{2+} levels increase, (in calcium-dependent activation of eNOS) (Figure 3) calmodulin detaches eNOS from caveolin permitting the enzyme to become active. Apart from the increases in intracellular [Ca^{2+}] , other mechanisms (calcium-independent activation of eNOS), leading to the phosphorylation of eNOS (especially phosphorylation of Ser1177) and hence to the activation of eNOS occur via protein kinases pathways, such as protein kinase A (PKA) and cGMP dependent protein kinase II (PKG). The relative contributions of different kinase pathways remain under active investigation, but it is clear that extra-cellular stimuli (shear stress, estrogen, ET-1 and VEGF) activate distinct kinase pathways leading to eNOS phosphorylation and regulating the eNOS state at transcriptional level and stability (Dimmeler et al. 1999, Haynes et al. 2000; Sandoo et al. 2010) (Table 3). eNOS generates NO upon the conversion of L-arginine to L-citrulline in the endothelium (Figure 2). NO has the ability to diffuse towards the underlying vascular smooth muscle to reduce vascular tone through activation of soluble guanylyl cyclase (sGC), and to prevent smooth muscle cell proliferation and migration thereby, maintaining the arterial wall in a quiescent state (Murad et al, 1978). Apart from its action on the smooth muscles, NO has another effect: NO has also been shown to prevent the expression of numerous pro-inflammatory and pro-atherothrombotic mediators such as monocyte chemoattractant protein-1 (MCP-1), tissue factors and adhesion molecules. Moreover, NO helps maintaining blood fluidity by preventing the adhesion and aggregation of platelets and the adhesion of monocytes (Lee et al, 2011). Diminished NO production or bioavailability has been implicated in the pathogenesis of essential and pulmonary hypertension and in multiple other vascular disorders including atherosclerosis (Voetsch et al. 2004). In portal hypertension: NO is diminished in the liver and increased in the splanchnic and systemic vascular beds. Many factors contribute to intra-hepatic reduced eNOS activity including increased oxidative stress, increased binding ability of caveolin-1 to eNOS and increased activity of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS causing un-coupling of NOS and leading to the generation of peroxynitrate (Shah et al. 1999; Laleman et al. 2005). Other factors play a role like decreased Akt activity and tetra-hydrobiopterin (BH4) level (BH4 acts as important catalytic agent in the oxidation of L-arginine by NO

synthases) (Alp and Channon 2004) (Figure 2), and impairment of the antioxidant system (Hu et al, 2013). In contrast to hypoactive hepatic endothelial cells, endothelial cells in the splanchnic and systemic circulation have been shown to produce NO which could modulate the vascular changes observed in cirrhosis. Increased activity of vascular Akt signaling (Fernandez-Varo et al, 2010), VEGF-induced NO production have been also implicated (Abraldes et al, 2006). Likewise, in portal hypertensive rats, NO production is increased in response to shear stress (Tazi et al, 2002) and finally the role of bacterial lipopolysaccharide (LPS) induced overproduction of pro-inflammatory cytokines like TNF-α and subsequent induction of inducible NOS (iNOS) in extrahepatic vasculature have been reported (Kajita et al, 2011; Moreau et al, 2002; Huang et al, 2009). It is worthy to mention, that the iNOS induced in the endothelial and other inflammatory cells following immunological activation (Geller et al. 1993), is calcium independent, mostly transcriptionally regulated and is not normally produced in most cell (Forstermann et al. 1994), iNOS generates NO in 100-1000 fold more than eNOS and the NO activity persists for many hours (Morris and Billiar 1994; Nathan and Xie 1994).

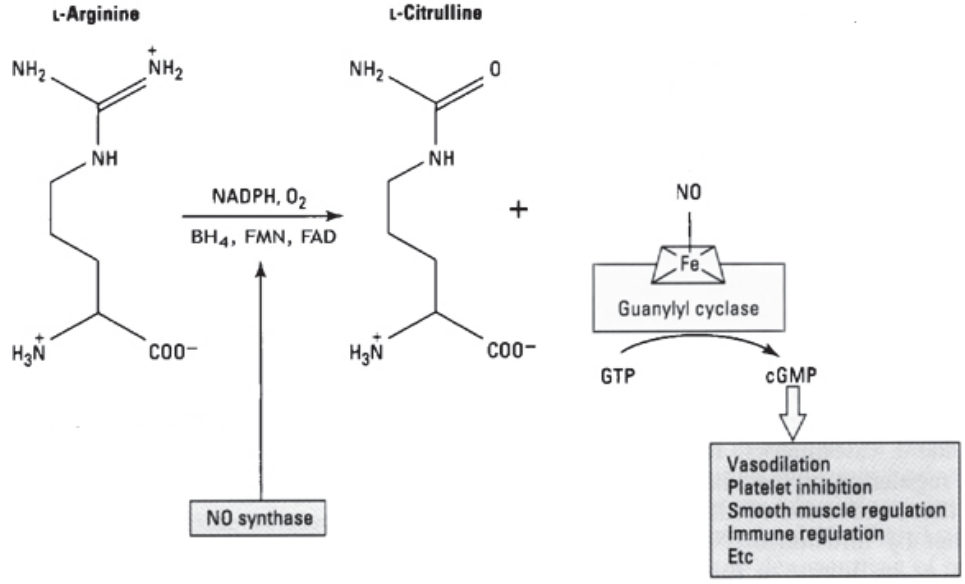


Figure 2. NO generation from L-Arginine and its functional properties. (Ghalayini 2004)

Name	location	main function
Neuronal NOS (nNOSorNOS1)	nervous tissue skeletal muscle type II	Cell communication
Inducible NOS (iNOS or NOS2)	Immune system Cardiovascular system	Immune defense against
Endothelial NOS (eNOS or NOS3)	Endothelium	Vasodilatation

Table 2. Three isoforms of NO synthase. (Stuehr and Griffith 1992)

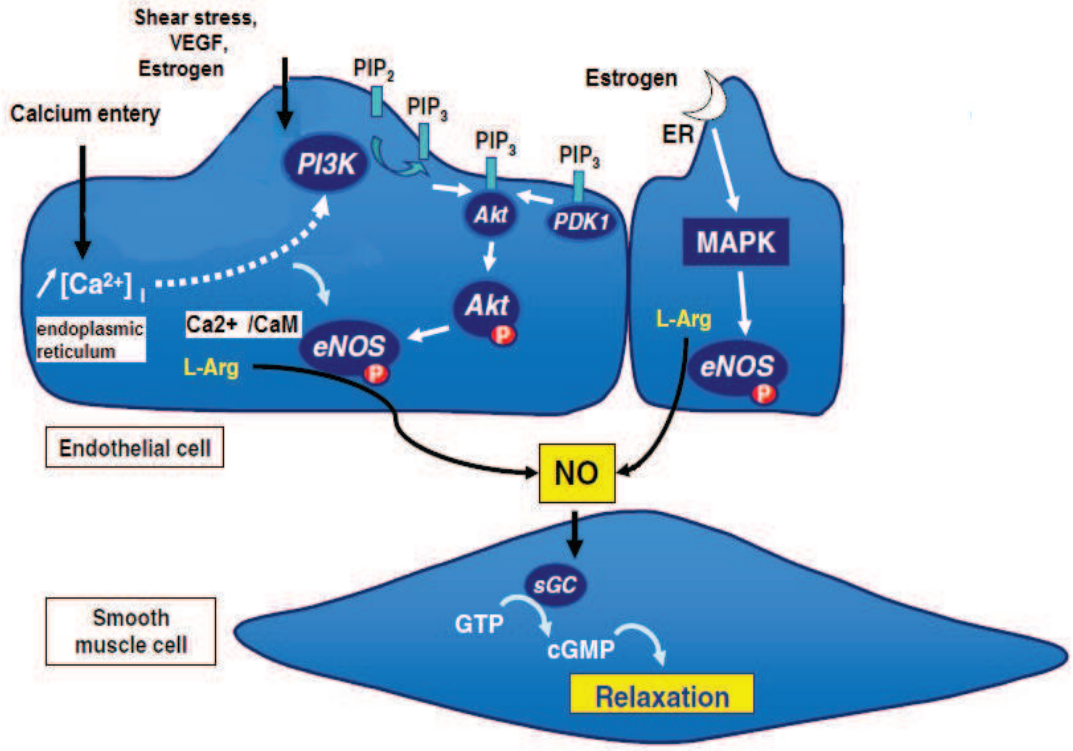


Figure 3. NO synthesis pathway through eNOS phosphorylation in calcium dependent and independent pathway. PI3K, phosphatidylinositol-3- kinase; PDK1, phosphoinositide-dependent kinase 1; eNOS, endothelial NO synthase; NO, nitric oxide; ER, estrogen receptor; CaM, calmodulin; sGC, soluble guanylyl cyclase modified. (Schini-Kerth et al. 2010)

Stimulus	Transcriptional Regulation	Posttranscriptional Regulation
Laminar shear stress	↑ transcription	↑ stability
Cyclic strain	↑ transcription	Unknown
Cell growth	NO effect	↑ stability
Hydrogen peroxide	↑ transcription	↑ stability
TGF-β1	↑ transcription	Unknown
Lysophsphatidylcholine	↑ transcription	Unknown
Oxidized linoleic acid	↑ transcription	↑ stability
TNF-α	↓ transcription	↓ stability
LPS	No effect	↓ stability
Hypoxia	↑ and ↓ transcription	↓ stability
Statins	No effect	↑ stability
Estrogen	↑ transcription	No effect
Protein kinase C	↑ transcription	No effect
Thrombin	Unknown	↓ Stability
Rho GTPase	No effect	↓ Stability
VEGF	Possibly ↑ transcription	↑ Stability
Histone deacetylase inhibition	↑ transcription	Probable ↓ stability
Hypercholesterolemia	Unknown	↓ Stability

Table 3. Physiological and pathophysiological stimuli shown to regulate eNOS expression and their mode of regulation: (increased :↑ , decreased:↓) (Searles 2006).

1.2.3. Endothelium-derived hyperpolarizing factor (EDHF)

Beside NO and prostacyclin, EDHF plays an important role in endothelium-dependent relaxation in most medium-to small sized arteries (Feletou and Vanhoutte 1996). EDHF is an important relaxing factor in the coronary artery as well as in small arteries and arterioles such as second and third-order mesenteric arteries (Shimokawa et al, 1996). After its discovery in 1987, the identity of EDHF is still in the center of controversy because it differs depending on the animal species and the type of blood vessel. In 1996, Shimokawa et al. reported that EDHF is more important in resistance vessels than NO and prostacyclin (Shimokawa et al, 1996). Also, in human arteries, endothelium-dependent vasodilation involves EDHF (Nakashima et al, 1993). EDHF is defined as a hyperpolarization of endothelial origin that is transmitted to the vascular smooth muscle leading to its relaxation. The EDHF component of the relaxation is evaluated in the presence of the combination of inhibitors of eNOS like L-NAME and COXs like indomethacin. To block also the EDHF mediated relaxation, we need to add the inhibitors, apamin and charybdotoxin for both calcium-dependent potassium channels of intermediate (IK_{Ca}) and small conductance (SK_{Ca}). In the EDHF-mediated response, IK_{Ca} and SK_{Ca} are activated so that potassium ions move from the intracellular compartment to the extracellular space of endothelial cells, which leads to their hyperpolarization (Figure 4). Thereafter, low concentrations of potassium ions in the extracellular space can activate inwardly rectifying K^+ (K_{IR}) channels and Na+/K+-ATPase to cause hyperpolarization of smooth muscle cells through the movement of potassium ions out of the smooth muscle cells (Edwards et al, 1998; Feletou and Vanhoutte 2006; Feletou et al, 2003). Another pathway leading to a direct transfer of the hyperpolarization from endothelial cells to smooth muscle cells occurs via myoendothelial gap junctions (meGJ) as reported previously by Edwards et al., 1998 (Edwards et al, 1998). Gap junctions are intracellular channels which can transfer signals from the endothelial cells to the underling smooth muscle cells (Sandoo et al, 2010) In addition, in some tissues, the hyperpolarization of the endothelial cells might be regulated by hydrogen peroxide (H_2O_2) (Matoba et al, 2002) or the activation of cytochrome P450 and the resulting generation of epoxyeicosatrienoic acids (EET), which are metabolites of arachidonic acid (Quilley and McGiff 2000). The EET results in increasing K^+ efflux from the smooth muscle cells resulting in hyperpolarization and relaxation (Figure 4). Hyperpolarization of smooth muscle cells leads to a decrease in cytosolic calcium concentration with subsequent relaxation.

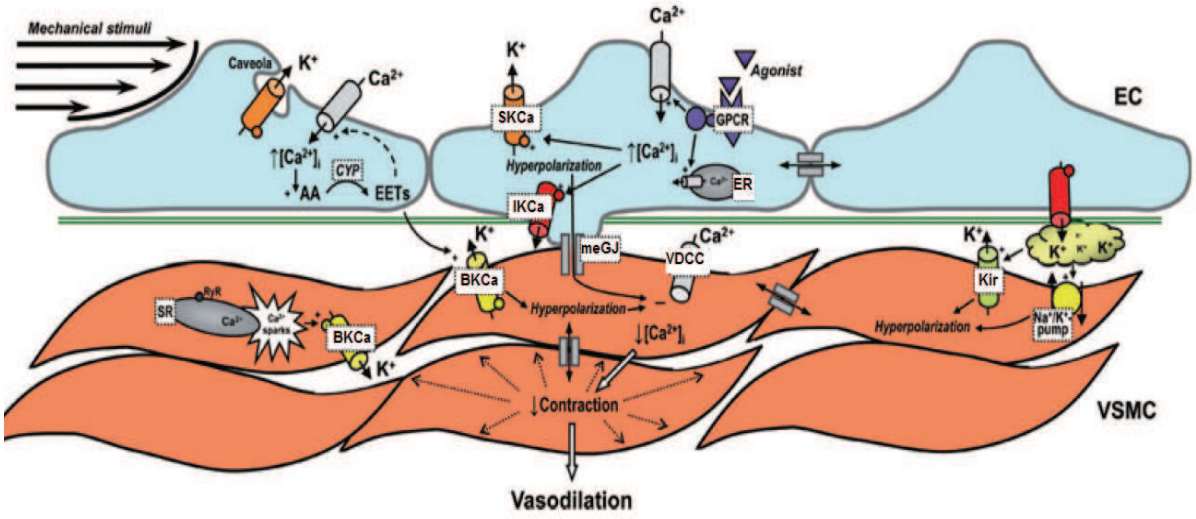


Figure 4. Hypothesis describing the nature of EDHF pathway (Grgic et al, 2009).

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.

1.2.4. Prostacyclin (PGI_2)

Another important factor of endothelium-dependant relaxation is PGI_2. PGI_2 is a member of prostanoids (together with other prostaglandins and thromboxanes) which are products from the arachidonic acid metabolism (Figure 5). PGI_2 is a potent vasodilator, and effective endogenous inhibitor of platelet aggregation (Coleman et al, 1994; Moncada and Vane 1979). PGI2 is produced in the endothelium from prostaglandin H_2 (PGH_2) by the action of the enzyme prostacyclin synthase. PGI_2, PGG_2 and PGH_2 are major products of vascular cycloxygenase (COX). There are two isoforms of COX encoded by two separate genes. COX-1 is constitutively expressed and is present in many tissues, including endothelial cells. COX-2 is not constitutively expressed, but can be induced rapidly and transiently in many cells, including vascular endothelial cells and smooth muscle cells, under the effect of physical stimuli and pro-inflammatory agents (Marnett et al, 1999; Hong and Deykin 1982; Topper et al, 1996). PGI_2 elicits smooth muscle relaxation by stimulating adenylyl cyclase and formation of cyclic adenosine -3', 5'- monophosphate. Its vasodilator activity is determined by the expression of specific receptors which are prostaglandin I2 receptors of the G-protein

coupled receptor family in vascular smooth muscle cells (Coleman et al, 1994). The binding of PGI_2 to its receptors causes a change of conformation of the receptors, which will lead to an increase of cyclic adenosine-3', 5'-monophosphate (cAMP) levels in the vascular smooth muscle. cAMP then activates protein kinase A, which reduces intracellular Ca^{2+} by decreasing Ca^{2+} release from the endoplasmic reticulum and by stimulating its uptake by it (Kukovetz et al, 1979). Furthermore, PGI_2 is a potent inhibitor of platelet adhesion, aggregation, and degranulation (Mustard et al, 1980). In addition, PGI_2 facilitates the release of NO by endothelial cells (Shimokawa et al, 1988) and in turn, the action of PGI_2 in vascular smooth muscle cells and platelets is potentiated by NO (Delpy et al, 1996).

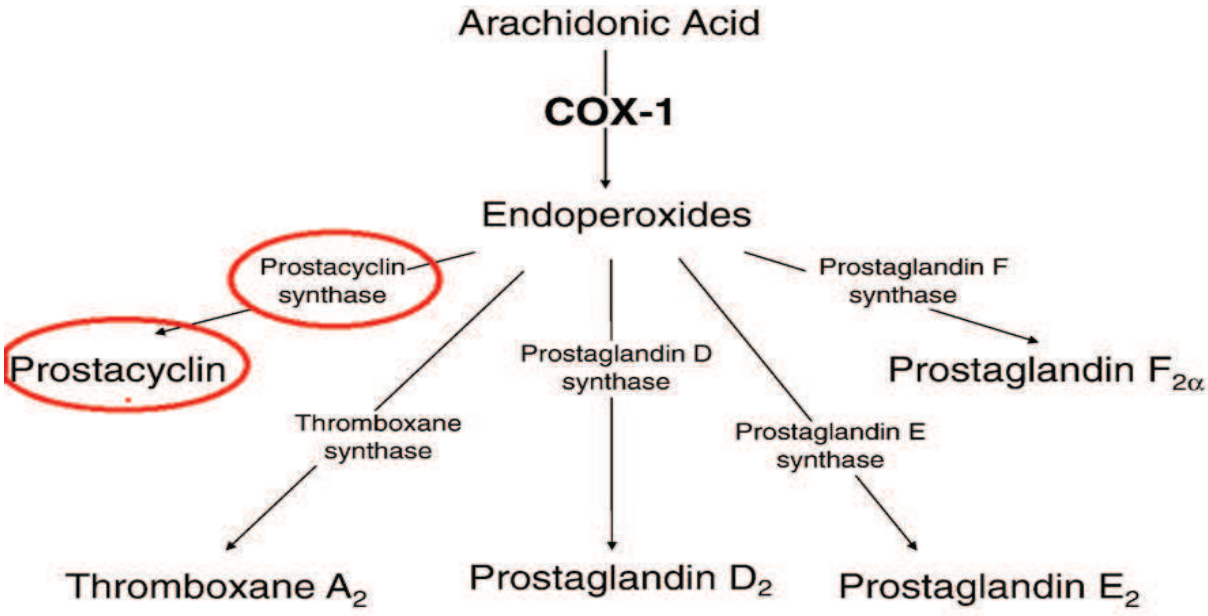


Figure 5. Arachidonic acid metabolism pathway. (Tang and Vanhoutte, 2009)

1.2.5. Summary of endothelium-derived relaxation

EDHF causes hyperpolarization of the endothelium via the activation of calcium dependant potassium channels of intermediate conductance (IK_{Ca}) and small conductance (SK_{Ca}). Furthermore, vascular smooth muscle relaxation is induced subsequently to the transmission of hyperpolarization through the myoendothelial junction (MGJ). NO is produced by eNOS which is activated by Src/PI3 kinase/Akt pathway or by the calcium calmodulin signaling and relaxes vascular smooth muscle via activation of soluble guanylyl cyclase (sGC). Moreover, PGI_2 bind to prostacyclin receptor (IP_1) receptor and induce

relaxation via adenylyl cyclase. Endothelin-1(ET-1) binds to ET-$_{B1}$ receptor on the endothelial receptor to activate eNOS (Figure 6).

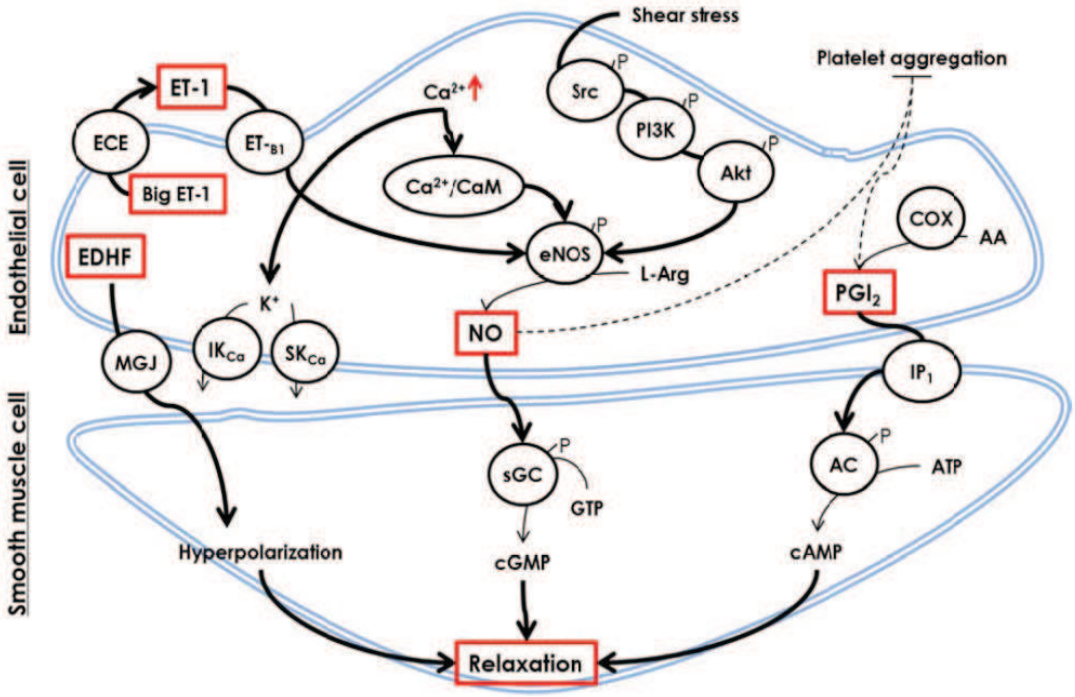


Figure 6. The pathways of endothelial-derived relaxation. AA, arachidonic acid; AC, adenylyl cyclase; ATP, Adenosine triphosphate ; Big ET-1, precursor of endothelin-1; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate ; eNOS, endothelium NO synthase; ECE, endothelin converting enzyme; ET-1, endothelin-1, ET-$_{B1}$ endothelin receptor; IP_1, prostacyclin receptor; GTP, guanosine triphosphate; NO , nitric oxide; PI3K, phosphatidylinositol 3-kinse; Src, Src family. Dash line represents inhibition.

1.3. Endothelium-derived contracting factors (EDCF)

There is large heterogeneity in the formation of EDCF, depending on the stimuli, vascular bed and the age of the experimental animal used. Among the different contracting factors that are produced by the endothelial cells, we retain: thromboxane A_2 (TXA_2), prostaglandin H_2 (PGH_2) and also reactive oxygen species, endothelin-1 (ET1) and angiotensin II. (Figure 8) summarizes the different EDCF factors.

1.3.1. Reactive oxygen species (ROS)

Under normal conditions ROS are produced and released from endothelial cells for vascular homeostasis. However, in different pathological conditions like hypertension, diabetes mellitus, atherosclerosis and in acute and chronic inflammatory diseases there is over-production of ROS causing vascular oxidative stress (Eisenberg and Ghigliotti 1999); (Mathis et al, 2012; Sedeek et al, 2012; Xi et al, 2007). ROS in the vasculature are produced by enzymes including cytochrome P450, cyclooxygenases (COXs including COX-1 and COX-2), lipoxygenases, uncoupled-eNOS, xanthine oxidase, NADPH oxidase, and also by the mitochondrial respiratory chain (Griendling and Ushio-Fukai 1997). Superoxide anions ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet-}$) and H_2O_2 are the major ROS. ROS may have direct vasoconstracting effects by facilitating the mobilization of cytosolic Ca^{2+} or promoting Ca^{2+} sensitization of the contractile elements (Jin et al, 1991); (Suzuki and Ford 1992). In addition, ROS in particular $O_2^{\bullet-}$ can also potentiate the contractile responses by reducing the bioavailability of NO (Rubanyi and Vanhoutte 1986) or by activating COXs in vascular smooth muscle cells (Hibino, Okumura et al. 1999) or associated with impairment of endothelium-dependent relaxation (Aubin et al, 2006; Liu et al, 2007). The effect of oxidative stress on EDHF pathway occur through reducing the activity of calcium dependent potassium channels (SK_{Ca} and IK_{Ca}) (Kusama et al, 2005) and also via modifying the passage of hyperpolarization from endothelial cells to underling smooth muscle cells through myoendothelial gap junctions (Griffith et al, 2005). $O_2^{\bullet-}$ can be catalyzed to H_2O_2 by superoxide dismutase (SOD). Thereafter, H_2O_2 is degraded to water (H_2O) and molecular oxygen (O_2) by catalase. Glutathione peroxidase (GPx) also degrades H_2O_2 to H_2O (Thorin-Trescases et al, 2010). This has been supported by earlier works where it has been shown that the antioxidants are able to improve the deleterious effect of oxidant stress on vascular endothelial function in vitro and in vivo in animals (Aubin et al, 2006; Liu et al, 2007) and as well as in humans (Kanani et al, 1999; Holowatz et al, 2007).

1.3.2. Thromboxane A_2 & prostaglandin H_2 (T_XA_2 & PGH_2)

T_XA_2 and PGH_2 are generated subsequently to the metabolism of arachidonic acid (AA) by COX-1 and COX-2 in endothelial cells (Oates et al, 1988). PGH_2 is the precursor of the prostaglandins E_2 ($PGE_{2\alpha}$), F_2 ($PGF_{2\alpha}$) and T_XA_2. T_XA_2 is synthesised from PGH_2 by thromboxane synthase in platelets, and produced during platelet activation to promote platelet aggregation, vasocontraction, and smooth muscle proliferation. In smooth muscle cells, it causes an increase of intracellular Ca^{2+} levels via binding to thromboxane-prostanoid (TP) receptors to induce vasocontraction (Coleman, Smith et al. 1994; Vanhoutte and Tang 2008) (Figure 3). In normal vessels, the regulation of T_XA_2 and PGI_2 is important, and has essential but opposing functions in the maintenance of vascular homeostasis. Especially, the ratio of both seems to be more important than the absolute amounts of both in pulmonary vascular disease (Caughey et al, 2001).

1.3.3. Angiotensin II (Ang II)

In the endothelial cells there is angiotensin converting enzyme (ACE) which is responsible for the conversion of Ang I to Ang II. The vast pharmacological effects of Ang II observed are mediated through ATI receptors. Ang II is a multifunctional hormone responsible for regulation of blood pressure and cardiovascular homeostasis. It regulates endothelial function and stimulates inflammatory, proliferative, fibrotic and thrombotic processes in the vasculature. It has potent effects on vascular tone, constricts smooth muscle cells, regulates vascular cell growth, apoptosis, fibrosis, matrix metalloproteinase production and extracellular matrix degradation (Griendling et al, 1997; Tomita et al, 1998; Yoo et al, 1998). It is well-known that Ang II leads to vasoconstriction and structural changes (Gibbons 1997). These effects are observed often in hypertension, atherosclerosis and portal hypertension (Kane et al, 2010) (Figure 7). The Ang II is produced from the biologically inactive decapeptide, Ang I, the metabolic product of the liver-synthesized angiotensinogen and the enzyme renin. Ang I has a half-life of a few seconds, as it is quickly converted mostly by angiotensin-converting enzyme (ACE) to the biologically active octapeptide, Ang II. In addition, Ang I can be converted to the biologically active Ang (1–7) by plasma or neutral endopeptidases or to Ang (1–9) by ACE2 and subsequently to Ang (1–7). Ang II can also be converted to Ang (1–7) by plasma and neutral endopeptidases. There are three subtypes of Ang II receptors: type 1 (AT1R), type 2 (AT2R) and type 4 (AT4R). AT1R are localised on cardiomyocytes, vascular smooth muscle and endothelial cells, nerve endings and conductive

tissues. AT2R are present in endothelial and vascular smooth muscle cells and in fibrous tissue of the heart (Regitz-Zagrosek et al, 1998). Effects of each receptor activation are summarized in (Figure 7). Now it is recognized that, in addition to the classical renin-angiotensin system RAS pathway in which ACE generates the powerful vasoconstrictor Ang II, there is an alternate arm of the RAS through which Ang II is cleaved by ACE2 to Ang (1-7), which stimulate Mas-receptor (MasR) (G-protein-coupled receptor) leading to cause vasodilatation (Figure 7, Grace et al, 2013).

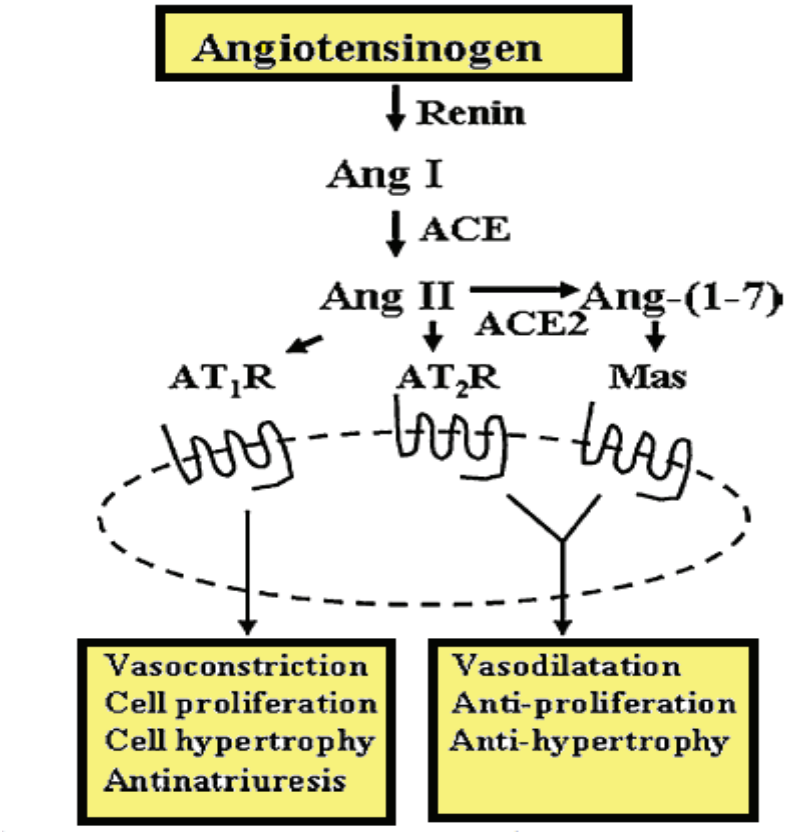


Figure 7. The renin-angiotensin system. (Grace et al, 2013)

Ang I , angiotensin I; ACE, angiotensin converting enzyme; Ang II, angiotensin II; ACE2, angiotensin converting enzyme type 2; AT1R, angiotensin receptor type 1; AT2R, angiotensin receptor type 2; Ang (1-7) , angiotensin (1-7).

1.3.4. Endothelin-1 (ET-1)

Endothelin (ET) are proteins that constrict the blood vessels and raise blood pressure. There are different ET isoforms expressed in the body, ET-1, ET-2 and ET-3 (Kedzierski and Yanagisawa 2001), the endothelial cells only release ET-1. ET-1 is produced by converting big ET-1 to ET-1 by the endothelin converting enzyme (Yanagisawa et al, 1988; Alonso and

Radomski 2003). Furthermore, the ET-1 production as well as its release is stimulated by pro-inflammatory cytokines such as interleukins and TNF-α and decreased by NO and PGI_2 (Alonso and Radomski 2003). ET-1 receptors subtypes have been identified both on endothelial cells (ET-$_{B1}$) and smooth muscle cells (ET-$_{A}$ and ET-$_{B2}$) (Sakurai et al, 1992). Moreover, the distribution of ET-1 receptors is dependent on the type of vascular bed : in veins ET-$_{A}$ to ET-$_{B}$ receptor ratio is reduced in comparison to arteries (Kedzierski and Yanagisawa 2001). Regarding the function of ET-1, when it binds to ET-$_{A}$ and ET-$_{B2}$ receptors, smooth muscle Ca^{2+} channels open allowing extracellular Ca^{2+} to enter into the cell. This causes vasoconstriction in a similar way as T_XA_2. Activation of ET-$_{B1}$ receptors on the endothelium causes vasodilatation by inducing the release of NO and PGI_2 (de Nucci et al, 1988;Cardillo et al, 2000). In endothelial dysfunction due to atherosclerotic arteries, endothelial cells ET-$_{B1}$ receptors are down-regulated, while smooth muscle cells ET-$_{B2}$ receptors are up-regulated, thus enhancing vasoconstriction (Dagassan et al, 1996; Sandoo et al, 2010; Bohm et al, 2002). In portal hypertension it has been reported from animal studies an over-production of ET-1 by the liver, which binds to ET-$_{B1}$ receptors which are up-regulated in the lung vascular endothelial cells, leading to an increase of eNOS expression and activity, and finally causing pulmonary vasodilation (Zhang et al, 2009).

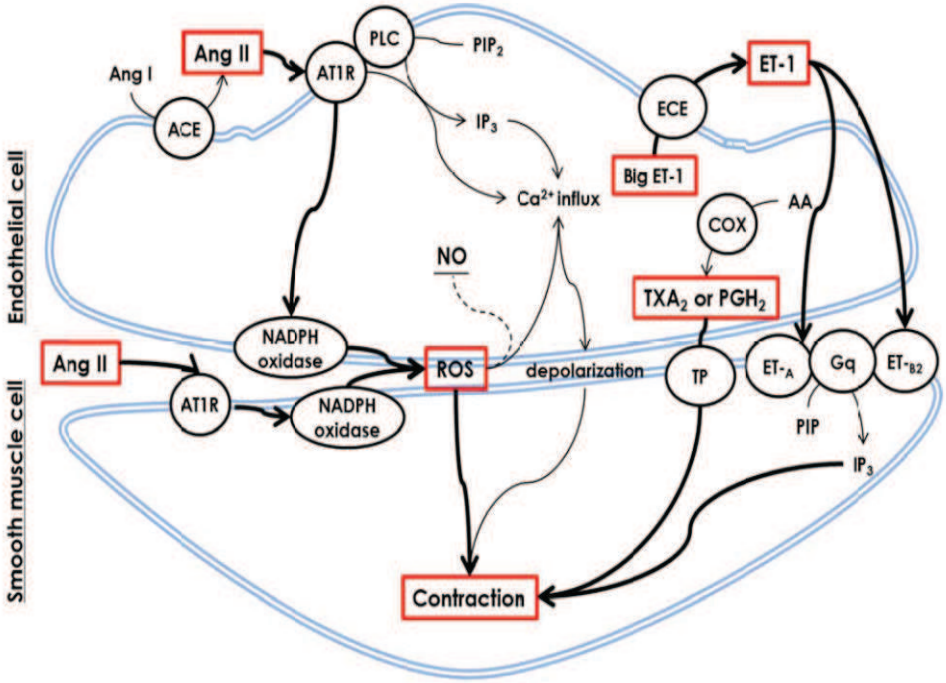


Figure 8. Pathways of endothelial derived contraction.

AA, arachidonic acid; ACE, angiotensin converting enzyme; Ang I, angiotensin I; Ang II, angiotensin II; ATR, angiotensin II receptor type I; Big ET-1, precursor of endothelin-1; ET-$_A$, endothelin receptor; ET-$_{B2}$, endothelin receptor; ECE, endothelin converting enzyme; ET-1, endothelin-1; Gq, Gq-protein; IP_3, inisitol triphosphate; PIP_2, phosphatidylinositol 4,5-biphophonate; PGH_2, prostaglandin H_2 ; ROS, reactive oxygen species ; TP, thromboxane prostanoid receptor; TXA_2, thromboxane A_2. Dash line represents an inhibitory effect.

CHPTER II

ENDOTHELIAL DYSFUNCTION AND PORTAL HYPERTENSION

Chapter two

2. Relationship between endothelial dysfunction and portal hypertension.

2.1. Introduction

Chronic liver diseases are characterized by a progressive vasodilatation, which is especially observed in the splanchnic and pulmonary beds. The vasodilatation is due to portal hypertension (PH) associated to either cirrhosis or extra-hepatic portal venous obstruction without cirrhosis (Abraldes et al, 2006).

Portal hypertension is a common clinical consequence of chronic liver disease which is associated with high morbidity and mortality rate (Hu et al, 2013). Portal hypertension is classified as either pre-hepatic, intra-hepatic or post-hepatic, with intra-hepatic PH being the form most frequently induced by cirrhosis, irrespective to the etiology (Buob et al, 2011). It has been reported that hepatic and non-hepatic endothelial dysfunction is a key factor that causes and worsens portal hypertension (Iwakiri and Groszmann 2007). In the hepatic microcirculation, hypoactive endothelial cells (ECs), contribute to an increase in intra-hepatic resistance mainly by decreasing production of nitric oxide (NO), which in turn leads to portal hypertension (Bosch 2007; Iwakiri and Groszmann 2006). Portal hypertension is associated with splanchnic and systemic and a hyperdynamic syndrome which in turn leads to an increase in portal vein blood flow further increasing portal hypertension. (Bosch 2007; Iwakiri and Groszmann 2006). While the endothelial cells are hypoactive in the intra-hepatic microcirculation, endothelial cells in the splanchnic and systemic circulation are hyperactive and have been shown to over produce NO. These two different phenotypic endothelial cells found in the intra- vs. extra-hepatic circulation contribute to the development and exacerbation of portal hypertension (Iwakiri and Groszmann 2007) (Figure 9).

An up-regulation of vascular endothelial growth factor (VEGF) and of endothelial nitric oxide synthase (eNOS) has been reported to be the initial event in portal hypertension (Abraldes et al, 2006). The mechanisms involved in the occurrence of the subsequent generalized vasodilatation and hyperdynamic syndrome are incompletely understood. Several lines of evidence support a role for an increased formation of nitric oxide (NO) in the occurrence of the general vasodilatation in chronic liver diseases in patients and also in the chronic bile duct ligated (CBDL) rat model (Nunes et al, 2001; Zhang et al, 2003; Chabot et al, 1996; Gomez et al, 2006). Other vasodilators have been reported to play a role: carbon monoxide (CO) produced as consequence of macrophage activation (Iwakiri and Groszmann

2006; Iwakiri and Groszmann 2007), EDHF (Cahill, Redmond et al. 2001; Heinemann and Stauber 1996; Mathie, Ralevic et al. 1996) may also be implicated and possibly prostacyclin.

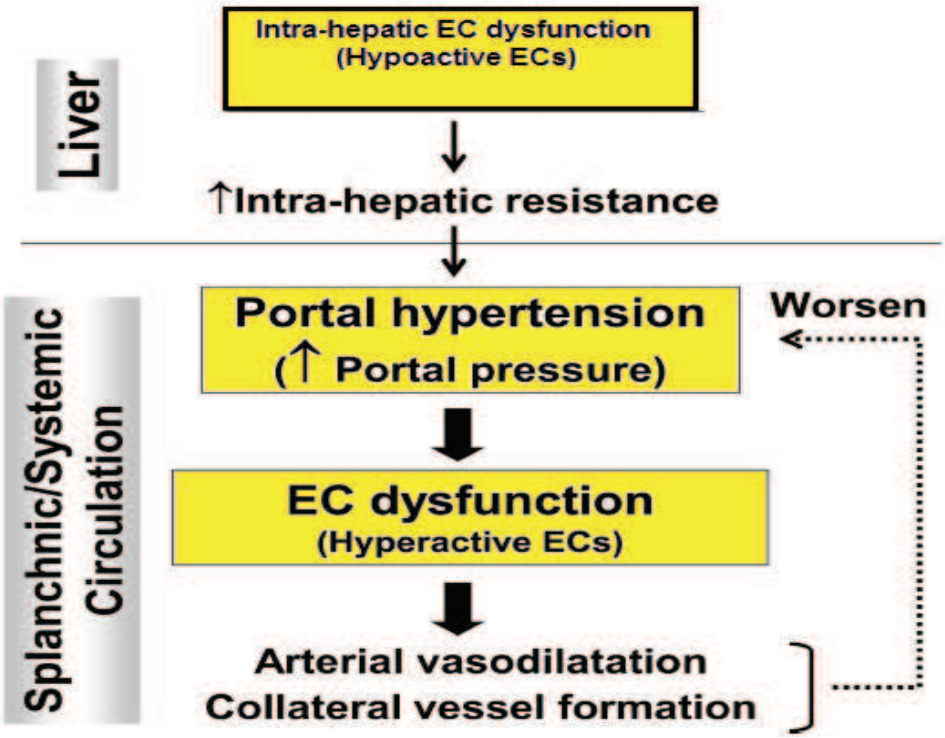


Figure 9. Overview of how portal hypertension develops. (Iwakiri 2012)

In addition to vasodilatation which has been related to the production of vasodilators (NO, CO, EDHF, prostacyclin) chronic activation of the renin-angiotensin-aldosterone system (RAS) has been observed in cirrhotic patients (Newby et al, 1998; Helmy et al, 2003) and in cirrhotic rats (Yang et al, 2002; Dal-Ros et al, 2010). Finally, chronic liver diseases are also characterized by an increased formation of reactive oxygen species (ROS).

Thus chronic liver diseases are characterized by an endothelial dysfunction related to a generalized vasodilation and a hyperdynamic syndrome with an increased cardiac output. The generalized vasodilation leads to complications and especially to a hepatopulmonary syndrome in approximately 10%-15% of patients awaiting liver transplantation (Rodriguez-Roisin et al, 2004). The hepatopulmonary syndrome (HPS) is characterized by arterial deoxygenation because of intrapulmonary vascular dilatation in the context of liver disorder. Its occurrence in patients with liver diseases is of prognostic value for their survival before and also after liver transplantation (Rodriguez-Roisin et al, 2004; Arguedas et al, 2003). It resolves in most cases with liver transplantation, but having an efficient approach to improve it is of great clinical importance because this could ameliorate survival or even delay liver

transplantation in some cases. Thus the aim of our studies was to assess treatments which could ameliorate endothelial dysfunction in a rat model of biliary cirrhosis.

2.2. Mechanisms of endothelial dysfunction in portal hypertension

2.2.1. Role of vasodilators

2.2.2. Nitric oxide

It has been suggested that NO has a major role in the pathogenesis of vasodilation and vascular hypocontractility associated with portal hypertension (Groszmann 1998). In a rat model of portal vein ligation leading to mild portal hypertension a significant increase in the expression of intestinal vascular endothelial growth factor (VEGF) with a subsequent increase in the expression of eNOS in the intestinal microcirculation (Abraldes et al, 2006) has been observed. The model of mild portal hypertension is most likely representative of the portal pressure changes observed in early stages of liver cirrhosis during which portal hypertension generally progresses slowly. Thereafter, with the worsening of portal hypertension, vasodilation in the arterial splanchnic circulation and hyperdynamic circulation occurs and other mechanisms such as shear stress may lead to an increased NO production. (Abraldes et al, 2006; Tsai et al, 2003; Iwakiri 2012). From animal studies, it has also been shown that an increase in the expression of eNOS occurs in the lung (Nunes et al, 2001). Pulmonary eNOS expression is closely related to the increased level of plasma ET-1. Over production of ET-1 occurs in the liver of cirrhotic rat. ET-1 binds further to the $ET\text{-}_B$ receptors whose expression is increased in the pulmonary vascular bed leading to an increase in eNOS expression and activity with subsequent pulmonary vasodilation (Ling et al, 2004). Thus in portal hypertension the general vasodilation has been in part related to the increased production of NO by eNOS in the splanchnic and the lung vascular beds.

Moreover, inducible NO synthase (iNOS) is also stimulated. This has been observed in the splanchnic circulation where iNOS is expressed by resident macrophages in rats with CBDL (Moreau et al, 2002; Morales-Ruiz et al, 1996). While the liver detoxification function is weakened, endotoxins and cytokines, especially TNF-α, can activate iNOS, leading to increased local or systemic NO level (Zhang et al, 2007; Umeda and Kamath 2009; Ferguson et al, 2006; Kajita et al, 2011; Theodorakis et al, 2003; Wei et al, 2005). Finally, it has also been demonstrated that intravascular lung macrophages probably play a key role in the

occurrence of the hepatopulmonary syndrome and in part by their ability to produce NO by iNOS stimulation (Thenappan et al, 2011).

Thus in portal hypertension an increased production in NO has been reported either related to the increased expression of eNOS and/or due to the stimulation of iNOS. Supporting this concept, Ferguson et al, observed that a selective iNOS inhibition by N-[3-(aminomethyl) benzyl] acetamidine, resulted in peripheral vasoconstriction in patients with cirrhosis (Ferguson et al, 2006). It has also been observed that cirrhotic patients with a hepatopulmonary syndrome had increased exhaled NO levels, a measure of pulmonary NO production, which normalized after orthotopic liver transplantation. In addition, methylene blue, an inhibitor of soluble guanylyl cyclase, has been reported to transiently improve hypoxemia in patients with hepatopulmonary syndrome (Tumgor et al, 2008; Zhang et al, 2005).

Thus from animal and human studies it has been reported that portal hypertension is related to increased production of NO thought to lead to a general vasodilation after the initial increased expression of eNOS in the intestinal microcirculation.

2.2.3. Prostacyclin

Shear stress (Topper et al, 1996) and pro-inflammatory substances (Marnett et al, 1999; Hong and Deykin 1982) are factors regulating in the local release of PGI_2**.** Few studies have been devoted to the possible role of an increased prostacyclin (PGI_2) level in the occurrence of hemodynamic abnormalities in cirrhosis. A pathogenesis role for PGI_2 in the hyperdynamic circulatory syndrome has been suggested in an experimental portal hypertension in rats (Munoz et al. 1999). In a rabbit model of chronic portal hypertension, elevated levels of PGI_2 have been observed probably due to an increased splanchnic production of the vasodilator (Sitzmann et al, 1994). In patients increased plasma levels of PGI_2 have been reported in cirrhotic patients with portal hypertension (Yin et al, 1995) as well as increased levels of 6-keto-PGF1α (a stable metabolite of PGI_2) in the plasma and the gastric mucosa of cirrhotic patients with portal hypertensive gastropathy (Ohta et al, 1995). Moreover, in the study done by Hou et al., in rats subjected to portal vein ligation increased plasma levels of PGI2 were observed along with an enhanced COX-1 (but not COX-2) expression in the superior mesenteric artery (Figure 10).

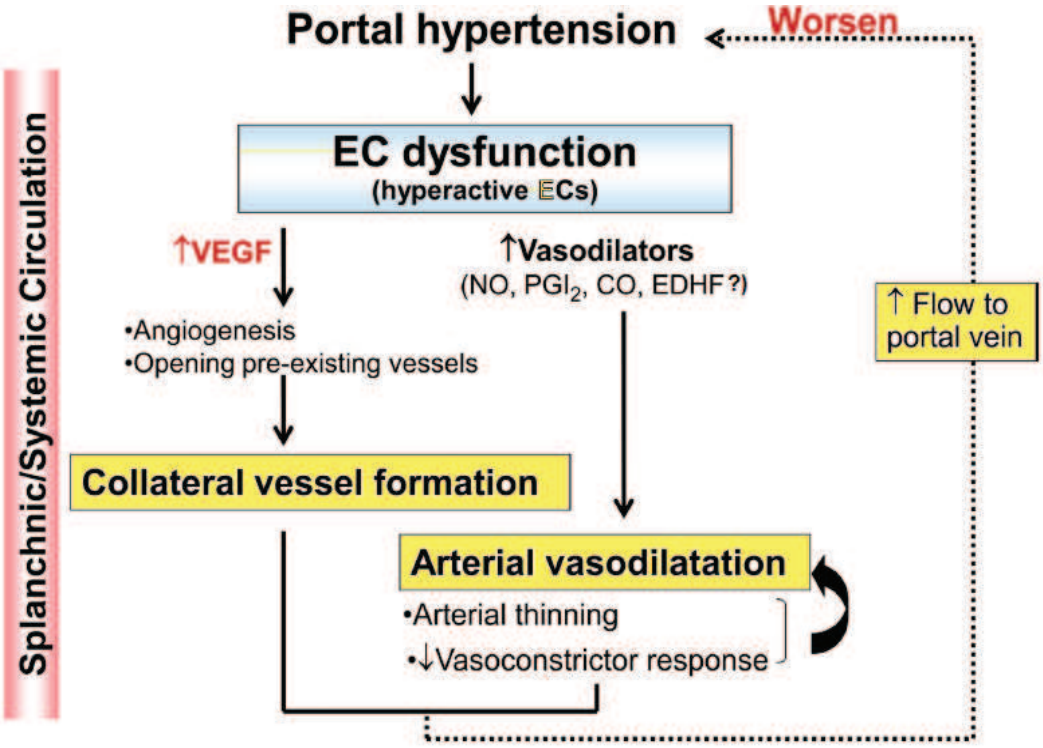


Figure 10. Overview of factors contributing to PH initiation and it is worsening. (Iwakiri 2012).

2.2.4. Carbon monoxide (CO)

CO is a vasodilator which has been shown to be increased in cirrhosis especially in the lungs of cirrhotic rats. The main pathway which produces CO is the heme metabolism. Heme is oxidized by the heme-oxygenase (HO) producing CO and biliverdin, the latter being reduced to bilirubin (Figure 11). Two isoforms of HO have been identified: HO-1 and HO-2. HO-1 is inducible by multiple agents like pro-inflammatory cytokines from inflammatory macrophage, whereas HO-2 is constitutively expressed (Maines et al, 1986). Like NO, CO is an endogenously produced gas molecule that activates soluble guanylyl cyclase, resulting in increased production of cGMP (Morita et al, 1995) and regulates vascular tone in a manner similar to NO (Morita and Kourembanas 1995; Cook et al, 1995). CO has a great affinity for hemoglobin, resulting in the formation of carboxyhemoglobin which was used by authors to measure the CO generation (De las Heras et al, 2003). A progressive increase in the expression of HO-1 in the aorta and mesenteric arteries of rats with biliary cirrhosis has been reported, whereas no change was observed in HO-2 expression. Moreover, the acute intra-peritoneal injection of zinc protoporphyrin, a selective inhibitor for HO to cirrhotic rats improved the hyperdynamic circulatory syndrome, suggesting the role of CO in arterial

vasodilatation in portal hypertension (Chen et al, 2004). It has been reported that HO-1 expression was increased in pulmonary intravascular macrophages (Zhang et al, 2003) in rats and an increased CO generation was observed in patients presenting with a hepatopulmonary syndrome compared with those without (Arguedas et al, 2005; Van Landeghem et al, 2009). Administration of HO-1 inhibitors partially improved vascular contraction to hypoxia, while administration of the NOS inhibitor, L-nitro-L-arginine methylester (L-NAME), brought HO-1 expression and vascular hypoxic vasoconstriction back to normal (Zhang et al, 2003; Carter et al, 2002). These evidences suggest that NO causes hypoxic contraction insensitivity, and induces the production of HO-1, thereby enabling an increase in CO production, which synergistically contributes to the pathogenesis of hepatopulmonary syndrome. The endogenous NOS/NO and HO-1/CO systems may relatively independently or synergistically contribute to the pathogenesis of hepatopulmonary syndrome (Umeda and Kamath 2009; Carter et al, 2002).

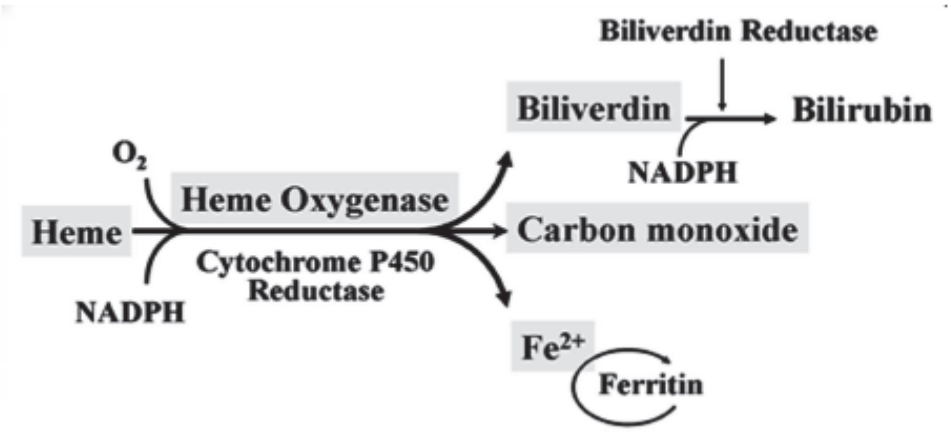


Figure 11. Production of CO by the enzymatic activity of Heme-oxygenase. (Araujo et al, 2012)

2.2.5. EDHF

Role of EDHF in the occurrence of vasodilation and hemodynamic abnormalities was suggested in rats with biliary cirrhosis (Barriere et al, 2000). These studies showed an EDHF-dependent inhibition of contraction in mesenteric arteries (Barriere et al, 2000) and in an isolated perfused lung (Carter et al, 2002). It has been reported that in normal resistance artery there may be a balance between EDHF and NO, with for example an up-regulation of EDHF-dependent relaxation in eNOS knockout mice (Waldron et al, 1999) or a decrease in EDHF (Bauersachs et al. 1996). On the contrary to these previous studies we find both a decreased

NO- and EDHF –dependent relaxation in our studies and this will be discussed in chapter four.

2.2.6. Intestinal endotoxemia and role of monocyte /macrophage activation

Bacterial translocation (BT) is defined as the migration of viable micro-organsims or bacterial endotoxins from the intestinal lumen to mesenteric lymph nodes and other extra-intestinal sites (Wiest et al, 2003). BT has been postulated as an important mechanism in the development of portal hypertension and hyperdynamic circulatory state (HCS) (Pinzone et al, 2012). The hyperdynamic circulatory state occurs as a consequence of splanchnic and systemic vasodilatation which leads to the subsequent activation of compensatory mechanisms (i.e., renin-angiotensin aldosterone, sympathetic nervous system, and anti-diuretic hormone) with an increased plasma volume and cardiac output. The hyperdynamic circulatory state contributes further to the worsening of portal hypertension, and is associated with systemic vascular complications of cirrhosis (Garcia-Tsao and Wiest 2004). The consequence of BT is an increased production of endotoxins, which results in an increase in inflammatory cytokines thought in part to stimulate iNOS in vessel walls, causing NO overproduction, vasodilatation and hyperdynamic circulatory state (Vallance and Moncada 1991). Among the inflammatory cytokines, TNF-α plays probably a role in the development of hyperdynamic circulatory state as suggested by a study by Lopez-Talavera, Cadelina et al. (1996) in which the inhibition of TNF-α ameliorated hyperdynamic circulatory state in cirrhotic rats. Moreover, in addition to BT, there is evidence of monocyte/macrophage activation in studies of liver cirrhosis and portal hypertension in experimental animals (Sztrymf et al, 2005; Zhang et al, 2007; Zhang et al, 2005; Neugebauer et al, 2008; Lopez-Talavera et al, 1996). Endotoxin induces and activates the monocyte/macrophage system *in vivo*, including liver Kupffer cells (specialized liver macrophages), splenic macrophages, pulmonary intravascular macrophages (PIMs), and blood mononuclear cells (Li et al, 2004; Gill et al, 2008). As far as the cirrhotic liver losses the bacterial detoxification capacity and due to portosystemic shunt the bacteria and toxins probably pass to lung in compensation. Subsequently, blood mononuclear cells, accumulate in the pulmonary vascular endothelium, and then differentiate into pulmonary intravascular macrophages (Zhang and Yang 2010). The function of pulmonary intravascular macrophages phagocytosis is enhanced to remove the bacteria and toxins from the blood. Upon presence of foreign bodies macrophages are activated and secrete a variety of active substances such as NO, CO, TNF-α, IL-1, IL-6, IL-8,

and other inflammatory mediators. These mediators play an important role in the occurrence and development of hepatopulmonary syndrome and hyperdynamic circulatory state. It has been shown, especially in the lung of cirrhotic rats that the administration of norfloxacin (an antibiotic) inhibits intestinal BT and reduces endotoxin injury to the lung and improves the symptoms of hepatopulmonary syndrome and hyperdynamic circulatory state (Wang et al, 2007; Wiest et al, 1999). Moreover, inhibiting TNF-α by pentoxifylline has been reported to reduce the number and activity of pulmonary intravascular macrophages, and to improve hepatopulmonary syndrome in cirrhotic rats along with decreased pulmonary intravascular macrophages adhesion and phagocytosis and iNOS expression in lung tissue (Sztrymf et al, 2004, Figure 12).

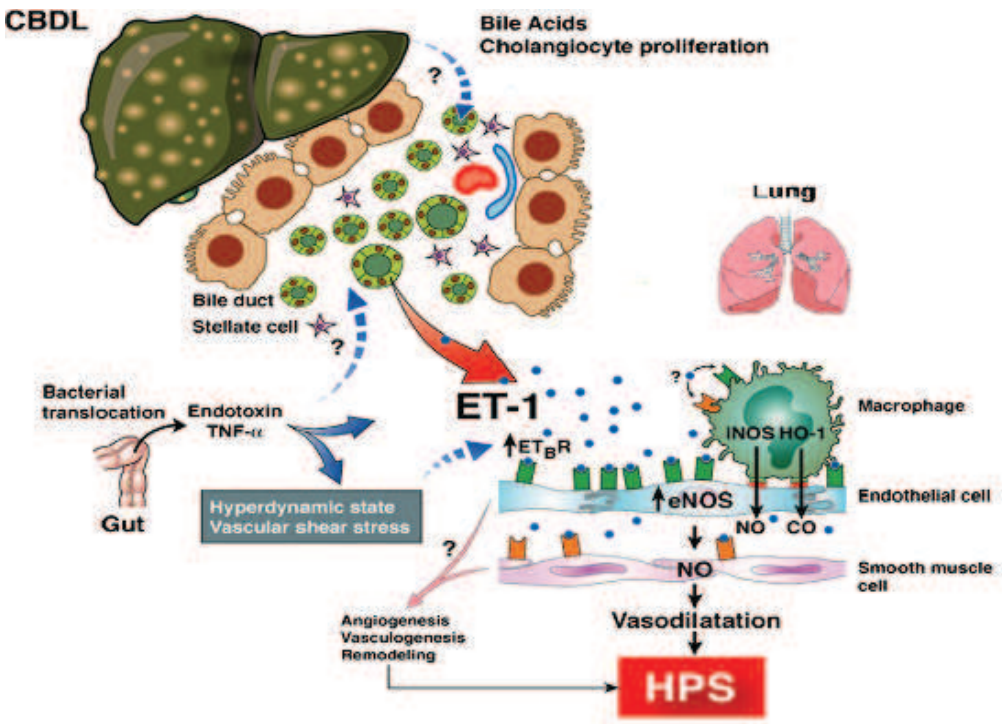


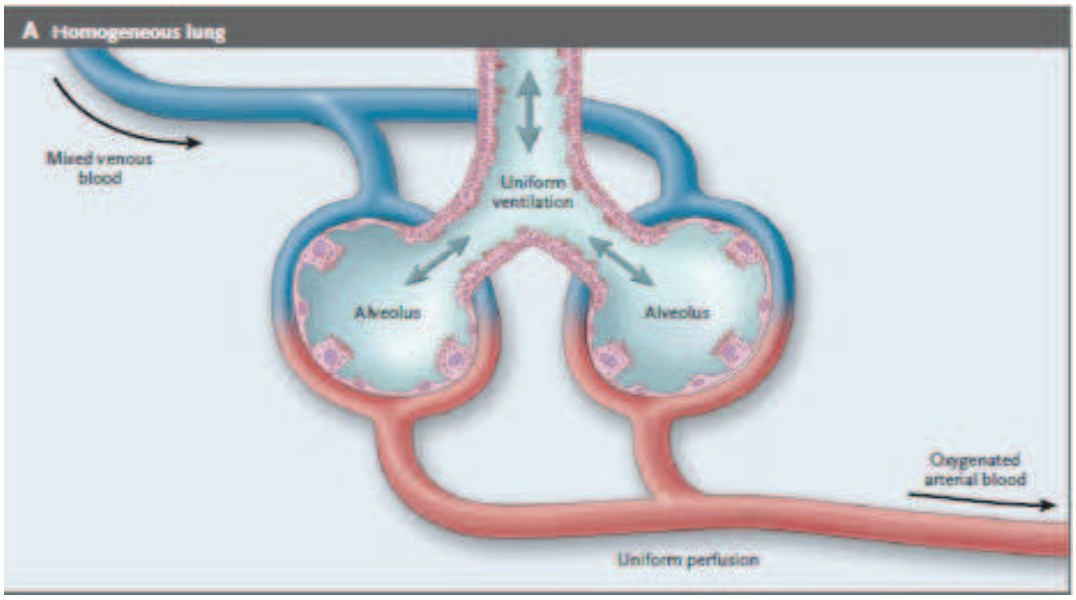
Figure 12. Potential mechanisms and target therapeutic options in hepatopulmonary syndrome (HPS) and hyperdynamic circulatory state. (Palma and Fallon 2006)

ET_BR= β endothelin receptor, ET_AR = α endothelin receptor

2.2.7. Angiogenesis in portal hypertension

In addition to the arterial vasodilatation in the splanchnic and systemic circulation, the development of collateral vessels as an adaptive response to an increase in portal pressure leads to worsening of portal hypertension and its complications (Bosch 2007). These

collateral vessels are formed by the enlargement of pre-existing vessels and by angiogenesis (Sumanovski et al, 1999). The process of angiogenesis is modulated by growth factors like VEGF. Studies using portal hypertensive rats by Abraldes et al. and Fernandez et al., reported that a sudden increase in portal pressure is sensed at the intestinal microcirculation and induces VEGF expression (Abraldes et al, 2006; Fernandez et al, 2005). Furthermore, anti-angiogenic agents such as blockers of VEGF receptor-2 and inhibitors of tyrosine kinases (Sorafenib and Sunitinib) lead to decrease in collateral circulation and reduce portal hypertension (Fernandez et al, 2004; Mejias et al, 2009). More recently, angiogenesis, has been attributed to both intrahepatic and extrahepatic component of portal hypertension (Thabut and Shah 2010). Angiogenesis has also been reported in the lung of experimental hepatopulmonary syndrome (Figure 13) (Zhang et al, 2009). In this latter study, increased numbers of lung microvessels, increased lung monocytes accumulation and activation of Akt and eNOS have been observed after CBDL in rats. Pentoxifylline treatment reduced the number of microvessels, lung monocytes accumulation, down-regulated pulmonary angiogenic factors, and improved the symptoms of hepatopulmonary syndrome (Zhang et al, 2009; Gill et al, 2008; Sztrymf et al, 2004). In addition, angiogenesis has been reported to be stimulated via VEGF-dependent pathways (Zhang et al, 2009) and especially VEGF-A (Zhang et al, 2009) produced in alveolar macrophages and blood vessel endothelial cells. VEGF angiogenic signaling occurs through interaction with specific VEGF receptors (VEGFR-1 and VEGFR-2). Subsequently, VEGFR-2 activation triggers downstream signaling in part through Akt and eNOS (Zhang et al, 2009).



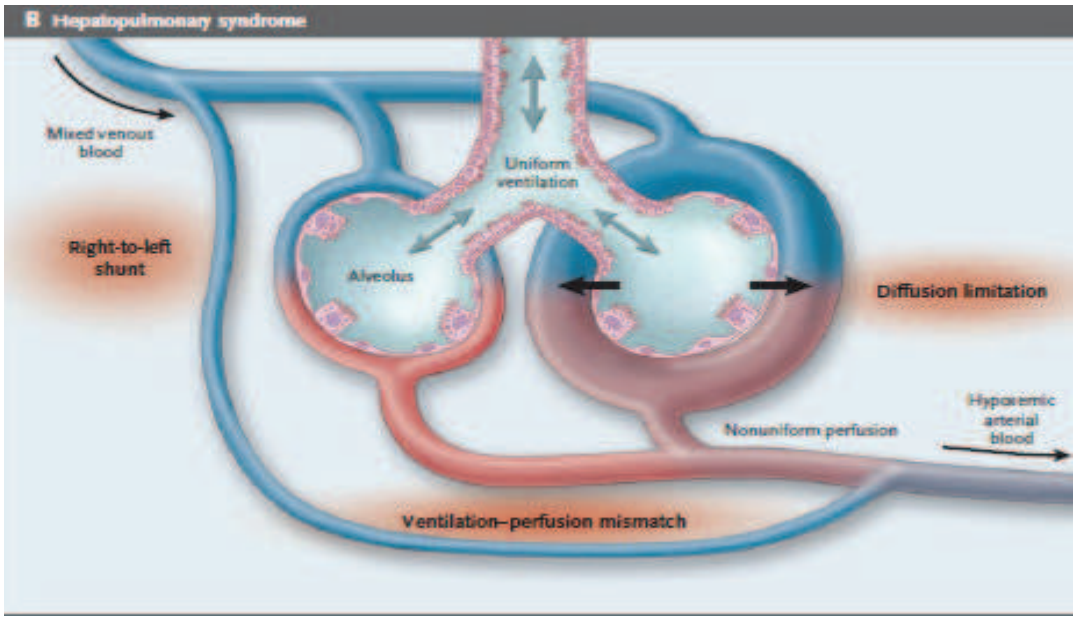


Figure 13. Role of angiogenesis in the pathology of HPS. (Rodriguez-Roisin and Krowka 2008)

2.2.8. Oxidative stress

Oxidative stress plays a role in the development of endothelial dysfunction in liver diseases in patients and in the CBDL rat model. Patients with cirrhosis have elevated level of circulating malondialdehyde (MDA), an indicator of oxidative stress (Hernandez-Guerra et al, 2006). In the intra-hepatic endothelial cells of the cirrhotic liver, there is an increased production of the superoxide anions ($O_2^{\bullet -}$), which reacts with NO to form peroxynitrite ($ONOO^-$). Thus, the bioavailability of intra-hepatic NO is decreased (Gracia-Sancho et al, 2008), favoring vasoconstriction which leads to an increased vascular resistance as an initiating step in portal hypertension (Iwakiri 2012). It was demonstrated that administration of vitamin C as an antioxidant to cirrhotic patients markedly attenuates portal pressure, and significantly reduces MDA levels, suggesting that increased oxidative stress in cirrhotic patients contributes to portal hypertension (Hernandez-Guerra et al, 2006). Furthermore, in CBDL- as well as partial portal vein ligated rats, N-acetylcysteine prevented the development of the hyperdynamic circulation. Moreover, by the using of antioxidant therapy a reduction in the increased urinary F_2-isoprostanes which were measured as a marker of oxidative stress, and increased SOD activity measured in lung homogenates have been observed (Fernando et al, 1998; Vercelino et al, 2008).

2.2.9. Renin-angiotensin system in portal hypertension

Up to date, the current understanding is that there is activation of the classical pathway of renin-angiotensin system (RAS) in patients with advanced liver disease and portal hypertension in response to vasodilatation and systemic hypotension (Schrier et al, 1988). Angiotensin II (Ang II), the main effector of the RAS, regulates key steps in the tissue remodeling process through angiotensin type 1 (AT1R) receptors (Mezzano et al, 2001; Mezzano et al, 2001). Recent research showed that, there is hepatic up-regulation of RAS, mediating fibrosis and stellate cell contraction in response to oxidative stress and systemic activation of RAS (Bataller et al, 2005; Grace et al, 2013; Asbert et al, 1992; Paizis et al, 2002; Bataller et al, 2003). The blockade of Ang II synthesis or its binding to AT1 receptors markedly ameliorates hepatic fibrosis in rats with CBDL (Ramalho et al, 2002; Yoshiji et al, 2002; Paizis et al, 2001). In an earlier study performed in our laboratory the angiotensin II receptor 1 (AT1) antagonist losartan improved endothelial dysfunction possibly by its antioxidant effect (Dal-Ros et al, 2010).

CHAPTER III

POLYPHENOLS

Chapter three

3.1. Polyphenols

Polyphenols, constitute of group of natural products, represent a secondary metabolite of plants and that have a great variability of chemical structures. One of the fundamental structural element is the presence of hydroxyl groups which can be found in free state or bound to other forms such as; ether, ester or heteroside. The polyphenols are abundant in vegetables, and are important part of human and animal nutrition. We can find polyphenols in large quantity in red wine, also in fruits, cereals, chocolate, legumes and the drinks like tea, and coffee (Bravo 1998).

3.2. Classification of polyphenols

Polyphenols composed of multiples hydroxyl groups (-OH) found on aromatic rings related to benzene (phenyl C6H5). They are mainly classified according to the chemical structure of the aglycons (the polyphenol deprived from its sugar moieties) into flavonoids, phenolic acids, stilbenes, and lignans.

Flavonoids; flavonoids are further divided into many subclasses: anthocyanins, flavan-3-ols (as monomers forming catechins and as polymers forming proanthocyanidins (condensed tannins), flavones, flavanones, flavonols, isoflavones, neoflavonoids and chalcones. They consist of a C6-C3-C6 backbone (Figure 14).

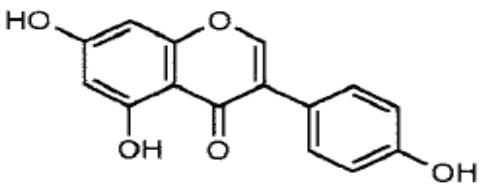
Anthocyanidins have a basic structure, known as flavylium cation, which contains positive charge in the C-ring, and by this positive charge it is distinguished from other flavonoids. In nature, highly unstable anthocyanidins are protected from degradation by glycosylation (anthocyanidins with sugar group(s) are called anthocyanins) generally with a number of sugars, in particular glucose, sophorose, rutinose, galactose, arabinose and xylose at different positions of the C-ring. Anthocyanins are water soluble pigments, providing the distinctive and vibrant palette of colors (red, blue and purple) found in fruits, vegetables, flowers, and other plant tissues or products.

Anthocyanins are widely distributed in human diet including red wine, certain varieties of cereals and certain vegetables (cabbage, beans, onions and radishes), but most abundant especially in fruit like berries. There are six main anthocyanidins distributed

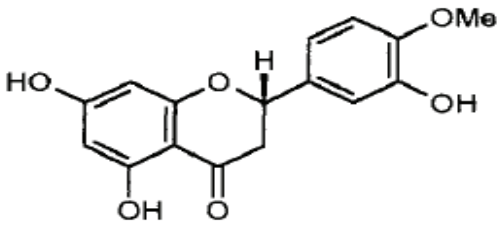
throughout the plant kingdom: cyanidin, malvidin, delphinidin, peonidin, petunidin and pelargonidin. Among them cyanidin is the most common anthocyanidin in food. Food contents of anthocyanins are generally proportional to color intensity and increase as the fruit ripens up to 2-4 g/kg fresh wt in blackcurrant or blackberries (Manach et al, 2004).

Phenolic acids are further divided into benzoic acid and cinnamic acid and consisting of a C1-C6 and C3-C6 backbones, respectively. They contain protocatechuic acid, vanillic acid, gallic acid and syringic acid as examples of phenolic acids in the former; and *p*-coumaric acid, caffeic acid, chlorogenic acid, cryptochlorogenic acid, neochlorogenic acid, ferulic acid and sinapic acid in the latter (Figure 14).

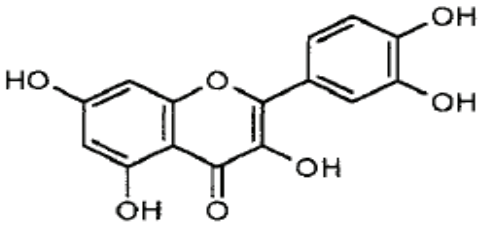
Stilbenes are diphenylethene barely present in human diet, a main member is resveratrol. Lignans consists of two phenylpropane units. This group of polyphenol is further metabolized by the intestinal microflora into enterodiol and enterolactone (Figure 14).



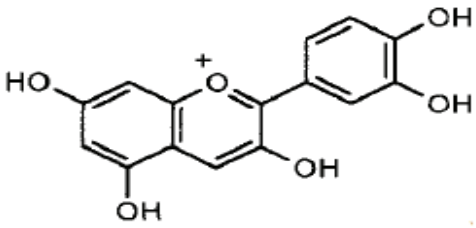
genistein
(isoflavone)



hesperetin
(flavanone)



quercetin
(flavonol)



cyanidin
(anthocyanidin)

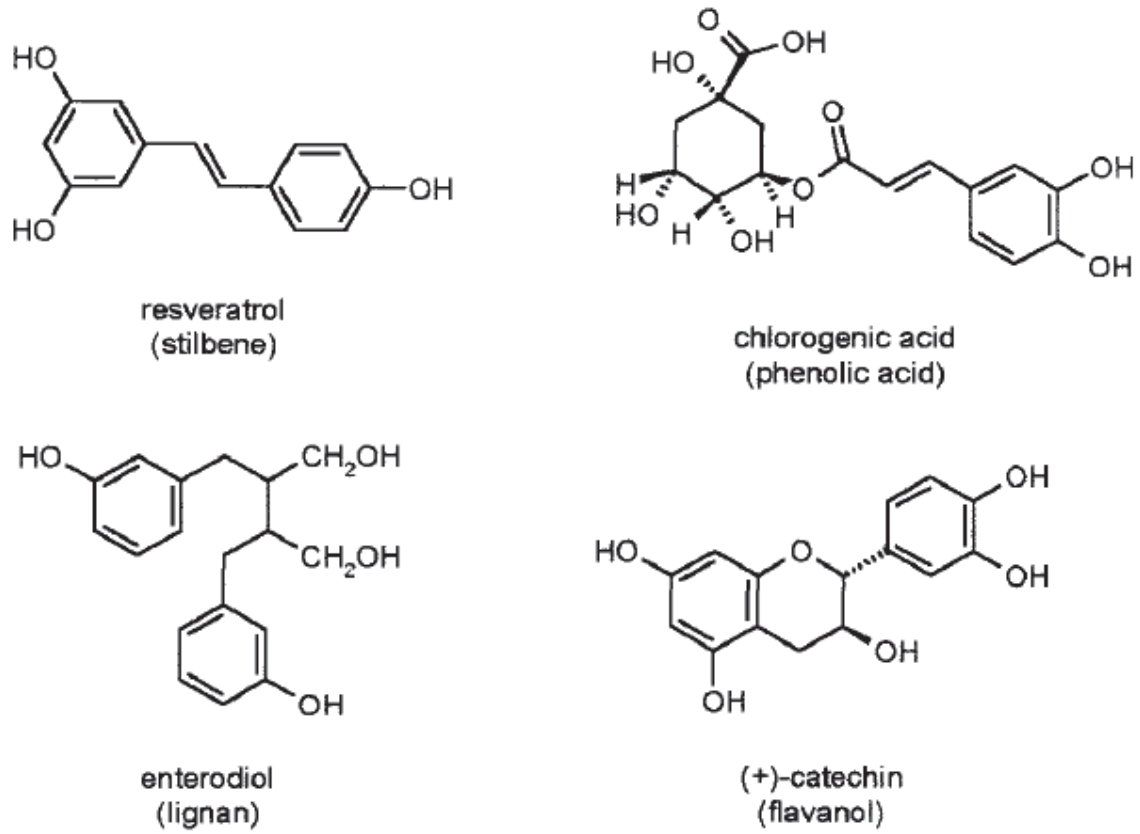


Figure 14. Structures of some polyphenols (Manach et al, 2004).

3.3. Absorption, metabolism and excretion of polyphenols

The metabolism of polyphenols is not yet well clarified. However, several studies were conducted in an effort to understand the metabolic and absorption processes of polyphenols *in vivo*.

In this respect, the metabolism of flavonoids is the most studied. This group is mainly characterized their presence in a glycosylated form (Crozier et al, 2000).

However, these flavonoids undergo deglycosylation in the intestinal lumen by lactase phlorizin hydrolase, an enzyme found in the brush border of the small intestine, yielding free aglycones. The latter compounds are hydrophobic and according to their solubility coefficients in the lumen, they can diffuse through the intestinal epithelial cells either passively or by facilitated diffusion (Azuma et al, 2002; Sriram and Abraham 2000). However, flavonoids can also be absorbed in the enterocytes of the small intestine via

membrane transporters such as sodium/D-glucose cotransporter 1 (SGLT1) without occurrence of any metabolism changes. Once within the cell, deglycosylation takes place by the action of cytosolic β-glucosidase to release aglycones (Day et al, 1998). Within the small intestine, most flavonoids undergo conjugation mainly by glucoronosyl transferases (UGTs) to form glucouromide conjugates. In small intestine models, such conjugate forms seem to be important for flavonoids to pass from the mucosal compartment to the blood compartment (Andlauer et al, 2000). Nevertheless, flavonoids can undergo further metabolic steps characterized by methylation or the replacement of glucoronide residues with sulfate groups, since the presence of both form were reported in peripheral blood (Donovan et al. 2001). These processes were shown to take place in the hepatic cells of the liver and to play a role in the stabilization of flavonoids (Monfardini and Veronese 1998; O'Leary et al, 2003). However, all conjugate flavonoids are then exported into the bile and eventually reenter the small intestinal lumen. At this stage, if flavonoids fail to undergo deconjugation steps, they will be transported to the colon.

The colon microorganisms have catalytic potential. Thereby, flavonoids could undergo deglucuronidation and subsequent sulfation. The resulting flavonoids are then reabsorbed thus leading to a continuous cycle referred to as the enterohepatic circulation (Franke et al 1998) Flavonoids are then delivered to different tissues by blood. Within tissues, flavonoids will be further deconjugated by the actions of β-glucuronidase and sulfatase activities. However, regarding anthocyanin the major polyphenolic composant of blackcurrant juice we used in our study, Passamonti et al., have proposed that the intact glycosides are the major circulating forms of anthocyanins, and the glycosides of anthocyanins may be transported by bilitranslocase at the gastric level, because the glycosides are good ligands for this carrier (Passamonti et al, 2002). Some more recent studies show that, in rats and mice, anthocyanins are absorbed from the stomach (Passamonti et al, 2005; Matuschek et al, 2006).

The excretion of flavonoids in urine varies following the type of flavonoids, but the yield from urine cannot be considered as a useful indicator of their bioavailability.

Other subtypes of polyphenols undergo similar metabolic pathways with some differences. For instance, unlike flavonoids, catechins and proanthocyanidins are mainly unglycosylated and therefore cannot get absorbed in the stomach but reach the small intestine intact (Rios et al, 2002). Figure 15, shows an example of the pharmacokinetic properties of the flavanol.

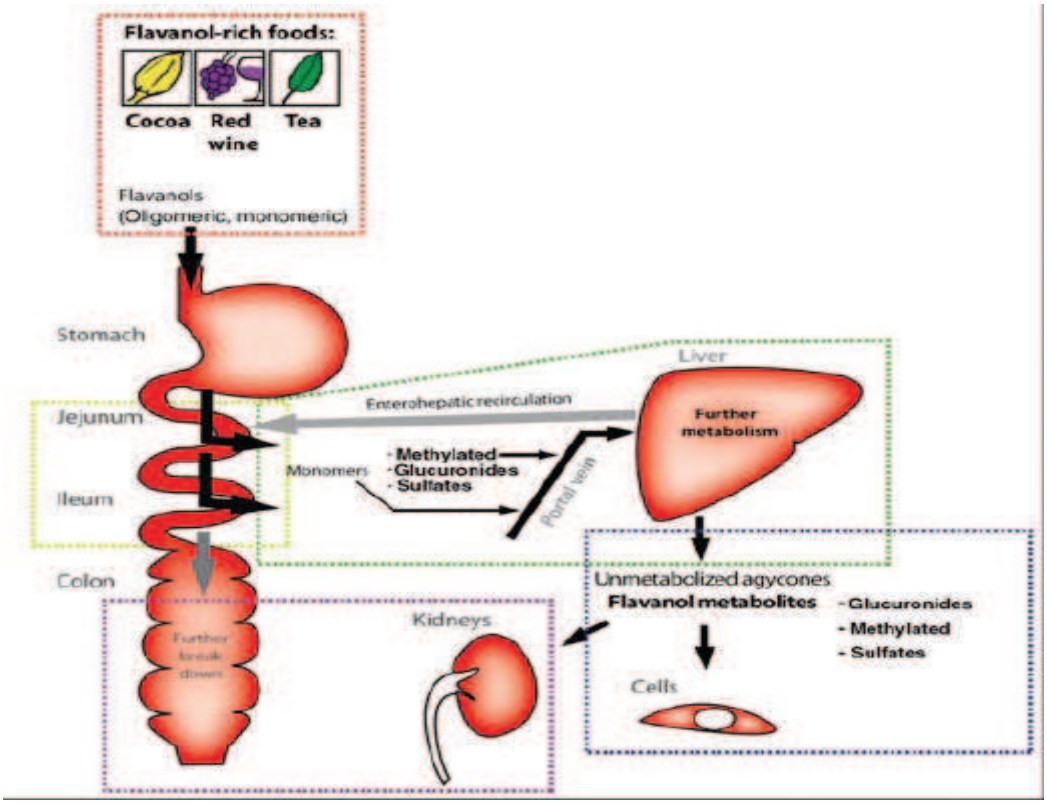


Figure 15. Schematic of pharmacokinetically relevant flavonol features pertaining to the composition in foods, absorption, metabolism, distribution and excretion. This can be applied to other flavonoids with exception some flavonoids are present in the form of glycosides, ester, or polymers that cannot be absorbed in their native form. Most of the glycosides probably resist acid hydrolysis in the stomach and thus arrive intact in the intestine (Heiss et al, 2010).

3.4. Beneficial effect of polyphenols in vascular function

Polyphenols are well known to protect the cardiovascular system by a variety of actions, including their ability to improve the lipid profile; dilate blood vessels by stimulating the endothelial formation of NO, and also in some blood vessels EDHF, and by their antioxidant properties, which will prevent the degradation of NO by superoxide anions (Ndiaye et al, 2003; Frankel et al, 1993; Fitzpatrick et al, 2000; Madeira et al, 2009; Mann et al, 2007). In addition, polyphenol-rich sources such as a red wine extract have been shown to reduce systolic blood pressure and to improve endothelial dysfunction in several experimental models of hypertension (Sarr et al, 2006; Lopez-Sepulveda et al, 2008; Madeira et al, 2009; Bernatova et al, 2002; Lee et al, 2011). Altogether, these findings support the view that polyphenol-rich natural products may protect the cardiovascular system, in part, by improving

the endothelial function and, hence, retarding the development of cardiovascular diseases. Polyphenols and tea polyphenols inhibit the expression of several NADPH oxidase subunits including NOX 1 and p22phox (Yu et al, 2010; Dal-Ros et al, 2011), and tea-polyphenols upregulated the expression of catalase in vascular cells and the arterial wall (Stangl et al, 2007). Atherosclerosis is characterized by the development of plaques which predominantly occur at the aortic roots, arch and abdominal aortas, and around the renal artery branches (Nakashima et al, 1994). In addition, endothelial cells at sites of atherosclerotic plaques have been shown to have high level of inflammatory responses (Feletou et al, 2010). Previous studies have shown that natural products such as a garlic extract and a grape seed extract decrease atherosclerotic plaque size in animal models (Jimenez et al, 2007; Chang et al, 1993).

3.5. The blackcurrant and its health benefit

The blackcurrant (Ribes nigrum L., Grossulariaceae), a small, perennial shrub native to central Europe and northern Asia, is cultivated throughout the world, including the United States. Modern laboratories have demonstrated the potent anti-inflammatory, antioxidant and antimicrobial effects of blackcurrant constituents on a myriad of disease states. The properties of the blackcurrants are conferred from its biochemical constituents, some of which include anthocyanins (specifically delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside), flavonols, phenolic acids and polyunsaturated fatty acids (Gopalan et al, 2012, Figure 16).

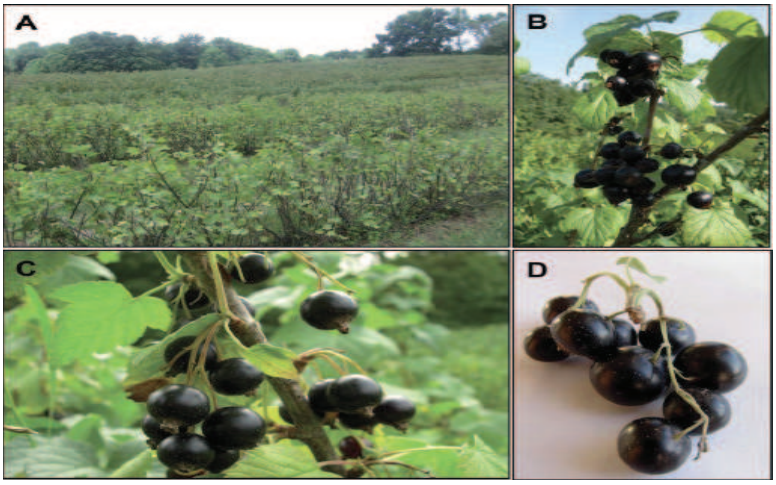
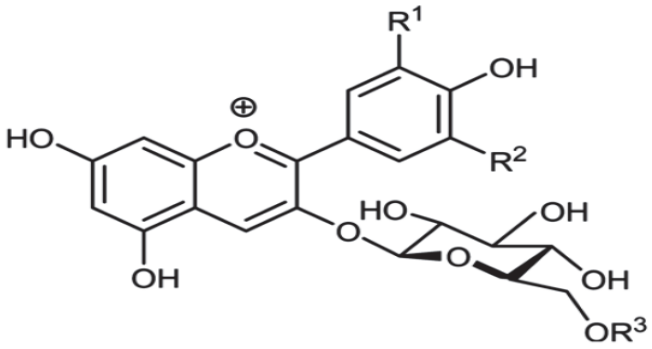


Figure 16. Various photographs of blackcurrants (Ribes nigrum) showing a commercial blackcurrant plantation (A), shrub with fruits (B and C), and isolated fruits (D) (Gopalan et al, 2012).

3.6. Phytochemical constituents of blackcurrant

Biochemical profiling of blackcurrants has revealed a myriad of constituents: flavonoids, polyunsaturated fatty acids (PUFA), structural and nonstructural carbohydrates, non-volatile organic acids, tannins and stilbenoids (Bordonaba and Terry 2008; Jaworska et al, 2011; Tabart et al, 2011). Blackcurrant flavonoids, a group of polyphenolic compounds with a diphenylpropane skeleton, include anthocyanins and flavonols (Mikkonen et al, 2001). Blackcurrants are an important source of anthocyanins with concentrations up to four-fold greater than other common fruits. Studies in various genotypes of blackcurrants reveal a great variation in the anthocyanin content of 80–280 mg per 100 g of fruit with maximum levels observed up to 300 mg per 100 g of fruit (Nour et al, 2013; Moyer et al, 2002). The aglycones (anthocyanidins) are salt derivatives of the 2-phenylchromenylium (flavylium) cation. Its positive charge differentiates anthocyanidins from other flavonoids. Anthocyanins are sugar derivatives of anthocyanidins. Four major anthocyanins have been identified in the blackcurrant: delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside (Figure 17) (Maatta et al, 2003; Delazar et al, 2010). Among PUFAs, g-linolenic acid (Figure 18) is an essential polyunsaturated fatty acid found in blackcurrants. g-linolenic acid has been isolated from the blackcurrant seed oil (Watson et al, 1993; Leventhal et al, 1994).



Compound	R^1	R^2	R^3
Delphinidin-3-*O*-glucoside	OH	OH	H
Delphinidin-3-*O*-rutinoside	OH	OH	rhamnosyl
Delphinidin-3-*O*-(6″-coumaroylglucoside)	OH	OH	coumaroyl
Delphinidin-3-*O*-sophoroside	OH	OH	glucosyl
Cyanidin-3-*O*-glucoside	OH	H	H
Cyanidin-3-*O*-rutinoside	OH	H	rhamnosyl
Cyanidin-3-*O*-sophoroside	OH	H	glucosyl
Cyanidin-3-*O*-(6″-coumaroylglucoside)	OH	H	coumaroyl
Petunidin-3-*O*-glucoside	OMe	OH	H
Petunidin-3-*O*-rutinoside	OMe	OH	rhamnosyl
Pelargonidin-3-*O*-glucoside	H	H	H
Pelargonidin-3-*O*-rutinoside	H	H	rhamnosyl
Peonidin-3-*O*-glucoside	OMe	H	H
Peonidin-3-*O*-rutinoside	OMe	H	rhamnosyl
Malvidin-3-*O*-glucoside	OMe	OMe	H
Malvidin-3-*O*-rutinoside	OMe	OMe	rhamnosyl

Figure 17. Chemical structures of the major anthocyanins present in blackcurrants. (Gopalan et al, 2012)

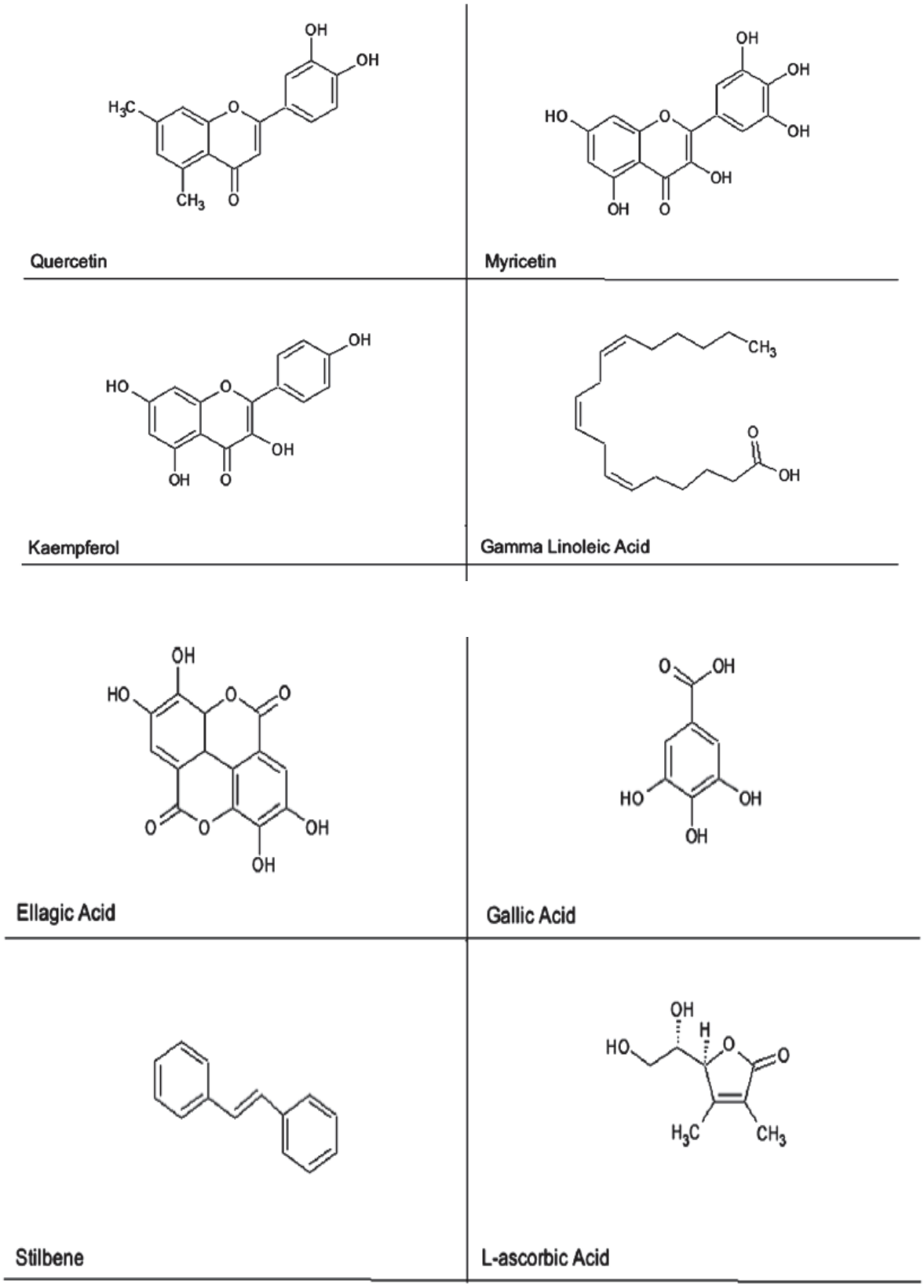


Figure 18. Chemical structures of the major phytoconstituents present in blackcurrants. (Gopalan et al, 2012)

3.7. Antioxidant properties

Multiple studies support the antioxidant ability of blackcurrants (Rubanyi, Ho et al. 1991). Data from additional studies demonstrate the degradation of hydrogen peroxide (H_2O_2) and NO, as well as impeding the propagation of lipid and protein peroxidation (Yoshiki et al, 2001; Viljanen et al, 2004). Furthermore, antioxidant enzymes, such as glutathione (GSH) peroxidase and superoxide dismutase, were also shown to increase significantly by the blackcurrant components (using commercially available antioxidant status kit) through an unknown mechanism (Nielsen et al, 2003). The specific antioxidant capacities of the blackcurrant fruit as a whole are mostly attributed to its phenolic and anthocyan content (Moyer et al, 2002; Ehala et al, 2005). This content can vary widely depending on the cultivar, season it was grown in, state of ripening and the particular part of the plant used (Tabart et al, 2006). Although the phenolic content is considered to contribute a more potent antioxidant activity than natural vitamins, high levels of vitamin C confers the additional inherent antioxidant properties to blackcurrants (Mikkonen et al, 2001; Borges et al, 2010). Following oral consumption, the antioxidant activity of anthocyanins in blackcurrants is affected by the pH of its environment, potentially due to a shift in its prototropic equilibrium (Estevez et al, 2010). Free radical scavenging activity was noted to attain maximum levels at a pH between 6.0 and 7.0, slightly more acidic than human serum (Matsumoto et al, 2002); (Young et al, 1999). This suggests that the anthocyanin antioxidant activity potentially varies depending on its location in the human body. Studies involving human subjects drinking blackcurrant juice revealed an increase in serum sulfahydryl group levels within two hours of consumption (Gopalan et al, 2012). Additional in vitro studies quantified a 94% inhibition of copper ion-induced low density lipoprotein (LDL) oxidation (Rosenblat, Volkova et al. 2010).

CHAPTER IV

AIM OF THE STUDY

Chapter four

4.1. AIM OF THE STUDY

Endothelial dysfunction is well-known to occur in portal hypertension following liver cirrhosis inhuman and/or experimental animals. It is characterized by impaired endothelium-dependent relaxation in studies using animal models of liver cirrhosis (Dal-Ros et al, 2010). A main cause of the endothelial dysfunction is an imbalance between endothelium-derived vasorelaxing factors (e.g., NO, EDHF, and PGI_2), and endothelium-derived vasocontracting factors (e.g., ROS, TXA_2, Ang II, and PGH_2).

1- As recent studies suggested that abnormal intestinal flora (pathogenic) contributes to a lot of deleterious complications of chronic liver diseases by enhancing intestinal inflammatory cell-dependent production of pro-inflammatory cytokines. The translocation of these pathogenic bacteria lead to recruitment of pulmonary intravascular macrophages which themselves produce more local inflammatory cytokines, which over all has also effect on vascular endothelial function. The possibility that probiotics (VSL#3) therapy may improve vascular reactivity of the mesenteric artery of rats with portal hypertension and, in particular, the endothelial function was examined.

2- On other hand a previous study by Dal-Ros *et al*. (Dal-Ros et al, 2010) concluded that impaired EDHF-mediated relaxation in CBDL is partly if not totally explained by an excessive vascular level of oxidative stress. And the enzymatic sources of this vascular oxidative stress are due to for example; NADPH oxidase, cytochrome P450, un-coupled eNOS etc. Therefore, the effect of a polyphenol-rich blackcurrant juice (PRBJ), a potent antioxidant product on vascular endothelial function in CBDL rat was tested.

3- Previous studies have suggested that an increased estradiol level in CBDL rats might contribute to pulmonary vasodilatation via excessive formation of the potent vasodilator NO (Aller et al, 2001; Aller et al, 2002; Yol et al, 2005). The last part of our study was to investigate the effect of fulvestrant, which is an estrogen receptor antagonist with no agonistic effect, in CBDL rat model of biliary cirrhosis with portal hypertension. Indeed, fulvestrant is expected to prevent the increased

estradiol-stimulated NO formation and, thus may improve the abnormalities observed in cirrhotic rat lungs.

4.2. EXPERIMENTAL PROTOCOLS

Various animal models of portal hypertension (PH), the main feature leading to the splanchnic and systematic vascular abnormalities in chronic liver diseases, exist. The common bile duct ligation (CBDL) (Fallon et al, 1997) model leads to liver biliary cirrhosis with a hyperdynamic state and a hepatopulmonary syndrome. The latter is not induced by the use of carbon tetrachloride CCl_4 (Cervinkova et al, 1987) to obtain liver cirrhosis. Other studies were devoted to the effect of PH induced by partial portal vein ligation which induces the same abnormalities than the CBDL model but without liver cirrhosis.

In our work we were interested to assess the endothelial dysfunction in portal hypertension and especially with its consequences on the splanchnic and lung vascular bed. Thus, we have chosen the CBDL rat model for our studies. CBDL leads to an acute obstructive jaundice in two weeks, and a progression to cirrhosis in about 4 weeks. CBDL stimulates the proliferation of biliary epithelial cells and oval cells (which are hepatocyte progenitors), resulting in proliferating bile ductules with an accompanying portal inflammation and fibrosis. Cholangiocyte proliferation starts after CBDL at the edge of the portal tract. During the first week of CBDL the hepatic microcirculation does not show any alterations with respect to the normal liver (Figure 19), (Chambliss and Shaul 2002).

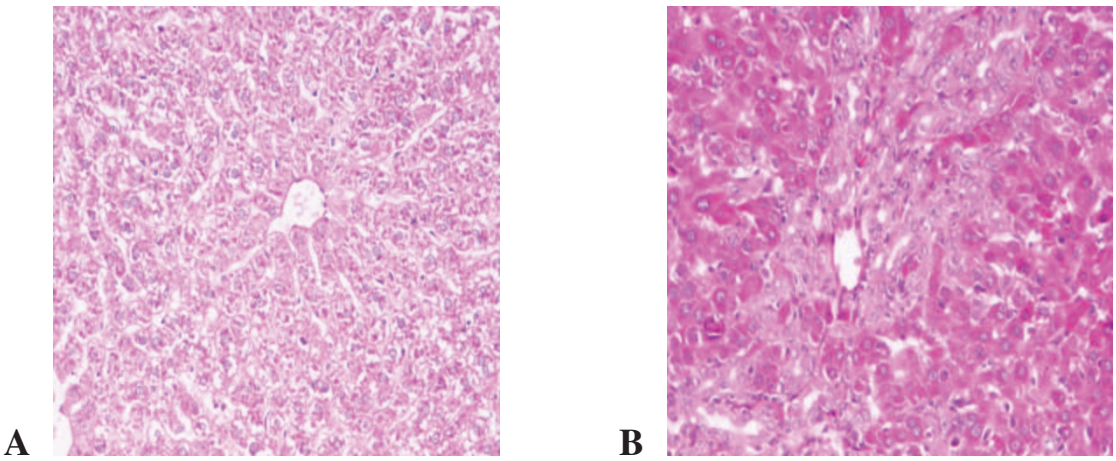


Figure 19. Histological study of liver section using heamatoxylin and eosin staining, sample taken at the end of study; A, sham: normal liver architecture**; B, CBDL:** disturbed liver architecture

4.3. CBDL model

Male Wistar rats (10–12 weeks old) were anesthetized with an intraperitoneal mixture of ketamine (80 mg.kg body wt-1) and xylazine (4 mg.kg body wt-1). Biliary cirrhosis was induced by CBDL. After laparotomy, the bile duct was isolated, double ligated, and resected in between the 2 ligatures. Sham rats had laparotomy and underwent mobilization of the common bile duct without ligation. After surgery, rats were housed in a thermoneutral environment, on a 12:12-hour photoperiod and were provided food and water ad *libitum* (Figure 20).

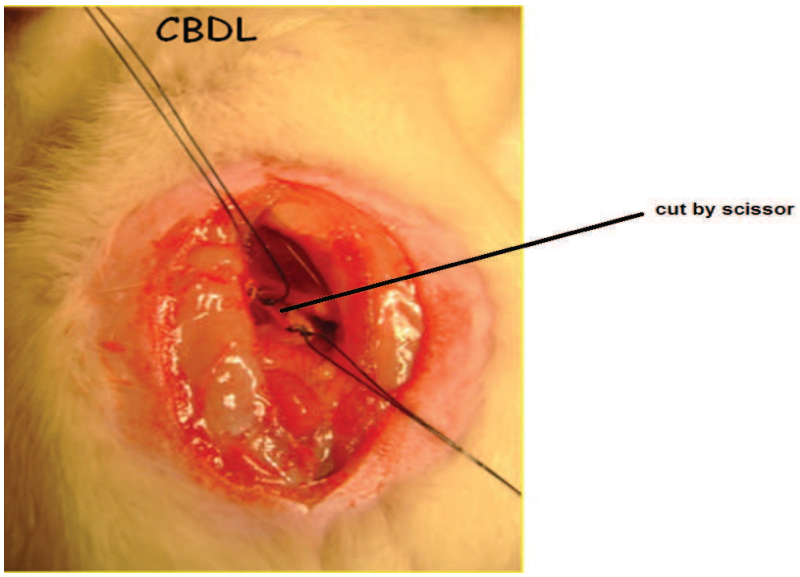


Figure 20. CBDL operation; consist of identification of the common bile duct and then put double ligature and cut between the ligature with scissor.

4.4. Animal scarification

Rats were anesthetized by 50 mg/kg intraperitoneal sodium pentobarbital in the all three studies. After anesthesia a large med-line incision made from xyphoid to pubic bone was performed. Blood was withdrawn from the heart or the aorta and collected for further measurements. The liver and spleen were removed and weighted. Liver samples (taken from the median lobe) from each rat were incubated in Bouin-Holland fixative and subsequently embedded in paraffin for histological analysis. Segments of the main superior mesenteric artery and thoracic aorta were cleaned, embedded in Tissu-Tek O.C.T. compound (Sakura Finetek France SAS, Villeneuve d'Ascq, France) or kept in -80°C for Western blot analysis or snap-frozen for immunofluorescence studies and the subsequence determination of the formation of ROS. In the fulvestrant study, after excision, the left lung was snap frozen and

the right lung was fixed by infusion of Bouin-Holland fixative at a pressure of 25 cm H_2O and embedded in paraffin for immunohistochemistry.

4.5. Vascular reactivity study in isolated organ chambers

The main superior mesenteric artery and the aorta were cleaned of connective tissue and cut into rings (2–3 mm in length). The mesenteric artery has been selected due to the fact that endothelium dependent relaxations involve both NO and EDHF, these vessels reflecting also the consequences of PH in the splanchnic circulation. In some preparations, the endothelium was removed by rubbing the intimal surface of rings with a pair of forceps. Rings were suspended in organ baths containing oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25 and d- glucose 11, pH 7.4, 37°C) for the determination of changes in isometric tension. The rings were stretched step by step until an optimal resting tension of 1 g was reached and then allowed to equilibrate for at least 60 min. After the equilibration period, the rings were exposed to Krebs bicarbonate solution containing a high concentration of potassium (80 mM) until reproducible contractile responses were obtained. After washing with Krebs bicarbonate solution, the rings were precontracted with phenylephrine (PE, 1 μM) to about 80% of the maximal contraction obtained by high K^+ solution before the addition of acetylcholine (ACh, 1 μM) to check the presence of a functional endothelium. After washout and a further 30-min equilibration period, rings were again contracted with PE before the application of increasing concentrations of either ACh, sodium nitroprusside (an exogenous NO donor) or levcromakalim (an ATP-sensitive K^+channel opener) to construct concentration-response curves. Sodium nitroprusside- and levcromakalim-induced relaxations were examined in endothelium denuded rings of mesenteric artery. In some experiments, rings were exposed to an inhibitor for 30 min before contraction with PE. The NO-mediated component of relaxation was determined in the presence of indomethacin (10 μM) and charybdotoxin (CTX, 100 nM) plus apamin (APA, 100 nM) to inhibit the participation of prostanoids and EDHF, respectively. The EDHF-mediated component of relaxation was determined in the presence of indomethacin (10 μM) and N□-nitro-L-arginine (L-NA, 300 μM) to inhibit the formation of prostanoids and NO, respectively. Relaxations are expressed as percentage of the contraction induced by PE (Figure 21).

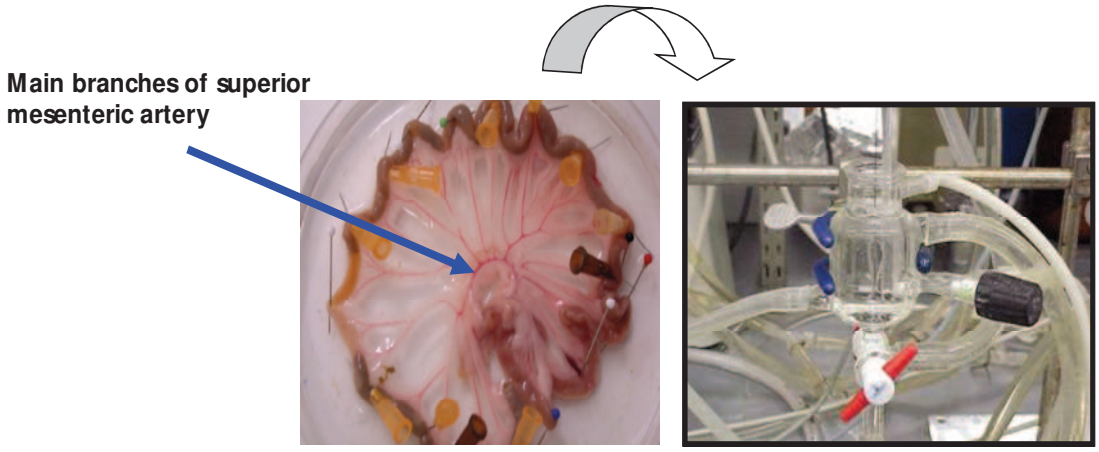


Figure 21. Study of the vascular reactivity.

Left: from the mesenteric circulation arrowed we take segments from main mesenteric artery. Right: organ bath used and segment of artery put in physiological tension with electrode attached and read changes in relaxation and constriction curve.

RESULTS

RESULTS

PART I

Article 1

Probiotics (VSL#3) prevent endothelial dysfunction in rats with portal hypertension: role of the angiotensin system

Sherzad K. Rashid [a], Noureddine Idris-Khodja [a], Cyril Auger [a], Mahmoud Alhosin[a], Nelly Boehm [b], Monique Oswald-Mammosser [c] and Valérie B. Schini-Kerth [a]

PLOS ONE, 2014; 10.1371/journal.pone.0097458

PART II

Article 2

Polyphenol-rich blackcurrant juice prevents endothelial dysfunction in the mesenteric artery of cirrhotic rats with portal hypertension: Role of oxidative stress and the angiotensin system

Rashid Sherzad[a], Idris Khodja Noureddine[a], Auger Cyril[a], Alhosin Mahmoud[a], Boehm Nelly[b], Oswald-Mammosser Monique,[a,c] and Schini-Kerth Valérie B[a].

Submitted to journal of Free Radical Biology and Medicine

PART III

Article 3

Effect of Fulvestrant, an estrogen receptor antagonist, on the cirrhotic rat lung

Oswald-Mammosser Monique, **Rashid Sherzad K.**, Boehm Nelly, Agin Arnaud, Geny Bernard, Schini-Kerth Valérie, Charloux Anne.

Submitted for publication

PART I

RESULTS:

PART I

STUDY 1: Probiotic VSL#3 as a candidate to prevent endothelial dysfunction in cirrhotic rats with PH.

The intestinal microbiota consists of more than 500 bacterial species that inhabit the human intestinal tract. In the healthy individuals, the composition of bacterial densities change in different parts of the intestine for example the lower part of ileum has higher bacterial concentration than the upper part of ileum (Madsen et al, 1999). The intestinal microbiota has been shown to play important immunomodulatory and homeostatic roles (Tlaskalova-Hogenova et al, 2004). The intestinal microbiota and the mucosal lining are closely related components of the intestinal epithelial barrier. Clinical observations and animal experiments have suggested that intestinal bacteria can trigger ongoing mucosal inflammation in some susceptible individuals (Madsen et al, 2000). It is known that in patients and animal with cirrhosis, gram-negative bacterial translocation plays a pivotal role with the development of spontaneous bacterial infections and the worsening of the hyperdynamic circulatory state further deteriorating the endothelial function (Wiest and Groszmann 1999; Albillos et al, 2003). Bacterial translocation in patient with advanced cirrhosis, is favored by gram-negative bacterial overgrowth, increased intestinal permeability, and impaired immunity in addition to the presence of ascites (Albillos and de la Hera 2002; Wiest and Garcia-Tsao 2005). In addition to splanchnic vasodilatation, it has been shown that systemic vasodilatation (as evidenced by lower mean arterial pressure) is greater in animals with bacterial translocation. These abnormalities correlate with increased levels of endotoxin, TNF-α, and nitric oxide (Wiest et al, 2003) (Figure 22).

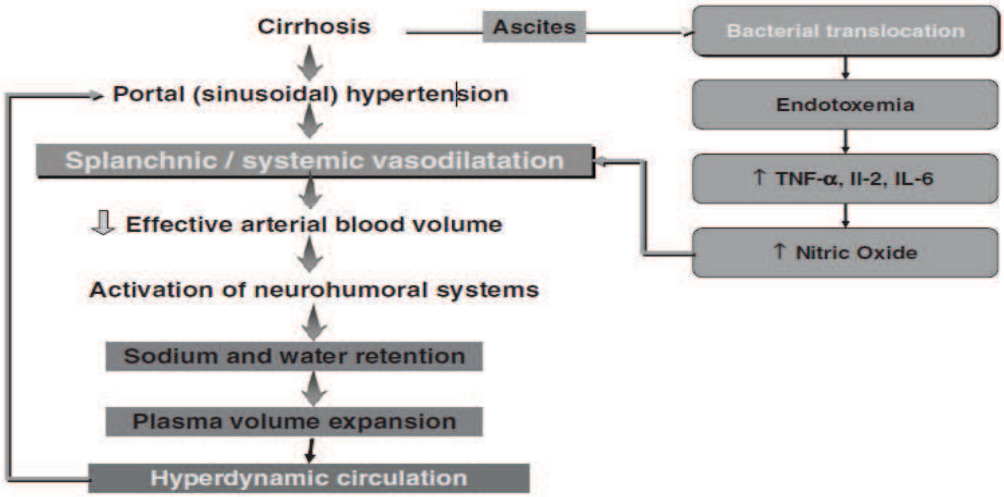


Figure 22. Bacterial translocation and the hyperdynamic circulatory state in cirrhosis.
(Fernandez et al, 2002)

Several studies assessed the effects of preventing bacterial translocation by using several strategies. Use of promotility agents, supplementation with oral bile acids, and selective intestinal decontamination have been employed to decrease the rate of gram-negative bacterial translocation (Grange et al, 1998; Soriano et al, 1992; Perez-Paramo et al, 2000; Pardo et al, 2000) selective intestinal decontamination involves the use of non-absorbable or poorly absorbable oral antibiotics to eliminate gram negative bacteria from the gut. Although this strategy has proven effective in decreasing the bacterial infections in cirrhotic patients, concern remains about the development of antibiotic resistance with long-term use**.**

We assessed in our first study the VSL#3 probiotics as an alternative to selective intestinal decontamination. By decreasing bacterial translocation, probiotics may play a role in normalizing the upregulated pro-inflammatory cytokines and endothelial dysfunction. It is known that probiotics can alter the composition of intestinal bacteria and minimize the progression of certain illnesses, for example, the probiotics can be used to prevent relapse of pouchitis, and ulcerative colitis (Bibiloni et al, 2005) and prevent antibiotics-induced diarrhea as well as Crohn's disease (Borruel et al, 2002; Gupta et al, 2000) see (Figure 23) which demonstrate the possible effect of probiotics on commensal bacteria. Probiotic mixture of VSL#3 contains 8 bacterial strains *Streptococcus thermophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei and Lactobacillus delbrueckii subsp. Bulgaricus* (Rehman et al, 2012). Hence, it is hypothesized that probiotics preserve epithelial barrier function. In vitro studies on epithelial monolayers showed that probiotics improve barrier function following *Escherichia coli* infection or incubation with pro-inflammatory cytokines (Bai et al, 2005; Parassol et al, 2005; Resta-Lenert and Barrett 2003). Probiotics also preserve the intestinal epithelial barrier in several in vivo models of mice, such as the IL-10 knockout colitis (Madsen et al, 2001) or models of sepsis (Ewaschuk et al, 2007); (Qin, Shen et al. 2005). Pathological bacterial translocation to mesenteric lymph nodes as a marker of impaired barrier function can also be effectively reduced by probiotic therapy (Ewaschuk et al, 2007; Osman et al, 2004; Qin et al, 2005; Schmitz et al, 1999).

Since bacterial translocation is one of the factors thought to lead to endothelial dysfunction in cirrhosis we have assessed the effects of VSL#3 in CBDL rats. The results of our study are

reported in the article entitled "Probiotics (VSL#3) prevent endothelial dysfunction in rats with portal hypertension: role of the angiotensin system" published in Plos One: May 15, 2014; 10.1371/journal.pone.0097458.

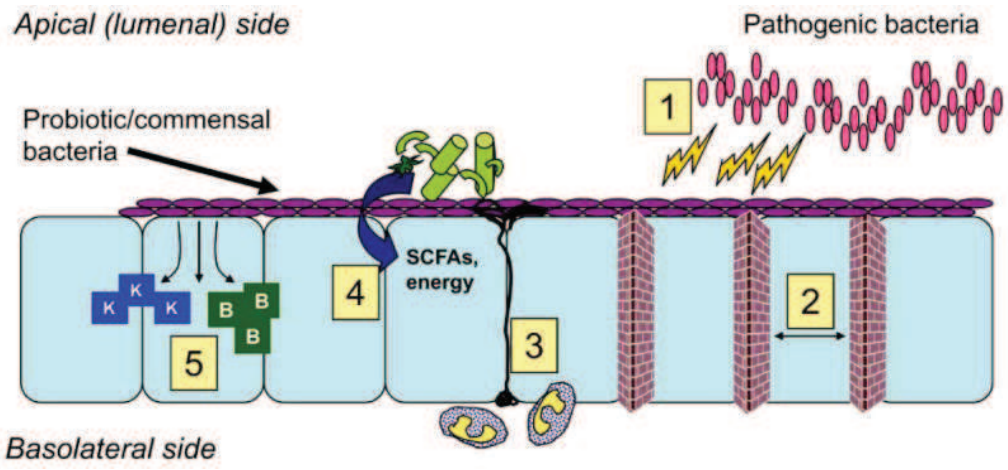


Figure 23: Beneficial effects of commensal and probiotic bacteria. (Petrof 2009)

Schematic of the many vital roles of bacteria in the intestine, commensal/ probiotic bacteria are purple, epithelial cells are in light blue, apical and basolateral sides of epithelial cells are also indicated. Intestinal bacteria serve an important role by 1) inhibiting pathogen growth through production of antimicrobials; 2) enhancing tight junction barrier function; 3) priming dendritic cell (drawn in black) and immune system; 4) assisting in digestion and breakdown of micronutrients from otherwise undigestable material (indicated in light green), synthesis of short chain fatty acids (SCFAs); 5) synthesizing key vitamins such as vitamin K and B vitamins.

Probiotics (VSL#3) prevent endothelial dysfunction in rats with portal hypertension: role of the angiotensin system

Sherzad K. Rashid [a], Noureddine Idris-Khodja [a], Cyril Auger [a], Mahmoud Alhosin[a], Nelly Boehm [b], Monique Oswald-Mammosser [c] and Valérie B. Schini-Kerth [a]

PLOS ONE, 2014; 10.1371/journal.pone.0097458

Chronic liver diseases with portal hypertension are characterized by a progressive vasodilatation associated with an endothelial dysfunction and vascular oxidative stress, which is especially pronounced in the splanchnic and pulmonary beds. It has been suggested that one of the pathophysiological mechanisms leading to the general vasodilatation is related to bacterial translocation and activation of rennin-angiotensin-aldosterone system.

The use of fermented milk is an old concept. Fermented milk products which contain probiotics have been used for centuries as nutrient element. According to Persian tradition, Abraham in the Old Testament owed his longevity to ingestion of fermented milk. King François Ier of France was reported to be cured of an illness after eating yogurt in the sixteenth century. Male Wistar rats received either control drinking water or water containing VSL#3 (50 billion bacteria/kg/day) for 7 weeks. After 3 weeks, the rats underwent surgery with either the resection of the common bile duct (CBDL rats) or sham surgery (sham rats). Thereafter, they were sacrificed after 4 weeks.

Both the nitric oxide (NO)- and the endothelium-dependent hyperpolarization (EDH)-mediated relaxations to acetylcholine in rings with endothelium were significantly reduced in CBDL rats compared to sham rats. In contrast, relaxations to sodium nitroprusside (NO donor) and levcromakalim (an ATP-sensitive K^+ channel opener) in rings without endothelium were minimally affected. Impaired endothelium-dependent relaxations were associated with a reduced vascular expression of Cx37, Cx40 and Cx43 and both $SK_{Ca,}$ IK_{Ca} and an increased expression of eNOS (endothelial NO synthase), cyclooxygenase-1, cyclooxygenase-2. In aortic sections of CBDL rats, an increased expression of NADPH oxidase subunits, angiotensin II, angiotensin-converting enzyme (ACE) and angiotensin type 1 receptors (AT1) are observed and an increased vascular formation of ROS and peroxynitrites was also observed. The endothelial dysfunction in CBDL rats was significantly prevented by VSL#3, and this effect was associated with the normalization of the vascular expression of Cx37, Cx40 and Cx43 and both $SK_{Ca,}$ IK_{Ca} and eNOS. VSL#3 treatment also reduced vascular oxidative stress in the aorta, and the increased plasma level of pro-inflammatory cytokines including TNF-α, IL-1α, MCP-1 and IL-4 in CBDL rats.

In conclusion, these findings indicate that VSL#3 ingestion prevented the endothelial dysfunction in the mesenteric artery of CBDL rats by improving both the NO and the EDHF components. The beneficial effect of the VSL#3 treatment is associated with the normalization of the CBDL-induced vascular oxidative stress the inflammatory response and the local angiotensin system possibly indicating a reduced bacterial translocation.

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Probiotics (VSL#3) Prevent Endothelial Dysfunction in Rats with Portal Hypertension: Role of the Angiotensin System

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Abstract

Aims: Portal hypertension characterized by generalized vasodilatation with endothelial dysfunction affecting nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH) has been suggested to involve bacterial translocation and/or the angiotensin system. The possibility that ingestion of probiotics prevents endothelial dysfunction in rats following common bile duct ligation (CBDL) was evaluated.

Methods: Rats received either control drinking water or the probiotic VSL#3 solution (50 billion bacteria.kg body $wt^{-1}.day^{-1}$) for 7 weeks. After 3 weeks, rats underwent surgery with either resection of the common bile duct or sham surgery. The reactivity of mesenteric artery rings was assessed in organ chambers, expression of proteins by immunofluorescence and Western blot analysis, oxidative stress using dihydroethidium, and plasma pro-inflammatory cytokine levels by flow cytometry.

Results: Both NO- and EDH-mediated relaxations to acetylcholine were reduced in the CBDL group compared to the sham group, and associated with a reduced expression of Cx37, Cx40, Cx43, IK_{Ca} and SK_{Ca} and an increased expression of endothelial NO synthase (eNOS). In aortic sections, increased expression of NADPH oxidase subunits, angiotensin converting enzyme, AT1 receptors and angiotensin II, and formation of ROS and peroxynitrite were observed. VSL#3 prevented the deleterious effect of CBDL on EDH-mediated relaxations, vascular expression of connexins, IK_{Ca}, SK_{Ca} and eNOS, oxidative stress, and the angiotensin system. VSL#3 prevented the CBDL-induced increased plasma TNF-α, IL-1α and MCP-1 levels.

Conclusions: These findings indicate that VSL#3 ingestion prevents endothelial dysfunction in the mesenteric artery of CBDL rats, and this effect is associated with an improved vascular oxidative stress most likely by reducing bacterial translocation and the local angiotensin system.

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Introduction

Advanced chronic liver diseases are characterized by generalized progressive vasodilatation, related to portal hypertension (PH), which is especially observed in the splanchnic and pulmonary beds. In a rat model of partial portal vein ligation, Albrades et al. [1] reported that the initial event in response to PH is an up-regulation of vascular endothelial growth factor and endothelial nitric oxide synthase (eNOS) in the intestinal microcirculation. Although animal models have led to a number of hypotheses, the exact mechanism involved in the occurrence of the generalized vasodilatation and hyperdynamic syndrome is incompletely understood [2–5]. Several lines of evidence support a role for an increased formation of nitric oxide (NO) in the general vasodilatation in chronic liver diseases in patients [6] and also in the chronic bile duct ligated (CBDL) rat model [2,4]. In addition, chronic liver diseases in patients and also in experimental PH are characterized by an increased vascular formation of reactive oxygen species (ROS) [7,8] and the activation of the renal renin-angiotensin-aldosterone system and the local angiotensin system [9–11]. Moreover, it has been suggested that bacterial translocation (BT) is an important step in the occurrence of the general vasodilatation and especially in the pulmonary bed [3,12]. In cirrhosis, BT is defined as the migration of bacteria and/or their

products from the intestinal lumen to mesenteric lymph nodes [13] as a consequence of intestinal bacterial overgrowth, impaired host defenses, and/or disruption of the gut mucosal barrier [14,15]. In cirrhotic rats, BT has been reported to lead to endotoxaemia, which stimulates the expression of TNF-α leading to an enhanced formation of the potent vasodilators NO and carbon monoxide [3]. Thus, reducing BT to improve the excessive oxidative stress and/or to reduce the increased vasodilator factors is an interesting target to improve the general vascular dysfunction in PH. Earlier studies have reported that treatment with either antiobiotics or pentoxifylline, an inhibitor of TNF-α, to prevent BT and/or its consequences improves the vasodilatation in CBDL rats [12,16] and also in patients with liver cirrhosis [17,18]. Due to the poor tolerance of pentoxifylline [18] and the increased risk of bacterial resistance with antibiotics, probiotics such as VSL#3 which are well tolerated [19,20] may be of interest. Thus, the aim of the present study was to evaluate the effect of the probiotic VSL#3 formulation on the vascular dysfunction in an animal model of biliary cirrhosis with PH with a special attention to oxidative stress and the local angiotensin system.

Materials and Methods

Ethics statement

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

Animal model

Male Wistar rats (10–12 week-old) were anaesthetized with an intraperitoneal mixture of ketamine (80 mg.kg body wt^{-1}) and xylazine (4 mg.kg body wt^{-1}). Biliary cirrhosis was induced by CBDL. After laparotomy, the bile duct was isolated, double ligated and resected between the two ligatures. Sham rats had laparotomy, underwent mobilization of the common bile duct without ligation. After surgery, rats were housed in a thermo-neutral environment, on a 12:12 h photoperiod and were provided food and drinking water *at libitum*. Eight CBDL rats and seven sham rats were treated three weeks before and 4 weeks following surgery with the probiotic formulation VSL#3 (VSL Pharmaceuticals, Inc, Towson, MD, USA; *Streptococcus thermophilus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus bulgaricus*) at a dose of 50 billion bacteria.kg body wt^{-1} daily, given in the drinking water. Seven CBDL rats and five sham rats receiving vehicle served as controls.

Four weeks after surgery, rats were sacrificed. The liver and spleen were removed and weighted. Liver samples (taken from the median lobe) from each rat were incubated in the Bouin-Holland fixative and subsequently embedded in paraffin for histological analysis. Segments of the main superior mesenteric artery and thoracic aorta were cleaned, embedded in Tissu-Tek O.C.T. compound (Sakura Finetek France SAS, Villeneuve d'Ascq, France) and snap-frozen for immunofluorescence studies and the determination of the formation of ROS. Samples of blood were taken by heart puncture, and, thereafter, plasma was stored at -80°C for subsequent serological analysis.

Assessment of liver cirrhosis and portal hypertension

Sections of liver samples (5 µm thick) were stained with hematoxylin, eosin and Masson's trichrome stain and evaluated by light microscopy. Histologically, liver of chronic bile duct ligation showed evidence of intrahepatic tubular duct hypertrophy with severe fibrosis. Portal hypertension is reflected by an increase in the spleen weight.

Vascular reactivity studies

Vascular reactivity studies on the main superior mesenteric artery were performed as described previously [11]. Briefly, the main superior mesenteric artery was cleaned of connective tissue, cut into rings (2–3 mm in length) and suspended in organ baths containing oxygenated Krebs bicarbonate solution for the determination of changes in isometric tension. After equilibration and functional tests, rings were contracted with phenylephrine (PE, 1 µM) before construction of concentration-response curves in response to acetylcholine (ACh), sodium nitroprusside or levcromakalim. In some experiments, rings were exposed to an inhibitor for 30 min before contraction with PE. Relaxations were expressed as percentage of the contraction induced by PE.

Immunofluorescence studies

Frozen arteries were cryosectioned at 14 µm. Sections were air-dried for 15 min and stored at −80°C until use. Sections were first fixed with paraformaldehyde at 4%, washed and treated with 10% milk or 5% goat serum in PBS containing 0.1% Triton X-100 for 1 h at room temperature to block non-specific binding. Mesenteric artery sections were then incubated overnight at 4°C with an antibody directed against either eNOS (1/100), small and intermediate conductance calcium-activated potassium channels (SK_{Ca}, IK_{Ca}, 1/200), or connexins (Cx37, Cx40 and Cx43; 1/100 to 1/200). Aortic sections were incubated with an antibody directed against either angiotensin II (1/500), AT1 receptors (1/400), angiotensin-converting enzyme (ACE, 1/200), nitrotyrosine (1/200), p47phox (1/200), p22phox (1/200) or cyclooxygenase-1 or -2 (COX-1, COX-2, 1/200). Sections were then washed with PBS, incubated with the secondary antibody (1/300, Alexa 488- or 637-conjugated goat anti-rabbit IgG) for 2 h at room temperature in the dark before being washed with PBS and mounted in Dako fluorescence mounting medium (Dako France SAS, Les Ulis, France) and cover-slipped. For negative controls, primary antibodies were omitted.

Western blot analysis

Mesenteric artery and aortic segments were homogenized in extraction buffer (composition in mM: Tris/HCl 20 (pH 7.5; Q Biogene), NaCl 150, Na_3VO_4 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), a tablet of protease inhibitor (Complete Roche) and 1% Triton X-100 (Euromedex)). Total proteins (10 µg) were separated on SDS-polyacrylamide gels and transferred electrophoretically onto polyvinylidine difluoride membranes (Amersham). Membranes were blocked with blocking buffer containing 5% bovine serum albumin, Tris-buffered saline solution (Biorad) and 0.1% Tween 20 (Sigma) (TBS-T) for 1 h. Membranes were then incubated overnight at 4°C with an antibody directed against either eNOS (1/10000), SK_{Ca} (1/200), AT1 receptors (1/1000), p47phox (1/1000), p22phox (1/1000) or COX-2, (1/1000). Thereafter, membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody and signals were detected using enhanced chemiluminescence (Amersham).

Determination of vascular and mitochondrial ROS Formation

In situ formation of ROS was performed using the method previously described [21]. The redox-sensitive fluorescent dye dihydroethidium (DHE, 2.5 μM) was applied onto 25 μm unfixed cryosections of aorta for 30 min at 37°C in a light-protected humidified chamber. To determine the sources of ROS, sections were incubated with either apocynin (NADPH oxidase inhibitor and antioxidant, 300 μM), L-NA (NO synthase inhibitor, 300 μM), sulfaphenazol (cytochrome P450 inhibitor, 100 μM), indomethacin (cyclooxygenase inhibitor, 10 μM) or inhibitors of the mitochondrial respiration chain (myxothiazol, 0.5 μM+rotenone, 1 μM+KCN, 1 μM) for 30 min at 37°C before the addition of dihydroethidium. The *in situ* formation of mitochondrial ROS was performed using MitoSox (Life Technologies SAS, Saint Aubin, France), a mitochondrion-specific dihydroethidium-derivative fluorescent dye. Briefly, 14 μm unfixed cryosections of aorta were incubated with MitoSox (5 μM at 37°C for 60 min) in a light-protected humidified chamber. Sections were then washed three times, mounted in DAKO and cover-slipped.

Image analysis

All samples from immunofluorescence and *in situ* ROS formation studies were observed using a confocal laser-scanning microscope (Leica SP2 UV DM IRBE). Quantification of fluorescence levels was performed using FIJI GPL v2 software (http://fiji.sc/Fiji).

Determination of plasma inflammatory cytokine levels

The determination of the plasma level of TNF-α, IL-1α, MCP-1 and IL-4 was performed using a commercial rat cytokine Kit Flowcytomix (eBioscience SAS, Paris, France), multiple analyte detection kit, according to the protocol supplied by the manufacturer. The raw data were analysed using "The FlowCytomixPro software".

Statistical analysis

Values are expressed as means ± SEM. Statistical analysis was performed using either Student's *t* test or an analysis of variance (ANOVA) followed by the Bonferroni post-hoc test as appropriate using GraphPad Prism (version 5 for Microsoft windows, GraphPad software, Inc, San Diego, CA, USA). A *P* value less than 0.05 was considered to be statistically significant.

Results

Characteristics and histologic findings

As shown in Table 1, four weeks after surgery, CBDL rats had a significantly increased spleen weight, and an enhanced bile duct proliferation as assessed by histological analysis of liver sections. Chronic intake of VSL#3 for seven weeks starting three weeks before the surgery reduced slightly but significantly the CBDL-induced increase in spleen weight whereas the liver weight was unaffected. The VSL#3 treatment alone had no effect on liver and spleen weight.

VSL#3 treatment prevents the CBDL-induced impaired EDH but not NO component of the relaxation to acetylcholine in mesenteric artery rings

ACh caused similar concentration-dependent relaxations in mesenteric artery rings from control and CBDL rats (maximal relaxations amounted to 97.4±1.4% and 94.9±2.4%, respectively, n = 6–8). However, the NO component of the relaxation as assessed in the presence of indomethacin and charybdotoxin plus apamin to prevent the formation of vasoactive prostanoids and EDH, respectively, was significantly impaired in the CBDL group (Figure 1A). In addition, the EDH-mediated component of the relaxation as assessed in the presence of indomethacin plus N^G-nitro-L-arginine (an inhibitor of eNOS), was also significantly reduced in the CBDL group (Figure 1B). Although the VSL#3 treatment did not affect the CBDL-induced impaired NO component, it significantly improved the EDH component (Figures 1A and B). In addition, both the sodium nitroprusside (an NO donor) and the levcromakalim (an ATP-sensitive K channel opener) induced endothelium-independent relaxations were similar in all groups (Figures 1C and D).

VSL#3 treatment prevents CBDL-induced up-regulation of eNOS and down-regulation of SK_{Ca}, IK_{Ca}, Cx37, Cx40, and Cx43 in the mesenteric artery

Immunofluorescence studies indicated that the eNOS signal is observed predominantly at the luminal surface of mesenteric artery sections in the control group, and that this signal is significantly increased in the CBDL group (Figure 2A). Pronounced fluorescence signals for SK_{Ca} and $IK_{Ca,}$ two calcium-activated potassium channels involved in EDH [11], are observed throughout the arterial wall in the control group whereas both signals are significantly reduced in the CBDL group (Figure 2A). In addition, the fluorescence signal of connexins Cx37, Cx40 and Cx43, which form myoendothelial gap junctions transmitting the hyperpolarization from endothelial cells to the underlying smooth muscle cells to cause relaxation [11], is observed predominantly at the luminal surface and also to some extent in the vascular smooth muscle (Figure 2B). The fluorescence signals of Cx37, Cx40 and Cx43 are significantly reduced in the CBDL group (Figure 2B). The VSL#3 treatment prevented partially but significantly the CBDL-induced up-regulation of eNOS, and the down-regulation of SK_{Ca} and IK_{Ca}, and of Cx37, Cx40 and Cx43 (Figure 2). The VSL#3 treatment of sham rats was without effect except for SK_{Ca}, which was significantly reduced (Figure 2). An increased eNOS protein level and a decreased SK_{Ca} level were also observed in the CBDL group as assessed by Western blot analysis (Figure 2C). These effects were significantly prevented by the VSL#3 treatment (Figure 2C). In addition, IK_{Ca} and Cx37 protein levels were below the detection level in all groups.

VSL#3 treatment reduces the CBDL-induced vascular oxidative stress involving several sources in the aorta

CBDL markedly increased the DHE fluorescence signal, the MitoSOX fluorescence signal and the nitrotyrosine fluorescence signal throughout the aortic wall, and the eNOS fluorescence signal at the luminal surface and eNOS protein level as assessed by Western blot analysis (Figures 3A and 4B). All these effects were significantly prevented by the ingestion of VSL#3 (Figures 3A and 4B). The VSL#3 treatment of sham rats affected only minimally these signals (Figures 3A and 4B).

The characterization of the cellular sources of oxidative stress has indicated that the CBDL-induced increased DHE fluorescence signal was markedly reduced by apocynin (an NADPH oxidase inhibitor and antioxidant), L-NA (an eNOS inhibitor), sulfaphenazol (a cytochrome P450 inhibitor), indomethacin (a cyclooxygenase inhibitor) and by a combination of inhibitors of the mitochondrial respiration chain (KCN, myxothiazol and rotenone) suggesting the involvement of NADPH oxidase, COXs, uncoupled eNOS, cytochrome P450, and the mitochondrial respiration chain (Figure 3C).

Table 1. Effect of VSL#3 ingestion on body, liver and spleen weight in both sham and CBDL rats.

	Initial body weight (*g*)	Final body weight (*g*)	Liver (*% total weight*)	Spleen (*% total weight*)
Sham	365±19.5	505±30.2	3.24±0.06	0.20±0.02
Sham+VSL#3	368±14.3	506±26.4	3.33±0.18	0.23±0.02
CBDL	369±16.3	485±30.5	6.69±0.63*	0.69±0.05*
CBDL+VSL#3	365±15.7	507±47.6	6.02±0.55	0.56±0.09#

Values are shown as mean ± SEM of 6 rats. *$P<0.05$ CBDL vs sham, and #$P<0.05$ for CBDL+VSL#3 vs CBDL.
doi:10.1371/journal.pone.0097458.t001

To obtain further evidence for a role of NADPH oxidase and COXs, their expression level was determined by immunofluorescence staining and Western blot analysis in the aorta. A significantly increased immunofluorescence signal of the NAPDH oxidase subunits p22phox and p47phox, and of COX-1 and COX-2 was observed in the aorta of the CBDL group compared to the sham group (Figure 4A). The VSL#3 treatment significantly reduced the CBDL-induced stimulatory effect for p22phox, p47phox and COX-2 whereas that for COX-1 was minimally affected (Figure 4A). The VSL#3 treatment of sham rats affected minimally all these signals (Figure 4A). In addition, an upregulation of p22phox and p47phox protein levels was also observed in the CBDL group as assessed by Western blot analysis whereas no such effect was observed in the CBDL+VSL#3 group (Figure 4B). In contrast, COX-2 protein levels were similar in all groups, and COX-1 protein levels were below the detection level (Figure 4B).

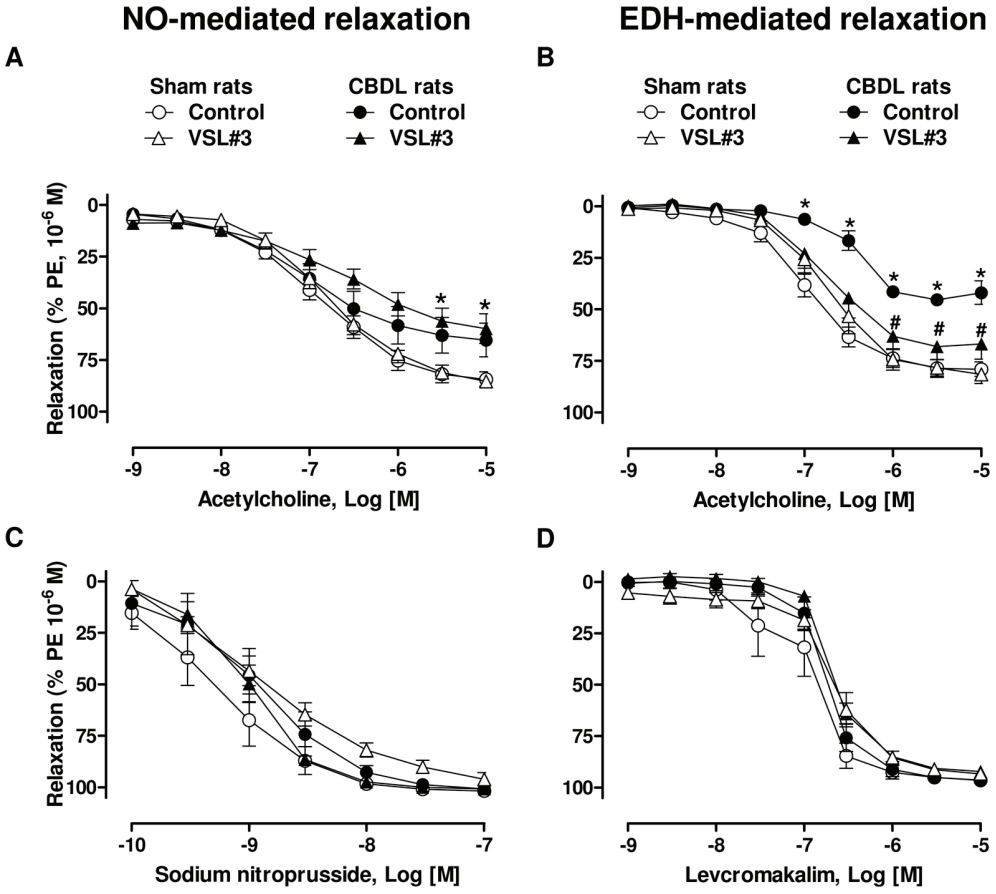

Figure 1. Ingestion of VSL#3 improves the CBDL-induced blunted EDH but not NO-mediated relaxations in mesenteric artery rings. Concentration-relaxation curves to acetylcholine in mesenteric artery rings with endothelium in sham, CBDL, sham+VSL#3 and CBDL+VSL#3 rats. A) The NO component of the relaxation was assessed in the presence of indomethacin (10 μM) and apamin plus charybdotoxin (100 nM each), and B) the EDH component in the presence of indomethacin and N^G-nitro-L-arginine (300 μM). C) Relaxations to sodium nitroprusside (an exogenous donor of NO) and D) levcromakalim (an ATP-sensitive K^+ channel opener) in mesenteric artery rings without endothelium are also shown. Results are shown as mean±SEM of 5-7 different rats; *$P<0.05$ CBDL vs sham, and #$P<0.05$ CBDL+VSL#3 vs CBDL.
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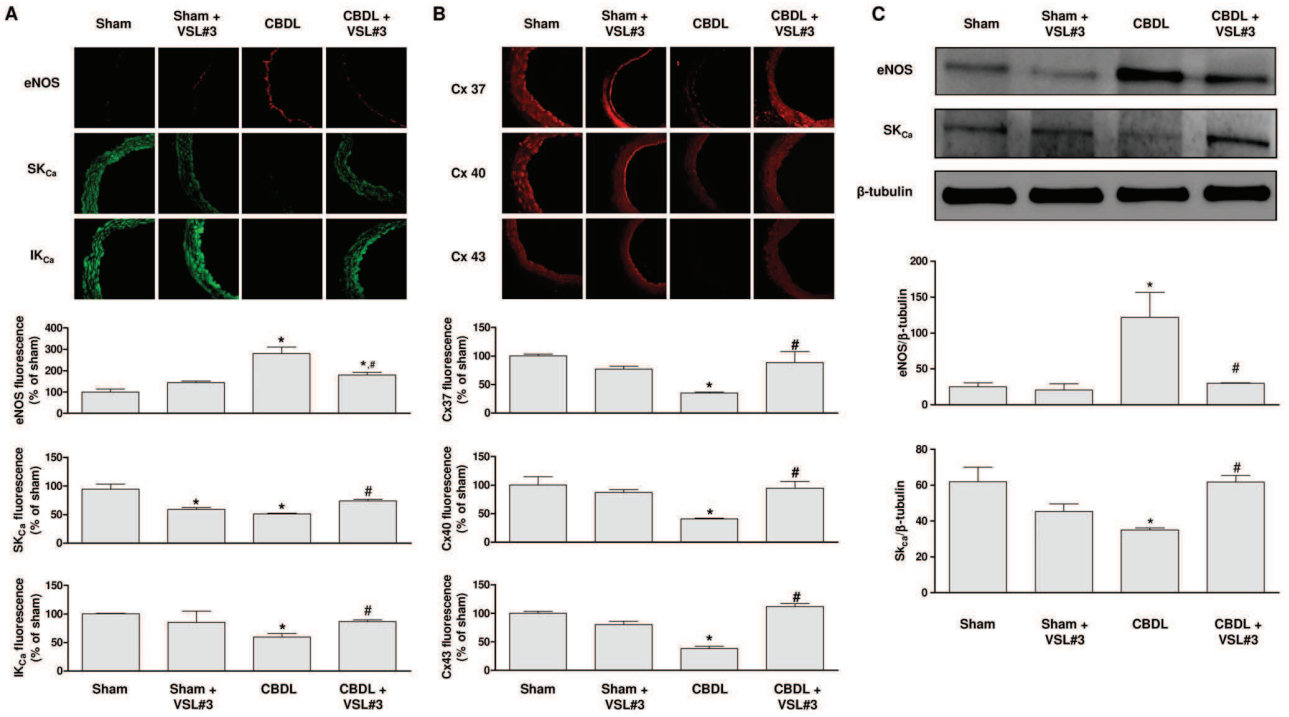

Figure 2. The VSL#3 treatment prevents the CBDL-induced A) up-regulation eNOS and down-regulation of SK_{ca} and IK_{ca}, and B) down-regulation of connexins (Cx37, Cx40 and Cx43) in the mesenteric artery. Upper panels show representative immunofluorescence staining, and lower panels corresponding cumulative data. C) Expression of eNOS and SK_{Ca} protein levels as assessed by Western blot analysis. Results are shown as mean±SEM of 4 different rats for A and B, and 3 for C. *$P<0.05$ for CBDL vs sham, and $^{\#}P<0.05$ CBDL+VSL#3 vs CBDL.
doi:10.1371/journal.pone.0097458.g002

VSL#3 treatment improves the CBDL-induced expression of the local angiotensin system

An increased immunofluorescence level of angiotensin II, AT1 receptors and ACE throughout the aortic wall was observed in the CBDL group (Figure 5). The stimulatory effect of CBDL was significantly prevented by the VSL#3 treatment (Figure 5). The VSL#3 treatment alone affected minimally all these signals (Figure 5). An upregulation of the AT1 receptor protein level was also observed in the CBDL group by Western blot analysis whereas no such effect was observed in the CBDL+VSL#3 group (Figure 4B).

VSL#3 treatment prevents the CBDL-induced increase in plasma pro-inflammatory cytokines

A significant increase in the plasma level of pro-inflammatory cytokines including IL-1α, MCP-1 and TNF-α was observed in the CBDL group, and this effect was significantly prevented by the VSL#3 treatment (Figure 6). In contrast, the plasma level of IL-4, which is a potent anti-inflammatory cytokine, was markedly reduced in the CBDL group, and this effect was significantly prevented by the VSL#3 treatment (Figure 6). The VSL#3 treatment of sham rats affected minimally all these cytokines (Figure 6).

Discussion

The present study indicates that the CBDL-induced endothelial dysfunction involves predominantly a reduced EDH component and, also, to some extent a decreased NO component in the mesenteric artery. The blunted EDH component is associated with a reduced expression of SK_{Ca} and IK_{Ca} levels, and Cx30, Cx37 and Cx40 levels, which are all involved in the EDH response, most likely as a consequence of the activation of the local angiotensin system leading to an increased vascular oxidative stress, and the formation of pro-inflammatory mediators. They further indicate that ingestion of the probiotic VSL#3 formulation effectively prevented the CBDL-induced endothelial dysfunction, at least in part, by targeting the vascular angiotensin system.

Biliary cirrhosis induced by chonic bile duct ligation in rats and evidenced by an increased spleen weight and enhanced bile duct proliferation is associated with an unaffected acetylcholine-induced endothelium-dependent relaxation in mesenteric artery rings. In contrast, when experiments were performed to study selectively either the NO component or the EDH component then a significant reduction of both components was observed in the CBDL group as observed previously in some but not all studies [11],[22],[23]. A severely reduced EDH component despite an unaffected ACh-induced relaxation has also been observed previously in aging-related endothelial dysfunction [24]. Such a difference is most likely explained by the fact that the NO component and the EDH component, although blunted, act in synergy to maintain a normal endothelium-dependent relaxation whereas such a compensating mechanism cannot take place when a single component is evaluated. Alternatively, the present findings due not rule out the possibility that vasoactive prostanoids contribute to preserve the acetylcholine-induced endothelium-dependent relaxation in the CBDL group. However, previous findings have indicated that although prostacyclin evoked relaxations in mesenteric resistance arteries from control rats, the prostanoid induced also contractile responses in arteries from spontaneously hypertensive rats presenting an endothelial dysfunction [25]. Moreover, chronic COX inhibition did not affect

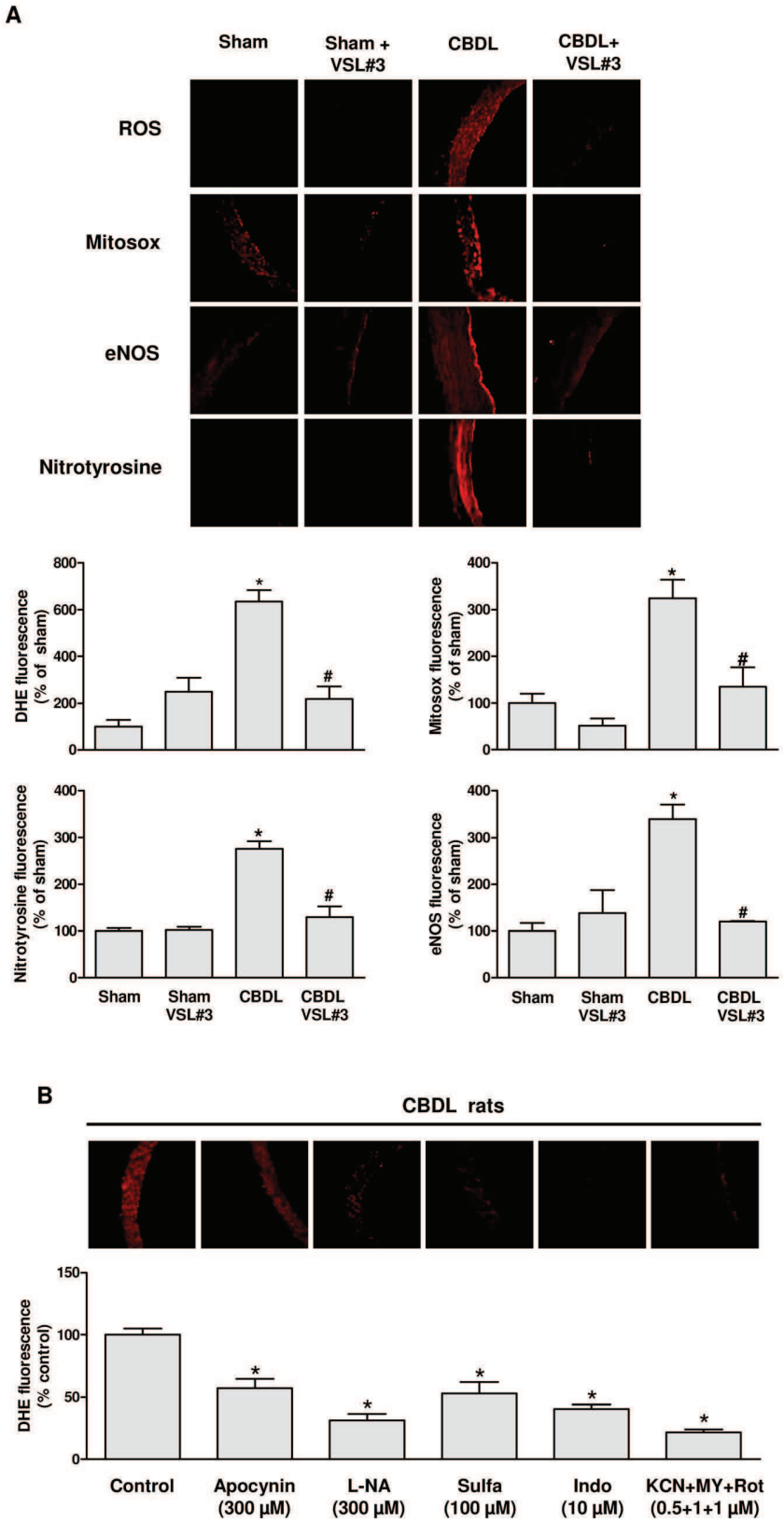

Figure 3. The VSL#3 treatment prevents the CBDL-induced oxidative stress in the aorta. (A) *In situ* determination of the formation of ROS, peroxynitrite (nitrotyrosine), and eNOS in aortic sections. Top: representative immunofluorescence staining; bottom: corresponding cumulative data for 4 to 5 rats/group. (B) To study the cellular sources of ROS, aortic sections were exposed either to apocynin (NADPH oxidase inhibitor and

antioxidant), L-NA (NO synthase inhibitor), sulfaphenazol (Sulfa, cytochrome P450 inhibitor), indomethacin (Indo, cyclooxygenase inhibitor) or the combination KCN, myxothiazol and rotenone (KCN+MY+Rot, inhibitors of the mitochondrial respiration chain) for 30 min before DHE staining. Top of each panel: DHE staining; bottom: corresponding cumulative data for 4 rats. Results are shown as mean±SEM. The lumen is on the right side of each image. A) *$P<0.05$ CBDL vs sham, and $^{\#}P<0.05$ CBDL+VSL#3 vs CBDL; B) *$P<0.05$ versus control.
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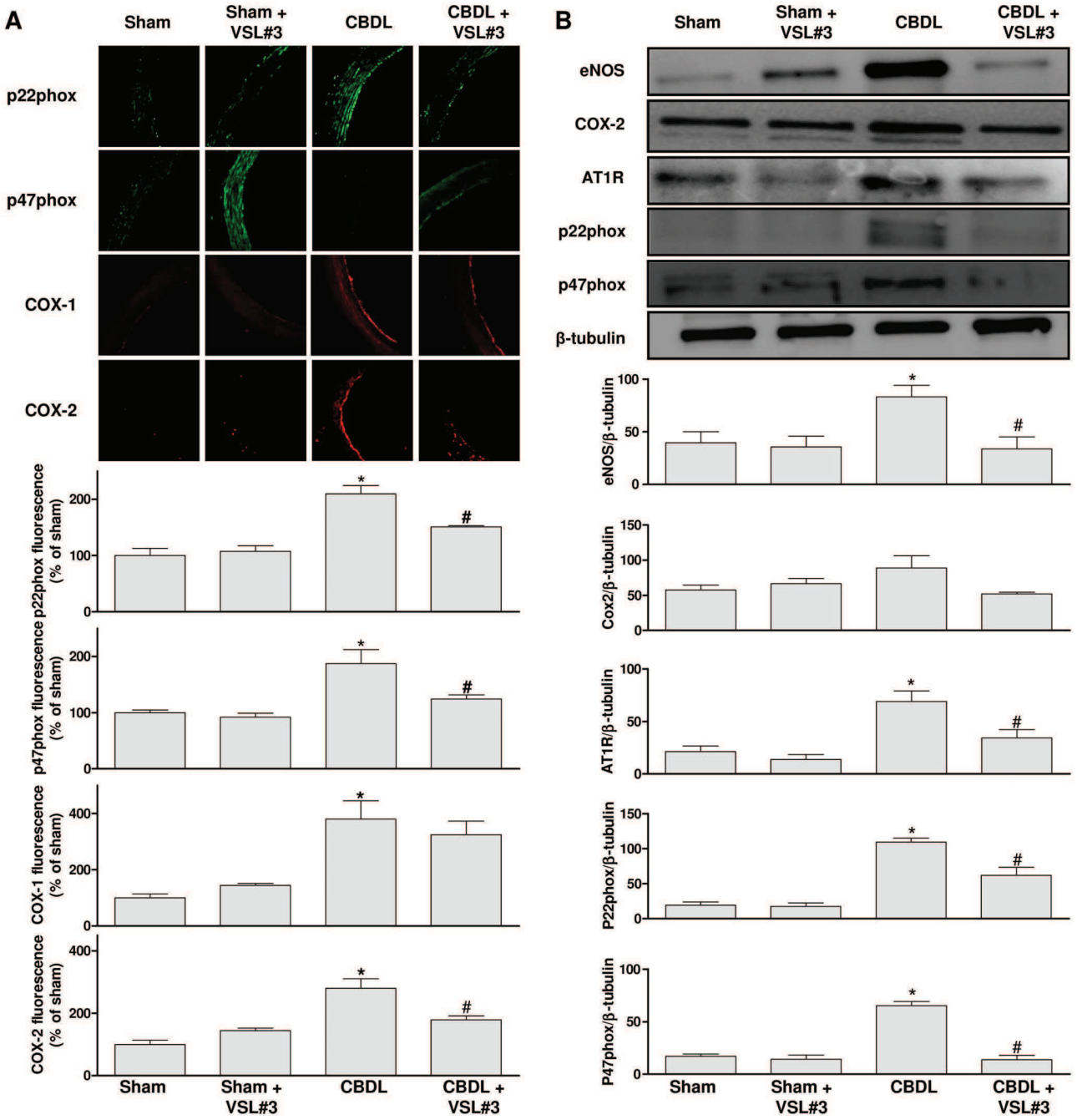

Figure 4. The VSL#3 treatment prevents the CBDL-induced up-regulation of the NAPDH oxidase subunits p22phox and p47phox, and COX-2 but not COX-1 in aortic sections. A) Representative immunofluorescence staining and corresponding cumulative data. B) Expression levels of target proteins as assessed by Western blot analysis in aortic segments. Results are shown as mean±SEM of 4 different rats. *$P<0.05$ CBDL vs sham, and $^{\#}P<0.05$ CBDL+VSL# 3 vs CBDL.
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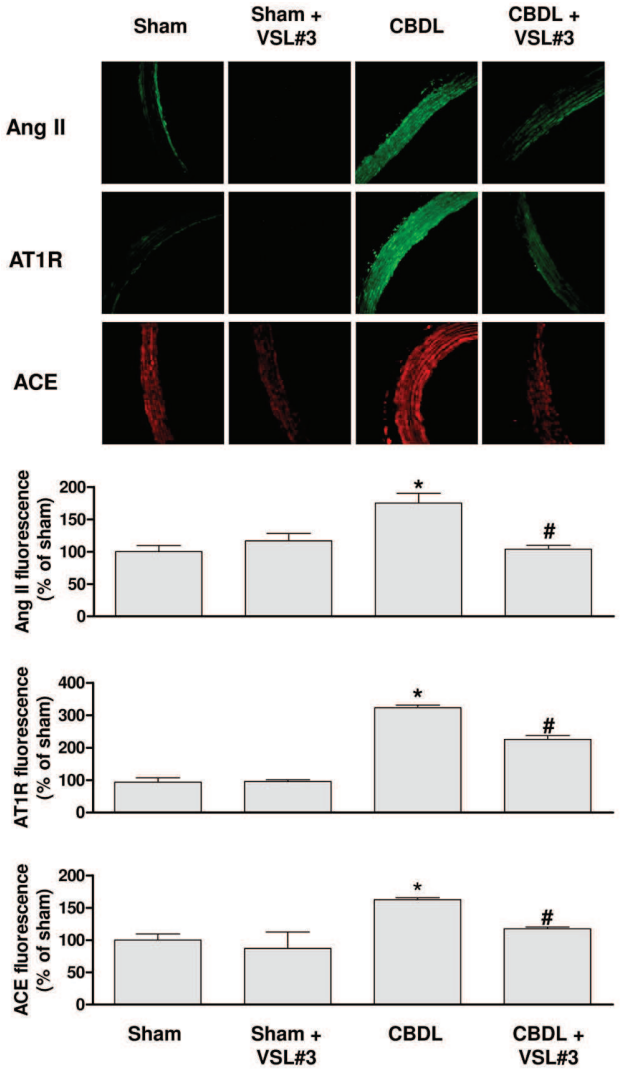

Figure 5. The VSL#3 treatment prevents the CBDL-induced up-regulation of several components of the local angiotensin system in the aorta. The expression level of angiotensin II (Ang II), AT1 receptors (AT1R) and angiotensin-converting enzyme (ACE) was determined by immunofluorescence staining. Upper panels show representative immunofluorescence staining; Lower panels corresponding cumulative data. Results are shown as mean±SEM of 4 different rats. *$P<0.05$ CBDL vs sham, and $^{\#}P<0.05$ CBDL+VSL#3 vs CBDL.
doi:10.1371/journal.pone.0097458.g005

the CBDL-induced decrease in mean arterial pressure in CBDL rats [26].

The impaired EDH-mediated relaxation is most likely the consequence of the decreased expression in IK_{Ca} and SK_{Ca} involved in EDH formation, and also of Cx37, Cx40 and Cx43, which form myoendothelial gap junctions transmitting the endothelial hyperpolarization towards the underlying smooth muscle [11]. In addition to the present findings, oxidative stress has been suggested to contribute to the impaired EDH-mediated relaxation [11]. The present findings further extent these previous observations by showing that oxidative stress is generated by several sources including NADPH oxidase, uncoupled eNOS, cytochome P450, COXs and the mitochondrial respiration chain. Moreover, an upregulation of several pro-oxidant enzymes including NADPH oxidase, COX-1, and COX-2 is observed in the arterial wall of CBDL rats. Besides the EDH component, the NO component of the relaxation is also impaired in CBDL rats despite an increased expression of eNOS. This latter response is most likely part of a compensatory mechanism due to the degradation of the excessive formation of NO by superoxide anions as indicated by the increased peroxynitrite level.

The present findings support a key role for the angiotensin system in the CBDL-induced endothelial dysfunction. Indeed, an increased expression of ACE, Ang II and AT1R was observed throughout the arterial wall of CBDL rats. They are also consistent with the fact that losartan, an AT1 receptor antagonist, prevented the CBDL-induced endothelial dysfunction and oxidative stress [11]. A major novel finding of the present study is that ingestion of the probiotic VSL#3 formulation improved the endothelial dysfunction in CBDL rats along with a pronounced improvement of the local angiotensin system and the level of oxidative stress in the arterial wall.

Previous studies have shown that amongst factors involved in the general vasodilatation in cirrhosis is bacterial translocation [3,5,12]. Indeed, an alteration of the gut microflora associated with disruption of the gut mucosal barrier leading to bacterial translocation has been observed in 45–75% of animals with experimental cirrhosis [14,15]. This response, in turn, promotes the subsequent inflammatory response, which will contribute to the hyperdynamic circulatory state in cirrhosis [5,12,16]. Indeed, treatments preventing bacterial translocation using either antiobiotics or pentoxifylline (an inhibitor of TNF- α) reduced the excessive vasodilatation in CBDL rats [5,12,16]. However, the long-term use of antibiotics and pentoxifylline is hazardous due to the risk of inducing bacterial resistance with antibiotics and the poor tolerance of pentoxifylline as reported in a pilot study by Tanikella et al. [18]. In contrast, chronic use of probiotics has not been associated with major side effects [19,20]. Furthermore, previous studies have indicated that intake of probiotics such as the VSL#3 formulation leads to major changes in the composition of the gut flora in both experimental animals and in humans. Indeed, intake of VSL#3 in patients with pouchitis was associated with an increased intestinal bacterial diversity and a reduced fungal diversity in comparison with patients treated with a placebo [27]. VSL#3 treatment also prevented the antibiotic-induced decrease in several indigenous bacterial groups as assessed using a standardized human fecal microbiota in a computer-controlled model of large intestine [28]. Moreover, intake of the VSL#3 formulation in rats altered the species richness and diversity of the luminal intestinal microbiota [29]. However, the possibility that the beneficial effect of the VSL#3 treatment on the CBDL-induced endothelial dysfunction involves an improved bacterial translocation still remains to be determined.

The study of Loguercio et al. [20] indicated that administration of VSL#3 to alcoholic liver cirrhosis patients led to an improvement of the liver function and the increased plasma level of oxidative stress and TNF-α. In the present study, a relatively small but significant improvement of spleen weights was observed suggesting that VSL#3 may also have some effects on cirrhosis in CBDL rats, which, however, cannot account solely for the improved endothelial function.

In addition in a rat model of cirrhosis induced by CCl_4, Chiva et al. [30] reported that the addition of *Lactobacillus johnsonii* to an antioxidant treatment (vitamin C plus glutamate) reduced endotoxemia possibly due to an increased clearance of endotoxins by the monocyte-macrophage system. The VSL#3 treatment also

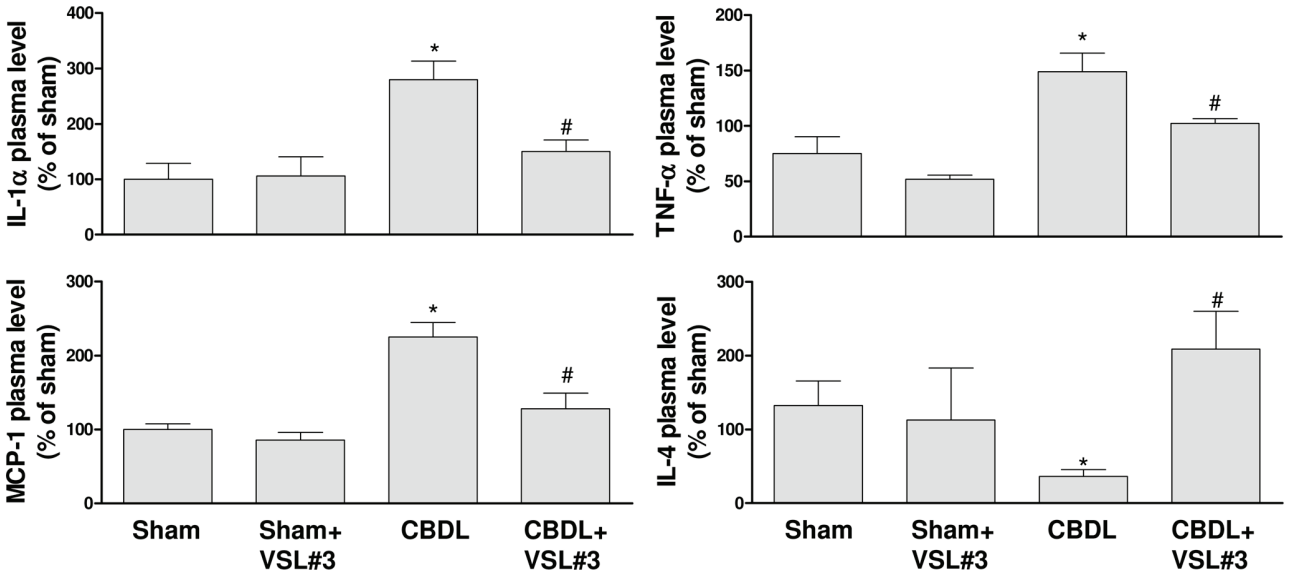

Figure 6. The VSL#3 treatment prevents the CBDL-induced increased formation of pro-inflammatory cytokines including IL-1α, MCP-1 and TNF-α, and decreased formation of the anti-inflammatory cytokine IL-4 in plasma. Results are shown as mean±SEM of 6 different rats. *$P<0.05$ CBDL vs sham, and $^{\#}P<0.05$ CBDL+VSL#3 vs CBDL.
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reduced the CBDL-induced pro-inflammatory response as indicated by the improvement of circulating levels of pro-inflammatory cytokines such as IL-1α and TNF-α. Thus, the protective effect of VSL#3 in CBDL rats may involve, besides an improvement of the gut flora composition, possibly also the stimulation of immunological defence mechanisms and perhaps also the recovery of the intestinal motility, which has been shown to be decreased in cirrhosis with bacterial translocation [31].

Another potential protective mechanism of VSL#3 is that upon fermentation, various probiotics (including those contained in VSL#3, [32]) are capable of releasing ACE inhibitory peptides. This has been observed *in vitro* [32,33] and also *in vivo* in hypertensive rats [34] and patients [35]. In an *in vivo* trial, the ingestion of milk fermented with probiotics led to a significant decrease in blood pressure [35]. In the present study, a normalized vascular expression level of ACE, angiotensin II and AT1R along with a reduced expression of NADPH oxidase was observed in CBDL rats treated with VSL#3. Since infusion of angiotensin II leads to an increased vascular expression of NADPH oxidase and oxidative stress [36], it is likely that the VSL#3 treatment may improve oxidative stress by reducing the local angiotensin system subsequent to the normalization of ACE expression. Alternatively, VSL#3 may also normalize ACE activity via the release of ACE inhibitory peptides as suggested by previous studies [32,35].

In conclusion, the present findings indicate that oxidative stress possibly due to bacterial translocation inducing a pro-inflammatory response and the local angiotensin system is involved in the endothelial dysfunction in CBDL rats, and that this effect is improved by the ingestion of the probiotic VSL#3 formulation.

Author Contributions

Conceived and designed the experiments: MOM VBSK. Performed the experiments: SKR NIK CA MA. Analyzed the data: SKR NIK CA. Contributed reagents/materials/analysis tools: NB. Wrote the paper: SKR MOM VBSK.

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Schematic presentation of the 1st article results:

Common bile duct ligation in rat, induces biliary proliferation at liver level and increase liver size, sign characterize the presence of biliary cirrhosis. This with splenomegaly represents a part of portal hypertension.

Rats received either control drinking water or the probiotic VSL#3 solution (50 billion bacteria.kg body $wt^{-1}.day^{-1}$) for 7 weeks. After 3 weeks, rats underwent surgery with either resection of the common bile duct or sham surgery. The reactivity of mesenteric artery rings was assessed in organ chambers, expression of proteins by immunofluorescence and Western blot analysis, oxidative stress using dihydroethidium, and plasma pro-inflammatory cytokine levels by flow cytometry.

Results: Both NO- and EDH-mediated relaxations to acetylcholine were reduced in the CBDL group compared to the sham group, and associated with a reduced expression of Cx37, Cx40, Cx43, IK_{Ca} and SK_{Ca} and an increased expression of endothelial NO synthase (eNOS). In aortic sections, increased expression of NADPH oxidase subunits, angiotensin converting enzyme, AT1 receptors and angiotensin II, and formation of ROS and peroxynitrite were observed. VSL#3 prevented the deleterious effect of CBDL on EDH-mediated relaxations, vascular expression of connexins, IK_{Ca}, SK_{Ca} and eNOS, oxidative stress, and the angiotensin system. VSL#3 prevented the CBDL-induced increased plasma TNF-α, IL-1α and MCP-1 levels, (Figure 24).

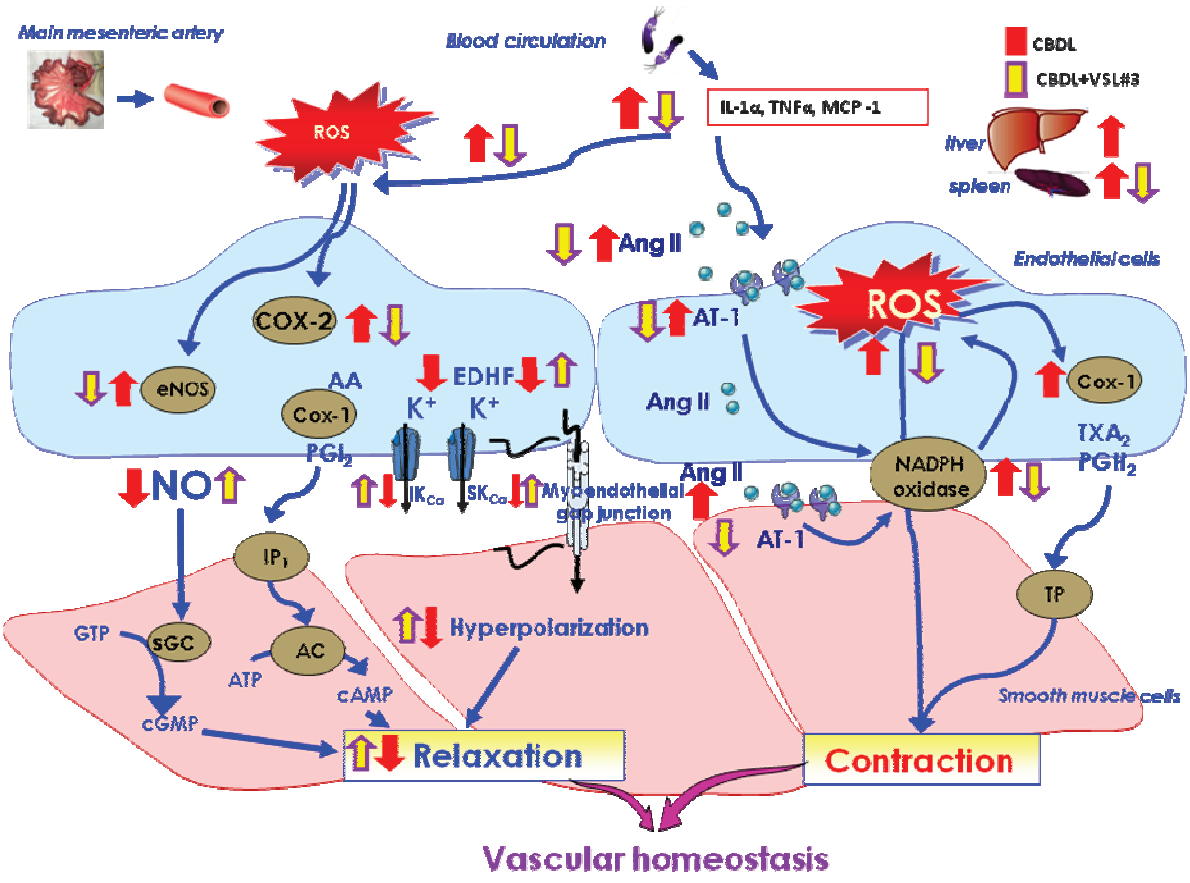

Figure 24. Schema presenting the effects of CBDL in rats and the therapeutic preventive effects of VSL#3 in the mesenteric arteries and both liver and spleen size. EDHF: endothelium derived hyperpolarizing factor, eNOS: endothelial NO synthase, ROS: reactive oxygen species, K^+: potassium, NO: nitric oxide, AT-1: angiotensin receptor type, AA: arachidonic acid, COX: cycloogynase, IK_{Ca} and SK_{Ca}: calcium dependent potassium channels of intermediate and small conductance, ⇑⇓: effects of CBDL, ⇑ increase and ⇓ decrease, ⇓⇑ : effects of CBDL+VSL# 3, ⇓ increase and ⇑ decrease.

PART II

RESULTS

PART II

Polyphenol-rich blackcurrant juice prevents endothelial dysfunction in the mesenteric artery of cirrhotic rats with portal hypertension: Role of oxidative stress and the angiotensin system

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There are numerous reports showing that polyphenols can play an important role in prevention of degenerative diseases such as cancer and attenuating inflammatory diseases like liver fibrosis. Polyphenols are well known to have antioxidant properties and also abilities to modulate the activity of a wide variety of enzymes and cell receptors. For these reasons we are interested to study the effect of polyphenols-rich blackcurrant in rat model of liver cirrhosis.

Chronic liver diseases with portal hypertension are characterized by a progressive vasodilatation associated with an endothelial dysfunction and vascular oxidative stress, which is especially pronounced in the splanchnic and pulmonary beds. It has been suggested that one of the pathophysiological mechanisms leading to the general vasodilatation is related to bacterial translocation and activation of rennin-angiotensin-aldosterone system.

The aim of the present study was to determine whether the ingestion of a polyphenol-rich Blackcurrant juice (PRBJ) improves the endothelial dysfunction and vascular oxidative stress in CBDL rats, and, if so, to determine the underlying mechanism.

Male Wistar rats (8 rats per group) received either control drinking water or water containing a dose of 60 mg/kg of polyphenol-rich Blackcurrant juice for 7 weeks. After 3 weeks, the rats underwent surgery either with ligation and resection of the common bile duct (CBDL rats) or sham surgery (sham rats). Thereafter, the rats were sacrificed after 4 weeks. Reactivity of mesenteric artery rings was assessed in organ chambers, the expression level of proteins in mesenteric artery and/or aorta sections by immunofluorescence, and the vascular formation of reactive oxygen species (ROS) by dihydroethidium (DHE). Plasma levels of pro-

inflammatory cytokines including TNF-α, IL-1α, MCP-1 and IL-4 were evaluated by flow cytometry.

Both the nitric oxide (NO)- and the endothelium-dependent hyperpolarization (EDHF)-mediated relaxations to acetylcholine in rings with endothelium were significantly reduced in CBDL rats compared to sham rats. In contrast, relaxations to sodium nitroprusside (NO donor) and levcromakalim (an ATP-sensitive K^+ channel opener) in rings without endothelium were minimally affected. Impaired endothelium-dependent relaxations were associated with a reduced vascular expression of Cx37 and SK_{Ca} and an increased expression of eNOS (endothelial NO synthase), cyclooxygenase-2 and inducible NOS. In aortic sections of CBDL rats, an increased expression of NADPH oxidase subunits, angiotensin II, angiotensin-converting enzyme (ACE) and angiotensin type 1 receptors (AT1) are observed as well as an increased vascular formation of ROS and peroxynitrites. The endothelial dysfunction in CBDL rats was significantly prevented by PRBJ, and this effect was associated with the normalization of the vascular expression of Cx37 and $SK_{Ca,}$ and eNOS. PRBJ treatment also reduced vascular oxidative stress in the aorta, and the increased plasma level of pro-inflammatory cytokines including TNF-α, IL-1α, MCP-1 and IL-4 in CBDL rats.

In conclusion, these results indicate that PRBJ ingestion prevented the blunted NO- and EDHF-mediated endothelium-dependent relaxation in the mesenteric artery of CBDL rats most likely by preventing the excessive oxidative stress in the arterial wall and attenuating the activation of local angiotensin system.

Polyphenol-rich blackcurrant juice prevents endothelial dysfunction in the mesenteric artery of cirrhotic rats with portal hypertension:

Role of oxidative stress and the angiotensin system

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Abstract:

Chronic liver diseases with portal hypertension are characterized by a progressive vasodilatation associated with an endothelial dysfunction and vascular oxidative stress, and have been suggested to involve bacterial translocation and the angiotensin system. Since blackcurrant berries are a rich natural source of antioxidants, the aim of the present study was to determine the possibility that ingestion of a polyphenol-rich blackcurrant juice (PRBJ) prevents endothelial dysfunction in a rat model of cirrhosis induced by chronic bile duct ligation (CBDL), and, if so, to determine the underlying mechanism.

Male Wistar rats received either control drinking water or water containing 60 mg/kg gallic acid equivalents (GAE) of PRBJ for 7 weeks. After 3 weeks, rats underwent surgery with CBDL or sham surgery.

Both the acetylcholine-induced nitric oxide (NO)- and the endothelium-dependent hyperpolarization (EDH)-mediated relaxations in mesenteric artery rings are significantly reduced in CBDL rats compared to sham rats, and associated with a reduced vascular expression of connexion Cx37 and small conductance calcium-dependent K^+ channel (SK_{Ca}), and an increased expression of endothelial NO synthase (eNOS). An increased formation of reactive oxygen species (ROS), and expression level of nitrotyrosine, p22phox and p47phox NADPH oxidase subunits, cyclooxygenase-2, inducible NOS, angiotensin II, angiotensin-converting enzyme (ACE) and angiotensin type 1 receptors (AT1) are observed in aortic sections in the CBDL group. Chronic intake of PRBJ prevented the CBDL-induced impaired EDH-mediated relaxation, oxidative stress and expression of different target proteins in the arterial wall. In addition, PRBJ prevented the CBDL-induced increase in the plasma level of pro-inflammatory cytokines (IL-1α, MCP-1 and TNFα) and decrease of the anti-inflammatory cytokine level, IL-4.

Altogether, these observations indicate that regular ingestion of PRBJ prevents the CBDL-induced endothelial dysfunction and, in particular, the blunted EDH component in the mesenteric artery by improving vascular oxidative stress and the angiotensin system.

Keywords: Portal Hypertension; Endothelial Dysfunction; Blackcurrant; Polyphenols; Angiotensin II; Oxidative Stress; Inflammatory Cytokines.

Introduction

Advanced chronic liver diseases with portal hypertension are characterized by a generalized vasodilatation with endothelial dysfunction, which is especially observed in the splanchnic and pulmonary beds (Abraldes, Iwakiri et al. 2006). The mechanism contributing to the generalized vasodilatation still remains poorly characterized (Nunes, Lebrec et al. 2001; Zhang, Ling et al. 2003; Ling, Zhang et al. 2004; Sztrymf, Rabiller et al. 2004; Zhang, Han de et al. 2007). In a rat model of partial portal vein ligation, Albrades et al (Abraldes, Iwakiri et al. 2006) reported that the initial event subsequent to the induction of portal hypertension is an up-regulation of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) in the intestinal microcirculation. Indeed, an increased formation of NO has been involved in the endothelial dysfunction in chronic liver diseases in patients (Rolla, Brussino et al. 1998; Gomez, Barbera et al. 2006), and also in the chronic bile duct ligated (CBDL) rat model (Nunes, Lebrec et al. 2001; Ling, Zhang et al. 2004; Zhang, Ling et al. 2003). In addition, endothelial dysfunction in chronic liver diseases in patients and also in experimental portal hypertension has also been shown to involve a high level of oxidative stress in the arterial wall (Chen, Mo et al. 1997; Dal-Ros, Oswald-Mammosser et al. 2010). The increased formation of reactive oxygen species (ROS) has been suggested to be initiated by bacterial translocation and the subsequent development of endotoxaemia, and also to the chronic activation of the systemic and local angiotensin system (Yang, Lin et al. 2002; Bode and Bode 2005; Helmy, Newby et al. 2003), which promotes the NADPH oxidase-mediated formation of superoxide anions (Sarr, Chataigneau et al. 2006; Dal-Ros, Oswald-Mammosser et al. 2010; Yu, Sanchez-Lozada et al. 2010). Moreover, oxidative stress has been shown to contribute to endothelial dysfunction in CBDL rats by affecting the endothelium-dependent hyperpolarization component (Dal-Ros, Oswald-Mammosser et al. 2010), and possibly also by promoting an inflammatory response subsequent to the expression of pro-inflammatory cytokines such as IL-1 and TNF-α (Bode and Bode 2005; Zhang, Han de et al. 2007; Yu, Sanchez-Lozada et al. 2010). The aim of the present study was to evaluate the possibility that chronic intake of a natural rich source of antioxidants such as the polyphenol-rich backcurrant juice prevents endothelial dysfunction in an animal model of biliary cirrhosis with portal hypertension, and, if so, to characterize the underlying mechanism.

Material and Methods

Ethics statement

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

Animal models

Male Wistar rats (10-12 weeks old) were anaesthetized with an intraperitoneal mixture of ketamine (80 mg.kg body wt^{-1}) and xylazine (4 mg.kg body wt^{-1}). Biliary cirrhosis was induced by CBDL. After laparotomy, the bile duct was isolated, double ligated and resected between the two ligatures. Sham rats had laparotomy, underwent mobilization of the common bile duct without ligation. After surgery, rats were housed in a thermo-neutral environment, on a 12:12 h photoperiod and were provided food and water *ad libitum*

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Protocols

Eight CBDL rats and seven sham rats received three weeks before surgery and four weeks thereafter a polyphenol-rich blackcurrant juice (PRBJ, 60 mg GAE.kg body wt^{-1} daily) in the drinking water, and seven CBDL rats and five sham rats received only drinking water. Four weeks after surgery, rats were sacrificed. After excision, the mesenteric artery and the aorta were placed in Krebs-Ringer bicarbonate solution for the subsequent determination of vascular reactivity using organ chambers. The liver and spleen were removed and weighted. Liver samples (taken from the median lobe) from each rat were incubated in Bouin-Holland fixative and embedded in paraffin for subsequent histological analysis. Segments of the main superior mesenteric artery and the thoracic aorta were cleaned, embedded in Tissu-Tek O.C.T. compound and snap-frozen for subsequent immunofluorescence studies and the determination of the level of oxidative stress. Samples of blood were taken from heart puncture, and thereafter plasma was stored at -20°C for subsequent determination the levels of cytokines.

Assessment of liver cirrhosis and portal hypertension

Sections of liver samples (5 µm thick) were stained with hematoxylin, eosin and Masson's trichrome stain and evaluated by light microscopy. Histologically, liver of chronic bile duct ligation showed evidence of intrahepatic tubular duct hypertrophy with severe fibrosis. Portal hypertension was reflected by an increase in the spleen weight.

Vascular reactivity studies

Vascular reactivity was performed using the main superior mesenteric artery as described previously (Dal-Ros, Oswald-Mammosser et al. 2010). Briefly, the main superior mesenteric artery was cleaned of connective tissue, cut into rings (2-3 mm in length) and suspended in organ baths containing oxygenated Krebs bicarbonate solution for the determination of changes in isometric tension. After equilibration and test of the presence of a functional endothelium, rings were contracted with phenylephrine (PE, 1 µM) before the construction of concentration-relaxation curve in response to acetylcholine (ACh) in endothelium-intact rings, and to either sodium nitroprusside or levcromakalim in endothelium-denuded rings. In some experiments, rings were exposed to an inhibitor for 30 min before addition of PE. Relaxations are expressed as percentage of the reversal of the contraction induced by PE.

Immunofluorescence studies

Frozen arteries were cryosectioned at 14 µm and, then, the sections were air-dried for 15 min and stored at -80°C until use. Sections were first fixed with paraformaldehyde at 4%, washed and treated with 10% milk or 5% goat serum in PBS containing 0.1% Triton X-100 for 1 h at room temperature to block non-specific binding. Sections were then incubated overnight at 4°C with an antibody directed against either eNOS (1/100), inducible NOS (1/200), connexin C37 (Cx37, 1/200), which is part of the myoendothelial gap junctions transmitting hyperpolarization to the smooth muscle cells, small conductance calcium-dependent K^+ channels (SK_{Ca}, 1/200), angiotensin II (Ang II, 1/500), angiotensin II type 1 receptor (AT1R, 1/400), angiotensin-converting enzyme (ACE, 1/200), nitrotyrosine (1/200), p47phox (1/200), p22phox (1/200) or cyclooxygenase-2 (COX-2, 1/200). Sections were then washed with PBS, incubated with the secondary antibody (Alexa 488- conjugated goat anti-rabbit IgG or Alexa 637-conjugated goat anti-rabbit, 1/300) in the same buffer for 2 h at room temperature in the dark, and washed before being mounted in Vectashield (Vector Laboratories, Inc. Burlingame, CA 94010) and cover-slipped. For negative controls, primary

antibodies were omitted. The samples were analyzed using a confocal laser-scanning microscope (Leica SP2 UV DM IRBE) with 20 × magnification lens. Quantification of fluorescence signals was performed using Image J software (http://rsweb.nih.gov/ij/).

Determination of vascular and mitochondrial ROS formation

The redox-sensitive fluorescent dye dihydroethidine (DHE) was used to evaluate the *in situ* formation of ROS as described previously by Sarr et al. (Sarr, Chataigneau et al. 2006). Dihydroethidine (2.5 µM, Sigma) was applied onto 25 µm unfixed cryosections of aorta for 30 min at 37°C in a light-protected humidified chamber. To determine the nature and the sources of ROS, sections were incubated with a pharmacological modulator for 30 min at 37°C before addition of DHE. MitoSox, a mitochondrion-specific hydroethidine-derivative fluorescent dye, was used to assess the mitochondrial formation of ROS. Briefly, 14 µm unfixed cryosections of aorta were incubated with MitoSox (5 µM at 37°C for 60 min) in a light-protected humidified chamber. After incubation, sections were washed three times, mounted in Vectashield and cover-slipped. Images were obtained with a Leica SP2 UV DM IRBE laser scanning confocal microscope. Quantification of staining levels was performed using FIJI GPL v2 software.

Determination of plasma cytokine levels

The determination of the plasma level of TNF-α, IL-1α, MCP-1 and IL-4 was performed using a commercial rat cytokine Kit Flowcytomix™ (eBioscience SAS, Paris, France), according to the protocol supplied by the manufacturer. The raw data were analysed using "The FlowCytomixPro software".

Products and chemicals

Antibodies were purchased as indicated: anti-rat Cx37 polyclonal antibody (Alpha Diagnostic, San Antonio, USA), anti-K_{Ca}2.3 (small conductance Ca^{2+}-activated K^+ channel 3, SK_{Ca}, Alomone Labs, Jerusalem, Israel), antibody directed against either p22phox, p47phox NADPH oxidase subunits or AT1 receptors (Santa Cruz Biotechnology, Santa Cruz, USA), purified mouse anti-eNOS, anti-iNOS, and COX-2 monoclonal antibody (BD Transduction Laboratories™, San Jose, USA), anti-nitrotyrosine (Millipore, Upstate Cell Signaling, USA),

ACE antibody (Abbiotec, San Diego, CA, USA), rabbit anti-angiotensin II (Peninsula laboratories, San Carlos, CA, USA), and Alexa fluor-488 or -637 labelled goat anti-rabbit IgG (Invitrogen, Molecular Probes). All chemicals were from Sigma-Aldrich except apamin (APA) and charybdotoxin (CTX) from Latoxan (Valence, France), and MnTMPyP from Alexis Biochemicals Corporation (San Diego, CA, USA). Blackcurrant juice concentrate (66.2 °Brix) was kindly provided by Eckes-Granini (Nieder-Olm, Germany). Final blackcurrant juice was reconstituted by dilution to 11.6 °Brix in distilled water (3.3 g/l polyphenols expressed as GAE measured by the Folin-Ciocalteu method).

Statistical analyses

Values were expressed as means±SEM. Statistical evaluation was performed using either Student's *t* test for paired data or an analysis of variance followed by the Bonferroni post-hoc test to identify significant difference as appropriate with GraphPad Prism (version 5 for Microsoft windows. GraphPad software, Inc, San Diego, CA, USA). Values of $P<0.05$ were considered to be statistically significant.

Results

Characteristics and histologic findings

As shown in Table 1, four weeks after surgery, CBDL rats had an increased liver and spleen weight and there was a pronounced bile duct proliferation (data not shown). Chronic intake of PRBJ 3 weeks before surgery and 4 weeks thereafter was associated with a slight but significant reduction of the weight of the spleen but not of the weight of the liver in CBDL rats (Table 1). The PRBJ treatment alone affected minimally liver and spleen weight in sham rats (Table 1).

PRBJ treatment prevents the CBDL-induced endothelial dysfunction in the mesenteric artery

ACh caused similar concentration-dependent relaxations in mesenteric artery rings from control and CBDL rats (maximal relaxations amounted to 97.4 ± 1.4% and 94.9 ± 2.4%, respectively, n = 6-8). However, the NO component of the relaxation as assessed in the

presence of indomethacin and charybdotoxin plus apamin to prevent the formation of vasoactive prostanoids and EDH, respectively, was slightly but significantly blunted in the CBDL group (Figure 1A). In addition, the EDH-mediated component of the relaxation as assessed in the presence of indomethacin plus N^G-nitro-L-arginine (a NOS inhibitor), was markedly reduced in the CBDL group (Figure 1B).

Although the PRBJ treatment did not affect the CBDL-induced impaired NO component, it significantly improved the EDH component (Figures 1A and B). The PRBJ treatment alone did affect neither the NO nor the EDH component in the sham group (Figures 1 A and B). Both sodium nitroprusside (a NO donor)- and levcromakalim (an ATP-sensitive K^+ channel opener)-induced endothelium-independent relaxations were similar in all groups (Figures 1C and D).

PRBJ treatment prevents CBDL-induced up-regulation of eNOS and down-regulation of SK_{Ca} and Cx37 in the mesenteric artery

Pronounced fluorescence signals for $SK_{Ca,}$ the small conductance calcium-activated K^+ channel involved in EDH (Dal-Ros, Oswald-Mammosser et al. 2010), and Cx37 involved in myoendothelial gap junction transmitting the hyperpolarization from endothelial cells to the underlying smooth muscle cell to cause relaxation (Dal-Ros, Oswald-Mammosser et al. 2010), were observed predominantly at the luminal surface and also to some extent in the vascular smooth muscle of the mesenteric artery in the sham group (Figure 2). The fluorescence signals of SK_{Ca} and Cx37 were significantly reduced in the CBDL group (Figure 2). In contrast to SK_{Ca} and Cx37, the eNOS fluorescence signal observed at the luminal surface of the mesenteric artery was significantly increased in the CBDL group (Figure 2). The PRBJ treatment significantly prevented the CBDL-induced down-regulation of both SK_{Ca} and Cx37 expression, and the up-regulation of eNOS (Figure 2). The PRBJ treatment alone had minor effects in the sham group (Figure 2).

PRBJ treatment reduces the CBDL-induced vascular oxidative stress involving several sources in the aorta

In the CBDL group, a markedly increased DHE fluorescence signal, MitoSOX fluorescence signal and nitrotyrosine fluorescence signal were observed throughout the aortic wall (Figure 3A). All these effects were significantly prevented by the PRBJ treatment (Figure 3A). The PRBJ treatment alone had no significant effect in the sham group (Figure 3A). The CBDL-induced vascular oxidative stress was significantly reduced by MnTMPyP (a membrane permeant superoxide dismutase mimetic) and PEG-catalase (a membrane permeant analogue of catalase), and slightly but not significantly by native SOD and catalase (Figure 3B). In addition, the CBDL-induced oxidative stress was also markedly inhibited by apocynin (NADPH oxidase inhibitor and antioxidant), L-NA (non-selective NOS inhibitor), sulfaphenazol (cytochrome P450 inhibitor), indomethacin (cyclooxygenase inhibitor) and by a combination of inhibitors of the mitochondrial respiration chain (KCN, myxothiazol and rotenone) (Figure 3C). These findings suggest that several sources of ROS contribute to the increased vascular level of oxidative stress in the CBDL group including NADPH oxidase, COXs, NOS, cytochrome P450 and the mitochondrial respiration chain.

To obtain further evidence for the role of NADPH oxidase, COX and NOS, their expression level was determined by immunofluorescence staining. An increased expression level of the NADPH oxidase subunits p22phox and p47phox was observed in the aorta, and COX-2 and inducible NOS in the mesenteric artery of the CBDL group compared to the sham group (Figure 4). The PRBJ treatment significantly prevented the CBDL-induced expression of these markers (Figure 4). The PRBJ treatment alone had only minor effects in the sham group (Figure 4).

PRBJ improves the CBDL-induced expression of the angiotensin system in the aorta

Previous findings indicating that losartan (an angiotensin type 1 receptor antagonist) prevented the CBDL-induced endothelial dysfunction and oxidative stress in the mesenteric artery of rats suggested the involvement of the angiotensin system (Dal-Ros, Oswald-Mammosser et al. 2010). To determine the possibility that the PRBJ treatment affects the vascular angiotensin system, the expression level of angiotensin II, AT1 receptors and ACE was assessed by immunofluorescence staining in aortic sections. The fluorescence staining of

Ang II, AT1 receptors and ACE was significantly increased in the CBDL group compared to that in the sham group (Figure 5). The PRBJ treatment prevented CDBL-induced up-regulation of Ang II, AT1R and ACE whereas it did not affected the signals in the sham group (Figure 5).

PRBJ reduces the CBDL-induced plasma level of pro-inflammatory cytokines

A significant increase in the plasma level of pro-inflammatory cytokines including IL-α, MCP-1 and TNF-α was observed in the CBDL group and this effect was significantly prevented by the PRBJ treatment (Figure 6). In contrast, the plasma level of IL-4, a potent anti-inflammatory cytokine, was markedly reduced in the CBDL group, no such effect was observed in the PRBJ-treated CBDL group (Figure 6). The PRBJ treatment alone affected only minimally the level of the different cytokines in the sham group (Figure 6).

Discussion

The present findings indicate that the CBDL-induced endothelial dysfunction affecting predominantly the EDH component in the mesenteric artery is associated with an increased vascular level of oxidative stress subsequent to the upregulation of the local angiotensin system and an increased level of circulating pro-inflammatory mediators. They further indicate that chronic ingestion of a blackcurrant juice, a polyphenol-rich natural source of antioxidants, prevented the CBDL-induced endothelial dysfunction, vascular oxidative stress and the pro-inflammatory response by improving the local angiotensin system.

Chronic bile duct ligation in rats is an experimental model of cirrhosis, which is characterized by an increased spleen weight reflecting portal hypertension, associated with an increased proliferation of biliary ducts. Consistent with previous observations (Dal-Ros, Oswald-Mammosser et al. 2010), CBDL leads to an endothelial dysfunction affecting predominantly the EDH component and also as shown in the present study to a slightly but significantly reduced NO component. The blunted EDH component is explained, at least in part, by a reduced expression of SK_{Ca} and Cx37 levels, and also as shown previously of IK_{Ca}, Cx40 and Cx43, which all contribute to the initiation and transmission of the hyperpolarization from endothelial cells to the underlying smooth muscle cells (Dal-Ros, Oswald-Mammosser et al.

2010). Moreover, the high level of oxidative stress in the arterial wall of CBDL rats appears to be the key mediator of the endothelial dysfunction. Indeed, increased levels of oxidative stress have been observed in blood of patients with decompensated liver cirrhosis (Chen, Mo et al. 1997), and also in the arterial wall of experimental models of portal hypertension including CBDL (Fernando, Marley et al. 1998; Vercelino, Tieppo et al. 2008; Dal-Ros, Oswald-Mammosser et al. 2010). Moreover, apocynin, an antioxidant and NADPH oxidase inhibitor, prevented the CBDL-induced impaired EDH-mediated component of the relaxation, vascular oxidative stress and the decreased expression of calcium-activated potassium channels and connexins (Dal-Ros, Oswald-Mammosser et al. 2010). A determinant role for oxidative stress is also supported by the fact that the antioxidant N-acetylcysteine prevented the development of the hyperdynamic circulation in portal hypertensive rats associated with a reduced urinary level of F_2-isoprostanes, a marker of oxidative stress (Fernando, Marley et al. 1998). The present findings further extent these previous observations (Dal-Ros, Oswald-Mammosser et al. 2010) by indicating that CBDL-induced vascular oxidative stress involves both the intracellular formation of superoxide anions and hydrogen peroxide. Furthermore, the pharmacological characterization of the sources of ROS has indicated the involvement of several sources including NADPH oxidase, uncoupled NOS, cytochrome P450, COXs and the mitochondrial respiration chain. Moreover, CBDL is associated with the upregulation of several sources of ROS including p22phox and p47phox NADPH oxidase subunits, COX-2 and both endothelial and inducible NOS in the arterial wall. Since apocynin has been shown to prevent the CBDL-induced upregulation of NADPH oxidase and eNOS, oxidative stress appears to be an early determinant stimulatory event to promote the upregulation of pro-oxidant enzymes, and, thus, to sustain oxidative stress (Dal-Ros, Oswald-Mammosser et al. 2010).

A major novel finding of the present study is that chronic ingestion of a polyphenol-rich blackcurrant juice, a natural source of antioxidants, prevented the endothelial dysfunction affecting the EDH component in CBDL rats most likely as a consequence of the improvement of the vascular level of oxidative stress and the prevention of the down-regulation of the expression of SK_{Ca} and Cx37. Blackcurrant juices is well known to contain high levels of anthocyanins predominantly delphinidin-3-rutinoside, delphinidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3-glucoside, which have potent antioxidant activity (Maatta, Kamal-Eldin et al. 2003; Wu, Beecher et al. 2004; Borges, Degeneve et al. 2010). However, PRBJ may also improve CBDL-induced oxidative stress indirectly as shown by the present

observations since it prevented the upregulation of several sources of ROS including NADPH oxidase, COX-2, and NOSs. Previous studies have also indicated that intake of several other polyphenol-rich sources such as a red wine extract prevented vascular oxidative stress and endothelial dysfunction in the angiotensin II-induced hypertensive rats (Sarr, Chataigneau et al. 2006) and in old rats (Idris Khodja, Chataigneau et al. 2012), and tea catechin in the Otsuka Long-Evans Tokushima fatty rat model of metabolic syndrome (Ihm, Lee et al. 2009).

Similarly to the EDH component, the NO component of the relaxation is impaired although only to some extent in the CBDL group despite an upregulation of eNOS expression in the arterial wall. This latter response is most likely part of a compensatory mechanism due to the degradation of the excessive formation of NO by superoxide anions leading to the formation of peroxynitrite. The PRBJ treatment improved the CBDL-induced upregulation of eNOS expression and vascular oxidative stress, which, however, did not result in an improvement of the NO-mediated relaxation in contrast to the enhanced EDH component. Such a difference is most likely explained by the fact that the EDH component is more sensitive to oxidative stress than the NO component as observed previously in vascular aging and angiotensin II-induced endothelial dysfunction and hypertension (Dal-Ros, Bronner et al. 2009; Idris Khodja, Chataigneau et al. 2012).

Although the mechanism underlying the CBDL-induced oxidative stress remains poorly characterized, several lines of evidence suggest the involvement of an increased translocation of bacteria from the intestinal tract to the blood circulation. Indeed, bacterial translocation has been observed in 45-75% of animals with experimental liver cirrhosis (Runyon, Borzio et al. 1995; Wiest, Das et al. 1999). Moreover, it has been shown to induce an inflammatory response, which, in turn, contributes to the hyperdynamic circulatory state observed in liver cirrhosis (Rabiller, Nunes et al. 2002; Sztrymf, Rabiller et al. 2004; Liu, Liu et al. 2012) . In addition, exposure of a human enteric epithelial cell line to *Escherichia coli* has been shown to induce a pro-oxidant response, which facilitates bacteria internalization and translocation (Schoultz, McKay et al. 2012). Consistent with those previous findings, the present ones indicate an increased plasma level of several pro-inflammatory cytokines including IL-1α, MCP-1 and TNF-α and a reduced level of the anti-inflammatory cytokine IL-4 in the CBDL group. The fact that the PRBJ treatment prevented the CBDL-induced vascular oxidative stress and the pro-inflammatory response suggests that the treatment may reduce bacterial translocation. Such a possibility is also supported by the fact that resveratrol, a natural antioxidant, attenuated bacterial translocation in mesenteric lymph nodes, liver and

spleen in rats following an intestinal ischemia/reperfusion injury (Ozkan, Yuzbasioglu et al. 2009).

Several lines of evidence suggest that the CBDL-induced vascular oxidative stress may also involve, besides bacterial translocation, an increased activation of the angiotensin system. Such a response will lead to an excessive formation of angiotensin II, a potent inducer of vascular oxidative stress by stimulating the activation of NADPH oxidase, a major enzymatic source of superoxide anions, and by increasing its expression (Rajagopalan, Kurz et al. 1996; Sarr, Chataigneau et al. 2006). Indeed, portal hypertension in both humans and experimental animals has been associated with an increased plasma renin activity and/or angiotensin II level (Yang, Lin et al. 2002; Helmy, Newby et al. 2003). Moreover, losartan, an AT1 receptor antagonist, has been shown to reduce portal hypertension in patients (De, Bandyopadhyay et al. 2003; Wagatsuma, Naritaka et al. 2006), and to improve the hyperdynamic circulation, the endothelial dysfunction and vascular oxidative stress in CBDL rats (Yang, Lin et al. 2002; Heller, Trebicka et al. 2005; Dal-Ros, Oswald-Mammosser et al. 2010). Consistent with an activation of the angiotensin system are the present findings indicating an upregulation of Ang II, AT1R and ACE in the arterial wall of CBDL rats. Furthermore, they also suggest that the beneficial effect of the PRBJ treatment on the CBDL-induced endothelial dysfunction involves the prevention of the activation of the angiotensin system as indicated by similar expression levels of Ang II, AT1R and ACE in the PRBJ + CBDL group and the sham group. Previous findings have also indicated that resveratrol decreases the expression of AT1R through sirtuin1 activation in vascular smooth muscle cells (Miyazaki, Ichiki et al. 2008). Potential mechanisms contributing to limit the activation of the angiotensin system by polyphenols involve their ability to inhibit angiotensin converting enzyme activity (Kang, Jeon et al. 2002; Actis-Goretta, Ottaviani et al. 2003) but possibly also their antioxidant properties since oxidative stress has been shown to induce AT1R and ACE expression (Banday and Lokhandwala 2011; Mu, He et al. 2013).

In conclusion, the present findings indicate that chronic ingestion of a blackcurrant juice prevents endothelial dysfunction and the inflammatory response in CBDL rats. The beneficial effect involves its ability to prevent vascular oxidative stress, in part, by preventing the activation of the local angiotensin system and possibly also bacterial translocation.

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Table 1. Effect of chronic PRBJ ingestion on body, liver and spleen weight in both sham and CBDL rats.

	Initial body weight (g)	Final body weight (g)	Liver (% total weight)	Spleen (% total weight)
Sham	365±19.5	505±30.2	3.24±0.06	0.20±0.02
Sham+PRBJ	365±23.7	538±29.8	3.14±0.13	0.19±0.01
CBDL	369±16.3	485±30.5	6.69±0.63*	0.69±0.05*
CBDL+PRBJ	367±13.8	500±31.2	5.91±0.8	0.56±0.12#

Values are shown as mean±SEM of 5 rats. *P<0.05 for CBDL vs sham, and #P<0.05 for CBDL+PRBJ vs CBDL.

Figure legends

Figure 1. Ingestion of PRBJ improves the CBDL-induced blunted EDH but not NO-mediated relaxations in rat mesenteric artery rings. A) The NO component of the relaxation was assessed in the presence of indomethacin (10 μM) and apamin plus charybdotoxin (100 nM each), and B) the EDH component in the presence of indomethacin and N^G-nitro-L-arginine (300 μM). C) Relaxations to sodium nitroprusside (an exogenous donor of NO) and D) levcromakalim (an ATP-sensitive K^+channel opener) in mesenteric artery rings without endothelium. Results are shown as mean±SEM of 5-7 different rats; $^*P<0.05$ for CBDL vs sham, and $^\#P<0.05$ for CBDL+PRBJ vs CBDL.

Figure 2. The PRBJ ingestion prevents the CBDL-induced up-regulation eNOS and down-regulation of both SK_{ca} and connexin Cx37 in the mesenteric artery. Upper panels show representative immunofluorescence staining, and lower panels corresponding cumulative data. Results are shown as mean±SEM of 4 to 5 different rats. $^*P<0.05$ for CBDL vs sham, and $^\#P<0.05$ for CBDL+PRBJ vs CBDL.

Figure 3. The PRBJ treatment prevents the CBDL-induced oxidative stress in the aortic wall. (A) *In situ* determination of the level of oxidative stress as assessed using dihydroethidine (DHE), MitoSox, and peroxynitrite (nitrotyrosine) in the aorta. Top: representative fluorescence staining; bottom: corresponding cumulative data for 4 to 5 rats/group. (B) Aortic sections from CBDL rats were exposed either to B) MnTMPyP (membrane-permeant superoxide dismutase mimetic), superoxide dismutase (SOD), PEG-catalase (membrane-permeant catalase) or catalase, and (C) apocynin (Apo, NADPH oxidase inhibitor and antioxidant), L-NA (NO synthase inhibitor), sulfaphenazol (Sulfa, cytochrome P450 inhibitor), indomethacin (Indo, cyclooxygenase inhibitor) or the combination KCN, myxothiazol (Myxo) and rotenone (Rot, inhibitors of the mitochondrial respiration chain) for 30 min before DHE staining. Top: representative ethidium staining; bottom: corresponding cumulative data for 4 rats. Results are shown as mean±SEM. $^*P<0.05$ for CBDL vs sham, and $^\#P<0.05$ for CBDL+PRBJ vs CBDL for A; $*P<0.05$ for CBDL with inhibitors vs CBDL in B and C.

Figure 4. The PRBJ treatment prevents the CBDL-induced up-regulation of the NAPDH oxidase subunits p22phox and p47phox in aortic sections, and COX-2 and inducible NOS (iNOS) in mesenteric artery sections. Left panels: Representative immunofluorescence staining; Right panels: corresponding cumulative data. Results are shown as mean±SEM of 4 different rats. $^*P<0.05$ for CBDL vs sham, and $^\#P<0.05$ for CBDL+PRBJ vs CBDL.

Figure 5. The PRBJ treatment prevents the CBDL-induced up-regulation of several components of the local angiotensin system in the aorta. The expression level of angiotensin II (Ang II), angiotensin II type 1 receptors (AT1R) and angiotensin-converting enzyme (ACE) was determined by immunofluorescence staining. Upper panels: Representative immunofluorescence staining; lower panels: corresponding cumulative data. Results are shown as mean±SEM of 4 different rats. $^{*}P<0.05$ for CBDL vs sham, and $^{\#}P<0.05$ for CBDL+PRBJ vs CBDL.

Figure 6. The PRBJ treatment prevents the CBDL-induced increase levels of pro-inflammatory cytokines including IL-1α, MCP-1 and TNF-α, and the decreased level the anti-inflammatory cytokine IL-4 in plasma. Results are shown as mean±SEM of 4 different rats. $^{*}P<0.05$ for CBDL vs sham, and $^{\#}P< 0.05$ for CBDL+PRBJ vs CBDL.

Figure 1

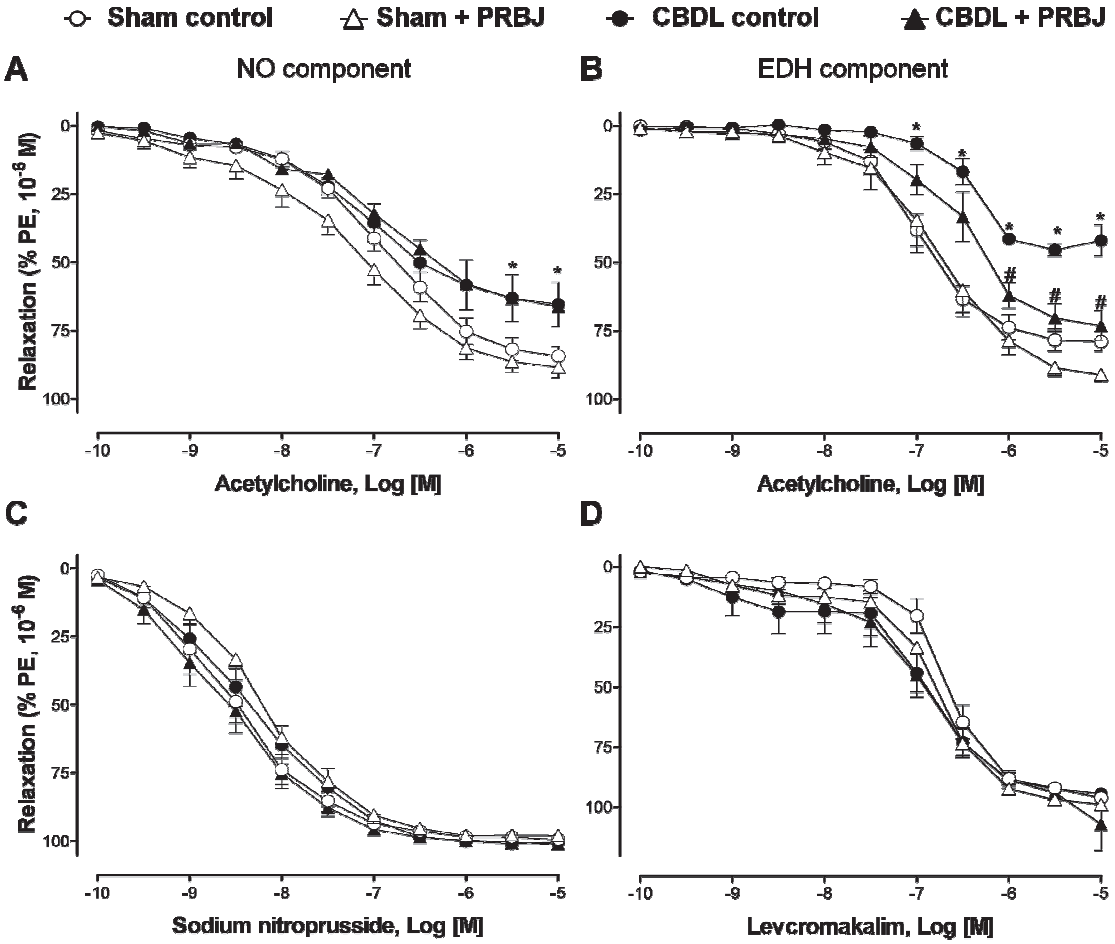
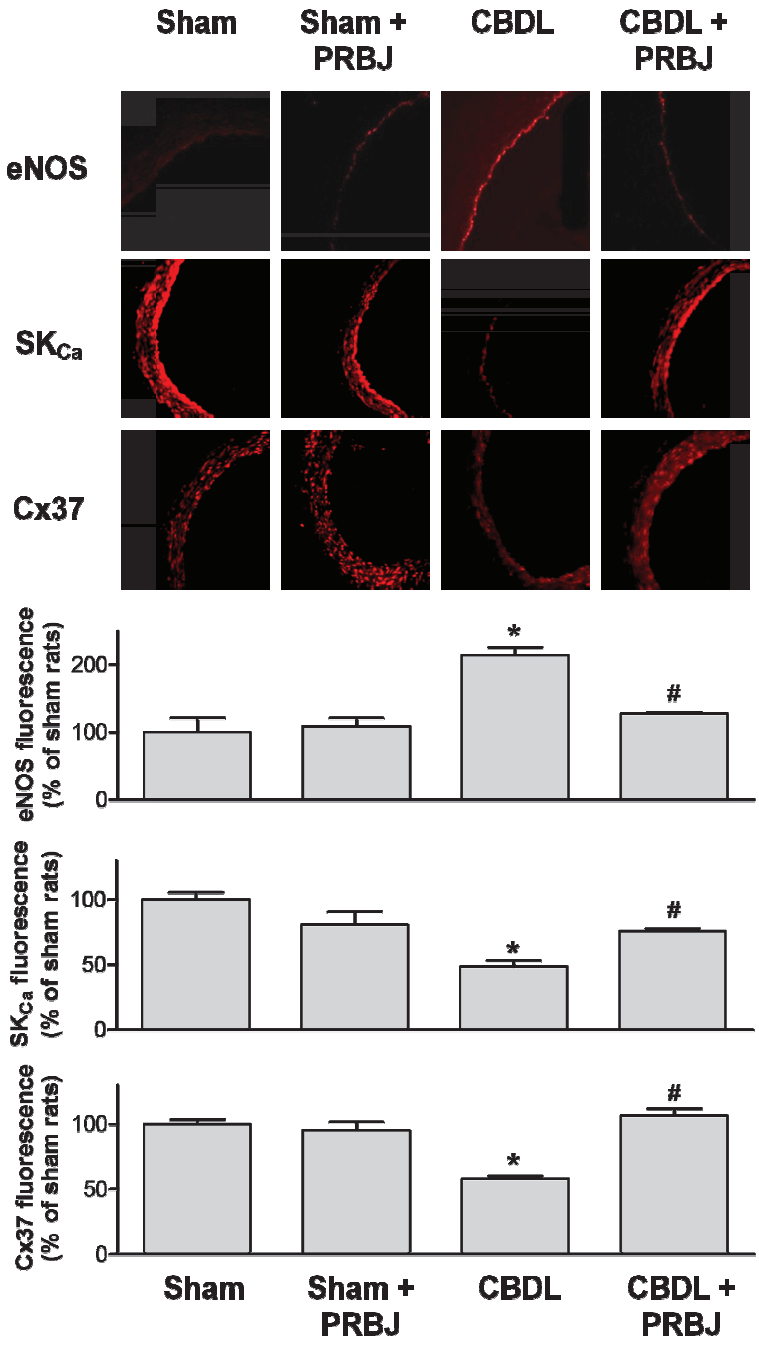

Sham control
Sham + PRBJ
CBDL control
CBDL + PRBJ
A
NO component
B
EDH component
Relaxation (% PE, 10-6 M)
Acetylcholine, Log [M]
C
D
Sodium nitroprusside, Log [M]
Levcromakalim, Log [M]

Figure 2


Sham
Sham + PRBJ
CBDL
CBDL + PRBJ
eNOS
SKCa
Cx37
eNOS fluorescence (% of sham rats)
SKCa fluorescence (% of sham rats)
Cx37 fluorescence (% of sham rats)
0
50
100
200
*
#
Sham
Sham + PRBJ
CBDL
CBDL + PRBJ

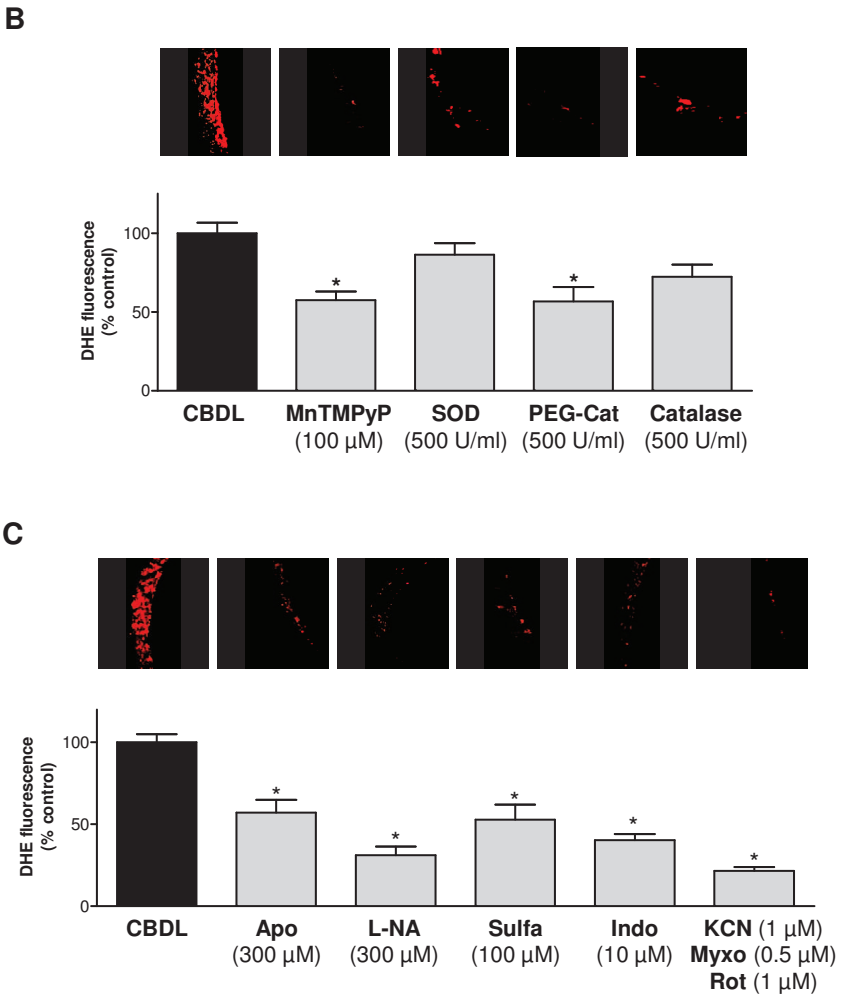

B
DHE fluorescence (% control)
100
50
0
*
*
CBDL
MnTMPyP (100 µM)
SOD (500 U/ml)
PEG-Cat (500 U/ml)
Catalase (500 U/ml)
C
DHE fluorescence (% control)
100
50
0
*
*
*
*
*
CBDL
Apo (300 µM)
L-NA (300 µM)
Sulfa (100 µM)
Indo (10 µM)
KCN (1 µM) Myxo (0.5 µM) Rot (1 µM)

Figure 3

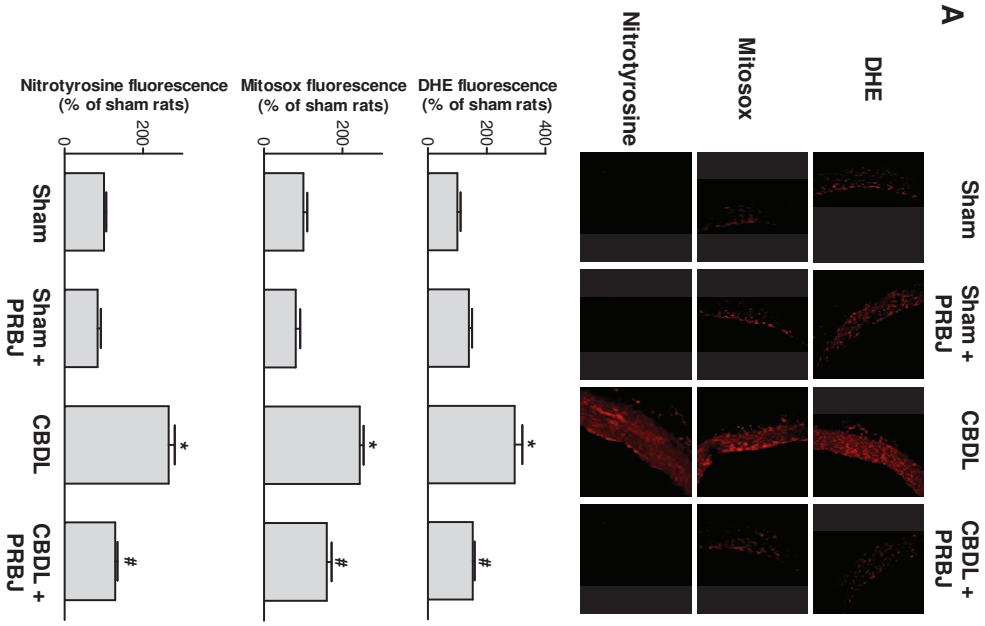

A
DHE
Mitosox
Nitrotyrosine
Sham
Sham + PRBJ
CBDL
CBDL + PRBJ
DHE fluorescence (% of sham rats)
Mitosox fluorescence (% of sham rats)
Nitrotyrosine fluorescence (% of sham rats)
0
200
400
*
#

Figure 3

Figure 4

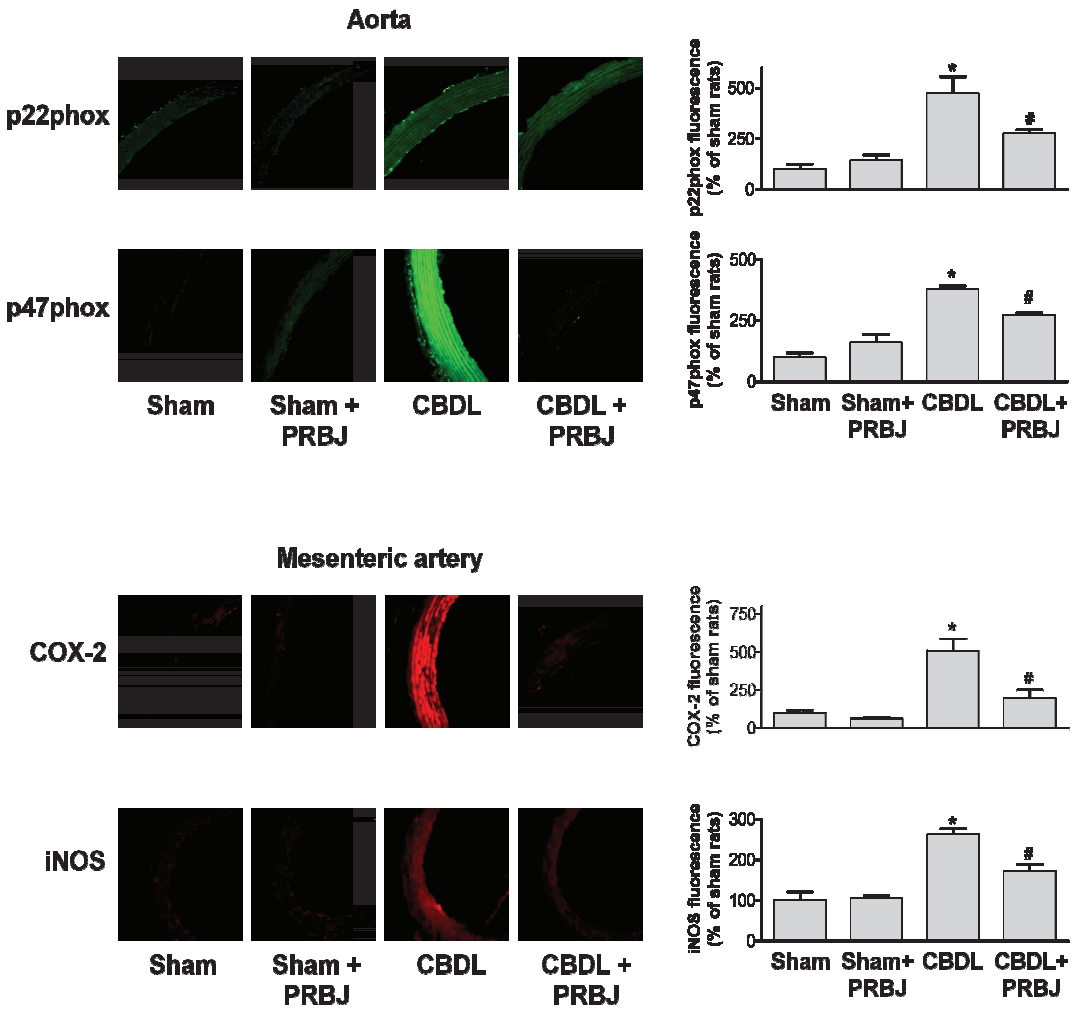

Aorta
p22phox
p47phox
Sham
Sham + PRBJ
CBDL
CBDL + PRBJ
p22phox fluorescence (% of sham rats)
p47phox fluorescence (% of sham rats)
Sham
Sham+ PRBJ
CBDL
CBDL+ PRBJ
Mesenteric artery
COX-2
iNOS
COX-2 fluorescence (% of sham rats)
iNOS fluorescence (% of sham rats)

Figure 5

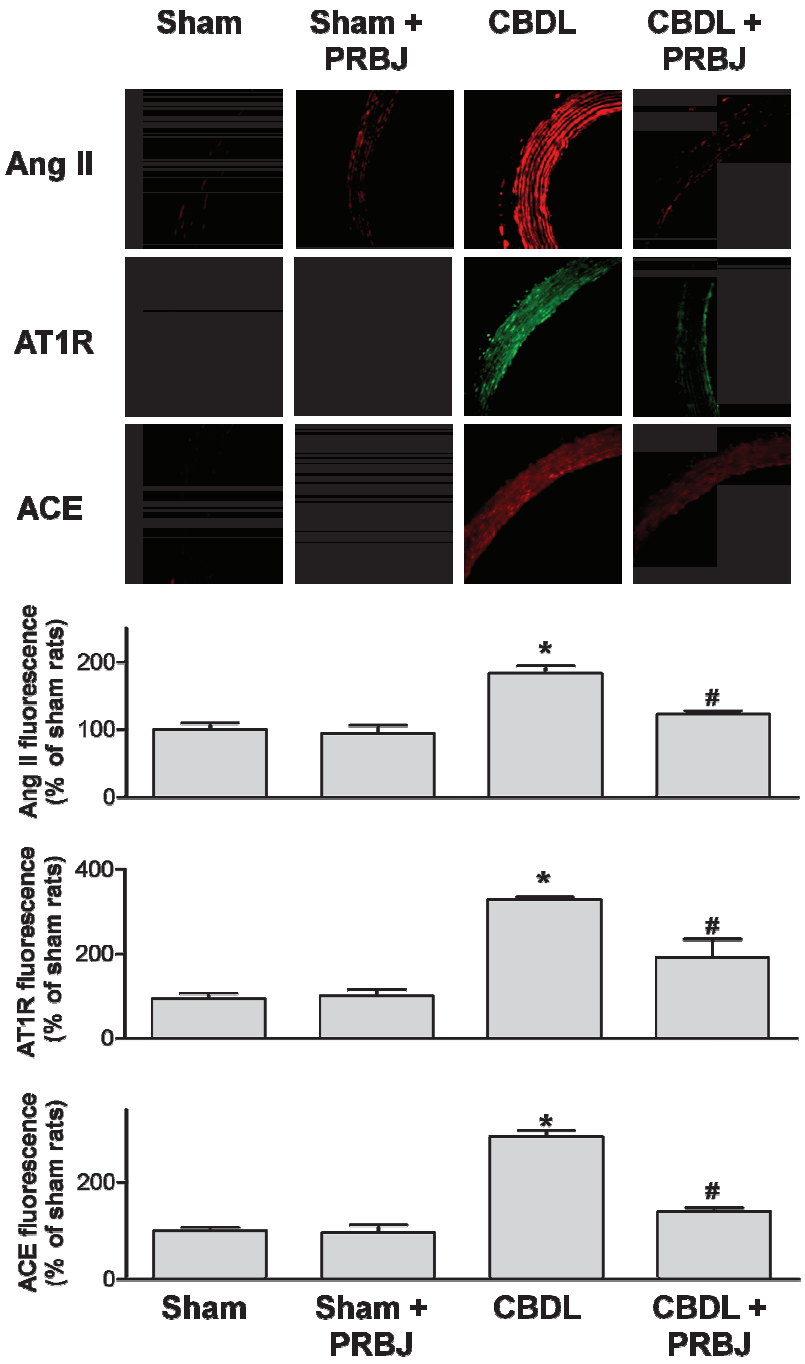
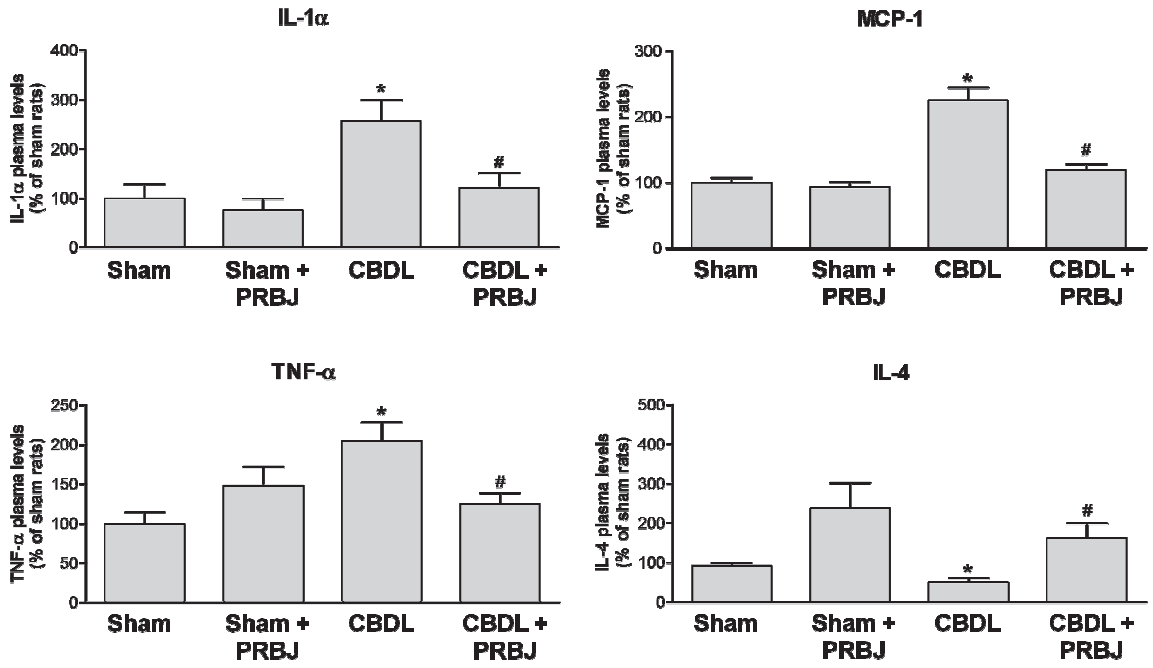

Sham
Sham + PRBJ
CBDL
CBDL + PRBJ
Ang II
AT1R
ACE
Ang II fluorescence (% of sham rats)
AT1R fluorescence (% of sham rats)
ACE fluorescence (% of sham rats)
0
100
200
400
*
#
Sham
Sham + PRBJ
CBDL
CBDL + PRBJ

Figure 6


IL-1α
IL-1α plasma levels (% of sham rats)
400
300
200
100
0
*
#
Sham
Sham + PRBJ
CBDL
CBDL + PRBJ
MCP-1
MCP-1 plasma levels (% of sham rats)
300
200
100
0
*
#
Sham
Sham + PRBJ
CBDL
CBDL + PRBJ
TNF-α
TNF-α plasma levels (% of sham rats)
250
200
150
100
50
0
*
#
Sham
Sham + PRBJ
CBDL
CBDL + PRBJ
IL-4
IL-4 plasma levels (% of sham rats)
500
400
300
200
100
0
*
#
Sham
Sham + PRBJ
CBDL
CBDL + PRBJ

Schematic presentation of the 2nd article results:

Chronic liver diseases with portal hypertension are characterized by a progressive vasodilatation (with endothelial dysfunction) which is especially observed in the splanchnic and pulmonary beds. The latter is referred as to the hepatopulmonary syndrome (HPS). It has been suggested that one of the physiopathologic mechanisms leading to the general vasodilatation and especially to the HPS is related to bacterial translocation which has been shown to improve in CBDL rats by changing the intestine microbiota. Therefore, the present study has evaluated the effect of ingestion of polyphenol-rich blackcurrant juice to prevent the endothelial dysfunction in rats following common bile duct ligation (CBDL), and, if so, to determine the underlying mechanism.

Male Wistar rats (8 rats per group) received either control drinking water or a dose of 60 mg/kg of polyphenol-rich blackcurrant juice (PRBJ) for 7 weeks. After 3 weeks, the rats underwent surgery with either the ligations and resections of the common bile duct (CBDL rats) or sham surgery (sham rats), and were followed for 4 weeks. Reactivity of mesenteric artery rings was assessed in organ chambers. The expression levels of connexins (Cx37) potassium channels (SK_{Ca}), endothelial NO synthase (eNOS), inducible endothelial NO synthase (iNOS), cyclo-oxygenase-2 (COX_2), NADPH oxidase subunits and nitrotyrosines were assessed by immunohistochemistry in mesenteric artery and/or aorta. The vascular formation of reactive oxygen species (ROS) was evaluated using dihydroethidine. Plasma levels of pro-inflammatory cytokines including TNF-α, IL-1α, MCP-1 and IL-4 were evaluated by 5plex-FlowCytomix.

Both the NO- and the EDHF-mediated relaxations to acetylcholine were significantly reduced in CBDL rats compared to sham rats, whereas relaxations to sodium nitroprusside (an exogenous donor of NO) and to levcromakalim (an ATP-sensitive K^+ channel opener) were similar. Impaired EDHF-mediated relaxations were associated with reduced vascular expression of Cx37 and SK_{Ca} and increased expression of eNOS. In aortic sections we found increased NADPH oxidase subunits, iNOS, COX_2 and increased vascular formation of ROS and peroxynitrites. The deleterious effect of CBDL on EDHF-mediated relaxations was significantly prevented by Blackcurrant juice through up-regulation of vascular expression of Cx37 and SK_{Ca} and down-expression of eNOS. Blackcurrant treatment also reduced vascular oxidative stress in aorta. Finally the plasma levels of pro-inflammatory cytokines were improved (Figure 25).

Taken altogether, these results indicate that Blackcurrant juice ingestion prevented the blunted EDHF-mediated relaxation and the increased vascular oxidative stress in the mesenteric artery of CBDL rats suggesting the contribution of endotoxemia and inflammation in the endothelial dysfunction.

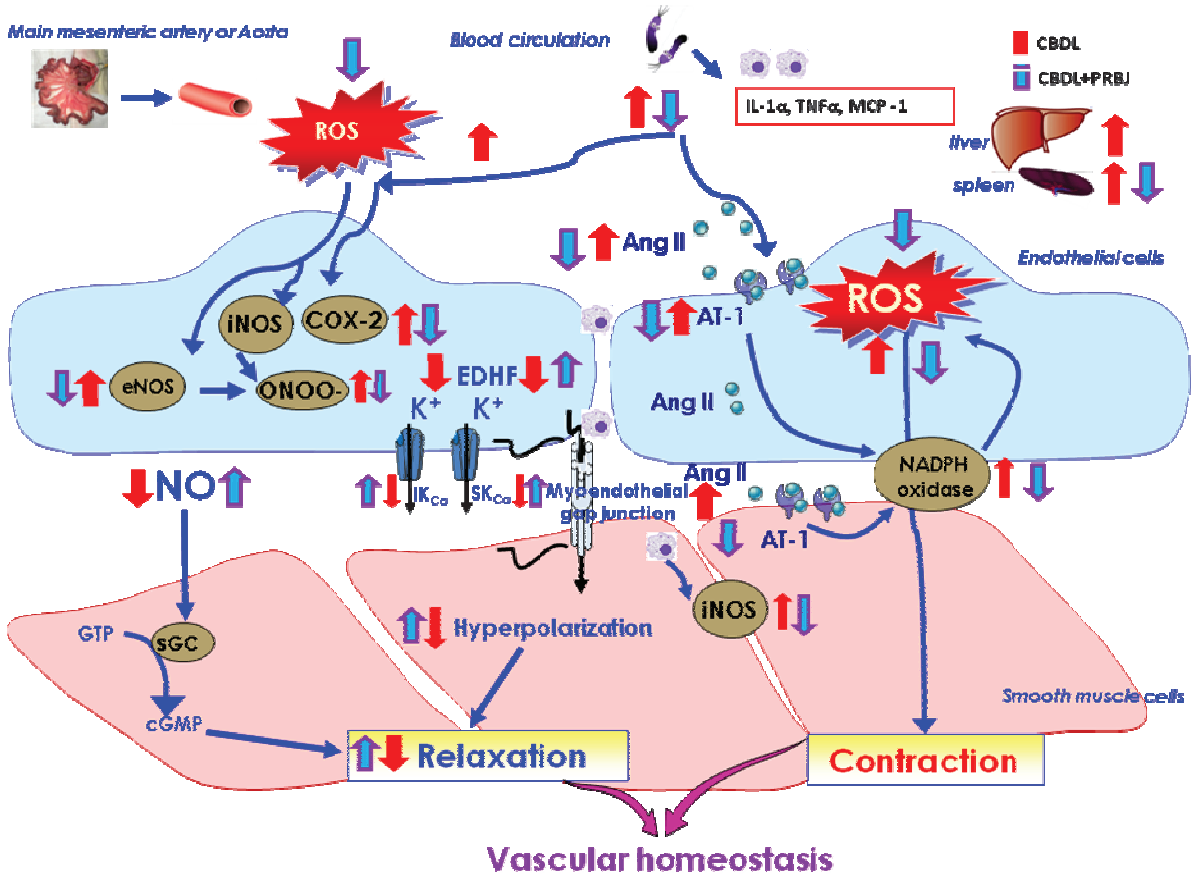


Figure 25. Schema presenting the effects of CBDL in rats and the therapeutic preventive effects of PRBJ in the mesenteric arteries and /or aorta and both liver and spleen size. EDHF: endothelium derived hyperpolarizing factor, eNOS: endothelial NO synthase, ROS: reactive oxygen species, K^+: potassium, NO: nitric oxide, AT-1: angiotensin receptor type, AA: arachidonic acid, COX: cycloogynase, IK_{Ca} and SK_{Ca}: calcium dependent potassium channels of intermediate and small conductance, ⇑⇓ : effects of CBDL, increase and decrease, ⇑⇓ : effects of CBDL+PRBJ, increase and decrease.

PART III

RESULTS

PART III

Effect of Fulvestrant, an estrogen receptor antagonist, on the cirrhotic rat lung

Oswald-Mammosser Monique, **Rashid Sherzad K.**, Boehm Nelly, Agin Arnaud, Geny Bernard, Schini-Kerth Valérie, Charloux Anne.

Submitted for publication

In the literature, hepatopulmonary syndrome (HPS) is a well-defined cause of hypoxemia in patients who have liver disease due to abnormal intrapulmonary vascular dilatation. The pulmonary symptoms are attributed to defect in the blood oxygenation that occurs as a result of dilation of pulmonary small vessels. Previous studies have suggested that an increased estradiol level in CBDL rats might contribute to pulmonary vasodilatation via excessive formation of the potent vasodilator NO (Aller, de Luis et al. 2001; Aller, Moya et al. 2002; Yol, Erikoglu et al. 2005). It is well documented that sex hormones play role in the regulation of vascular tone. Furthermore, in cirrhotic patients an imbalance in sex hormone levels have been reported, and for example, increased levels of progesterone and estradiol have been associated to hyperventilation in cirrhotic patients (Aller, Moya et al. 2002). Thus, our hypothesis was that an estrogen receptor antagonist like fulvestrant could improve the HPS in CBDL rats. This has been studied in article 3, which is submitted for publication.

Since HPS, occurring in 10 to 20 % of cirrhotic patients waiting for liver transplantation is of prognostic value, it is important to have an efficient treatment to improve the outcome of these patients before but also after surgery. Moreover, in some cases, improving this syndrome should lead to delay transplantation.

In our study, we investigated the possibility that fulvestrant, an estrogen receptor blocker could improve the HPS. Fulvestrant was administered, at the same day of surgery, as a single s.c. bolus dose of 10 mg/rat in a volume of 0.2 ml vehicle (Faslodex AstraZeneca). Sham rats received the same volume of castor oil. Four weeks after surgery, rats were killed. Blood was collected for hormonal and plasma nitrite determination, arterial blood was collected from the aorta in a heparinized syringe for the determination of carboxyhemoglobin (COHb) levels and haematocrit using an ABL Cooxymeter. Serum was stored at –80°C for

the subsequent determination of hormone and nitrite/nitrate levels. Liver, spleen and lung samples were obtained for histological and immunohistological studies.

Plasma nitrite and COHb levels were significantly increased in the CBDL group compared to the sham group, and these effects were not affected by fulvestrant treatment.

In the lung, the endothelial nitric oxide synthase (eNOS), and nitrotyrosine protein expressions were increased in CBDL rats as compared to sham rats, this effect was significantly reduced by fulvestrant. Furthermore, the HO-1 immunofluorescence signal was also significantly increased in lung sections of CBDL rats and this effect was prevented by fulvestrant treatment. In contrast there was an increased recruitment of macrophages in the lungs of CBDL rats compared to sham rats, an effect which was not affected by fulvestrant treatment. Macrophages in the lungs were mostly intravascular in CBDL rats. A significantly increased VEGF (the angiogenic factor involved in the development of HPS) immunostaining signal was observed in the lung sections of CBDL rats predominantly in the alveolar-capillary wall and also in the vessel lumen. These signals were not affected by fulvestrant treatment. Moreover, the diameter of small non-muscular lung vessels in CBDL lungs was increased, and this parameter was unaffected by fulvestrant.

In conclusion, the use of fulvestrant may not be an option to improve the lung abnormalities in CBDL rats since NO may not be biologically active and since other markers implicated in pulmonary abnormalities are not affected by fulvestrant.

Effect of the estrogen receptor antagonist fulvestrant on the cirrhotic rat lung

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Abstract

Background

It has been postulated that vasodilatation in the lung observed in cirrhosis and the subsequent hepatopulmonary syndrome, is explained in part by an increased estradiol level through an enhanced endothelial formation of nitric oxide (NO). In our study we assessed whether the estrogen receptor antagonist fulvestrant (F) could improve the lung abnormalities due to cirrhosis.

Methods

Cirrhosis was induced in rats by chronic bile duct ligation (CBDL). Four groups were studied: CBDL, CBDL+F, sham (S), S+F. Histological, immunohistochemical and western blot analyses were performed on lung samples.

Results

The endothelial nitric oxide synthase (eNOS), and nitrotyrosine protein expressions were increased in CBDL as compared to S, this effect was significantly reduced by fulvestrant. Surprisingly the level of pVASP (an indirect marker of NO formation and action) was decreased in CBDL, this was not affected by fulvestrant. In contrast, level of vascular endothelial growth factor, diameter of small lung vessels and number of macrophages in CBDL lungs were increased, these parameters were unaffected by fulvestrant.

Conclusions

Fulvestrant may not be an option to improve the lung abnormalities in cirrhosis since NO may not be biologically active and since key events contributing to the lung abnormalities are not affected by fulvestrant.

Keywords : angiogenesis; cirrhosis; fulvestrant; hepatopulmonary syndrome; nitric oxide

Abbreviations:

CBDL : chronic bile duct ligation

COHb : carboxyhemoglobin

eNOS : endothelial nitric oxide synthase

ER-alpha : estradiol receptor-alpha

F : fulvestrant

HO-1 : heme-oxygenase 1

HPS : hepatopulmonary syndrome

iNOS : inducible nitric oxide synthase

NO : nitric oxide

p-Akt : phosphorylated serine/threonine kinase Akt

p-VASP : phosphorylated vasodilator-stimulated phosphoprotein

S : sham

VEGF : vascular endothelial growth factor

Introduction

Chronic liver diseases are characterized by a progressive vasodilatation, which is especially observed in the splanchnic and the pulmonary beds. The vasodilatation is due to portal hypertension associated to either cirrhosis or extrahepatic portal venous obstruction without cirrhosis (Abraldes et al., 2006). In a rat model of partial portal vein ligation, Abraldes et al (Abraldes et al., 2006) reported that the initial event because of portal hypertension is an up-regulation of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) in the intestinal microcirculation. The mechanisms involved in the occurrence of the subsequent generalized vasodilatation and hyperdynamic syndrome are incompletely understood, although animal models have led to a number of hypotheses (Ling et al., 2004; Nunes et al., 2001; Sztrymf et al., 2004; Zhang et al., 2003). Several lines of evidence support a role for an increased formation of nitric oxide (NO) in the occurrence of the general vasodilatation in chronic liver diseases in patients and also in the chronic bile duct ligated (CBDL) rat model (Brussino et al., 2003; Chabot et al., 1996; Gomez et al., 2006; Ling et al., 2004; Nunes et al., 2001; Rolla et al., 1998; Schenk et al., 2000; Zhang et al., 2003). Moreover, from clinical and animal studies it has been suggested that an increased level of estradiol may be implicated in the occurrence of the vasodilatation most likely through subsequently to an increased NO formation (Aller et al., 2001; Aller et al., 2002; Yol et al., 2005). The generalized vasodilatation leads to complications and especially to a hepatopulmonary syndrome (HPS) in approximately 10%–15% of patients awaiting liver transplantation (Rodriguez-Roisin et al., 2004). The HPS, which is characterized by arterial deoxygenation because of intrapulmonary vascular dilatations, is of prognostic value for the survival before and also after liver transplantation (Arguedas et al., 2003; Rodriguez-Roisin et al., 2004). It resolves in most cases with liver transplantation (Arguedas et al., 2003) but having an efficient approach to improve this syndrome is of great clinical importance because this could ameliorate survival and possibly also delay liver transplantation in some cases. The aim of the present study was to investigate the effect of fulvestrant, which is an estrogen receptor antagonist with no agonistic effect, in an animal model of biliary cirrhosis with portal hypertension. Indeed, fulvestrant is expected to prevent the increased estradiol-stimulated NO formation and, thus, improve the abnormalities observed in cirrhotic rat lungs.

Material & Methods

The study was performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). The project has been approved by the HUS/CNRS committee (collaboration between the university hospital of Strasbourg and the research department CNRS for research project).

CBDL model

Male Wistar rats (10–12 weeks old) were anesthetized with an intraperitoneal mixture of ketamine (80 mg.kg body wt^{-1}) and xylazine (4 mg.kg body wt^{-1}). Biliary cirrhosis was induced by CBDL. After laparotomy, the bile duct was isolated, double ligated, and resected between the 2 ligatures. Sham rats had laparotomy and underwent mobilization of the common bile duct without ligation. After surgery, rats were housed in a thermoneutral environment, on a 12:12-hour photoperiod and were provided food and water ad libitum. Rats were divided into four groups: CBDL (n=10), CBDL + F (fulvestrant) (n=10), sham (S) (n=8), S + F (n=9). Fulvestrant was administered, at the same day than surgery, as a single s.c. bolus dose of 10 mg/rat in a volume of 0.2 ml vehicle (Faslodex AstraZeneca). Sham rats received the same volume of castor oil. Four weeks after surgery, rats were killed. Before the collection of blood for hormonal and plasma nitrite determinations, arterial blood was collected from the aorta in a heparinized syringe for the determination of carboxyhemoglobin (COHb) levels and haematocrit using an ABL Cooxymeter (ABL725, Radiometer, Copenhagen, Denmark). Serum was stored at –80°C for the subsequent determination of hormone and nitrite/nitrate levels. The liver and the spleen were removed and weighted. Liver samples from each rat were put in Bouin-Holland fixative and embedded in paraffin for histological analysis. After excision, the left lung was snap frozen and the right lung was fixed by infusion of Bouin-Holland fixative at a pressure of 25 cm H_2O and embedded in paraffin for immunohistochemistry.

Assessment of biliary cirrhosis and portal hypertension

Five-micrometer-thick sections of liver samples were stained with H&E and evaluated by light microscopy. Ten photographs were taken from each liver sample, and the area

occupied by the biliary ducts was evaluated from each photograph using the Image-J software (National Institutes of Health, Bethesda, MD; http://rsweb.nih.gov/ij/). The final result for each rat was the mean of the 10 evaluations and was expressed in percentage of the whole area of the photographs. Portal hypertension was reflected by an increased spleen weight.

Immunohistochemical studies and small vessels diameter

Immunolocalization of macrophages in lungs was performed using the mouse anti-rat CD68 monoclonal antibody (AbD Serotec) on 5 µm paraffin sections. Eight photographs were taken from each lung sample and the area occupied by the macrophages was evaluated from each photograph using the Image-J software (National Institutes of Health, Bethesda, MD; http://rsweb.nih.gov/ij/). On the same sections, we also evaluated the diameter of small, non-muscular vessels (ten vessels per rat). Only vessels round in shape were retained.

Immunohistochemical staining was performed on lung paraffin 10 µm sections fixed with 4% paraformaldehyde. Fixed sections were incubated with antibodies directed against either inducible heme oxygenase (HO-1) (1/ 500) or VEGF (1/300). Sections were then incubated with the secondary antibody (Alexa 488-conjugated or Alexa 637-conjugated goat anti-rabbit IgG). The preparations were then evaluated using a confocal laser-scanning microscope (Leica SP2 UV DM IRBE) with 20× magnification lens. Quantification of protein levels was performed using Image-J software (National Institutes of Health, Bethesda, MD; http://rsweb.nih.gov/ij/).

Western blot analysis

Frozen lung tissues were homogenized in extraction buffer (composition in mM: Tris/HCl 20 (pH 7.5; QBiogene), NaCl 150, Na_3VO_4 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogene)). Total proteins (25 µg) were separated on SDS-polyacrylamide (Sigma) gels and transferred electrophoretically onto polyvinylidine difluoride membranes (Amersham). Membranes were blocked with blocking buffer containing 5% bovine serum albumin, Tris-buffered saline solution (Biorad) and 0.1% Tween 20 (Sigma) (TBS-T) for 1 hour. For detection of targeted proteins, membranes were incubated with the respective primary antibody (phosphorylated vasodilator-stimulated phosphoprotein, p-VASP, Cell Signaling

Technology, 1/1,000; phosphorylated serine/threonine kinase Akt, p-Akt, Cell Signaling Technology, 1/1,000; eNOS, Cell Signaling Technology, 1/1,000; HO-1, Enzo life science, 1/1,000; estradiol receptor-alpha, ER-alpha, Cell Signalling Technology, 1/1,000; inducible NOS, iNOS, BD Transduction Laboratories 1/1,000; nitrotyrosine, Millipore, 1/1,000; VEGF, Santa Cruz Biotechnology INC, 1/2,000). Detection of either β-tubulin or β-actin protein was used for quantification. After incubation with a primary antibody, membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody and signals were detected using enhanced chemiluminescence (Amersham).

Nitrite determinations

Plasma nitrite levels were determined using the Griess reaction colorimetric method.

Hormonal levels measurements

Progesterone, testosterone and estradiol levels were determined using radioimmunoassays (Progesterone RIA, Coated Tube Radioimmunoassay, Orion Corporation Orion Diagnostica, Espoo, Finland; Testosterone RIA, Immunotech, Beckman Coulter, Marseille, France; Double antibody Estradiol RIA, Siemens Healthcare Diagnostics Inc, Los Angeles, USA).

Statistical analyses

Values were expressed as means ± SEM or median (minimal-maximal) values when normality test failed. Statistical evaluation was performed using an analysis of variance. If normality or equal variance tests failed, a Kruskal-Wallis one-way analysis of variance on ranks was performed. A Student-t test or a Mann-Whitney Rank Sum Test when normality test failed, were performed to compare 2 groups. Values of $P < 0.05$ were considered to be statistically significant.

Results

Characteristics of CBDL rats

Rats were sacrificed 4 weeks after surgery. CBDL rats showed a significant increase in liver and spleen weight and a clear bile duct proliferation (increased biliary density on the histologic analyses). These effects were not affected by fulvestrant treatment (Table I). Initial and final body weights did not differ between the four groups (Table I).

Hormone levels

Testosterone levels were significantly decreased in the CBDL group compared to the sham group and this effect was not affected by fulvestrant treatment (Table II). Estradiol levels were similar in the control and CBDL group (Table II). Although fulvestrant did not affect estradiol levels in sham it significantly increased estradiol levels in the CBDL group (Table II). Progesterone levels were similar in all groups (Table II).

Plasma parameters

Plasma nitrite and COHb levels were significantly increased in the CBDL group compared to the sham group, and these effects were not affected by fulvestrant treatment (Table III). The hematocrit level was significantly decreased in the CBDL group compared to the sham group; this was not affected by fulvestrant (Table III).

Western blot analysis

The expression level of eNOS, iNOS, nitrotyrosine and VEGF was significantly increased in lungs of the CBDL group compared to the control group (Figures 1 and 2). Fulvestrant treatment prevented the CBDL-induced upregulation of both eNOS and nitrotyrosine expression but did not affect that of iNOS and VEGF (Figures 1 and 2). p-VASP, an indirect marker of NO formation and action, was significantly decreased in the CBDL group and this effect was not affected by fulvestrant treatment (Figure 1). The level of pAkt, a marker of the VEGF-induced pro-angiogenic effect, was similar in the four groups (Figure 2). The expression level of ER-α was similar in the sham and the CBDL groups

(Figure 2). Although fulvestrant did not affect the ER-α expression level in sham, it significantly increased that in the CBDL group (Figure 2).

Immunofluorescence and histochemical analyses

There was an increased presence of macrophages in the lungs of CBDL rats compared to sham rats, an effect which was not affected by fulvestrant treatment (Table III). Macrophages in the lungs were mostly intravascular in CBDL rats (Figure 4). A significantly increased VEGF immunostaining signal was observed in the lung sections of CBDL rats predominantly in the alveolar-capillary wall and also in the vessel lumen (Figure 3). These signals were not affected by fulvestrant treatment (Figure 3). The HO-1 immunofluorescence signal was also significantly increased in lung sections of CBDL rats and this effect was prevented by fulvestrant treatment (Figure 3).

Small blood vessel diameter

The mean diameter of small non-muscular blood vessels was 21.8±5.7 μm in sham and 38.6±9.7 μm in CBDL groups, which were not affected by fulvestrant treatment (Table III).

Discussion

Consistent with previous findings (Brussino et al., 2003; Chabot et al., 1996; Gomez et al., 2006; Ling et al., 2004; Nunes et al., 2001; Rolla et al., 1998; Schenk et al., 2000; Zhang et al., 2003), the induction of CBDL in rats was associated with an upregulation of eNOS, iNOS and HO-1 protein levels in lungs. Treatment with fulvestrant prevented the CBDL-induced expression of eNOS and HO-1, and also reduced that of iNOS but this effect did not reach statistical significance. Surprisingly, p-VASP, which is an indirect marker of the NO-cyclic GMP pathway, was decreased in CBDL rats and this effect was not affected by fulvestrant. In addition, the expression level of VEGF, which has been reported to contribute to lung angiogenesis in cirrhotic rats (Zhang et al., 2009), was also increased in CBDL rats as assessed by both Western blot analysis and immunofluorescence staining, such an effect was not affected by fulvestrant. Furthermore, fulvestrant treatment did affect neither the increased diameter of small non-muscularized blood vessels nor the infiltration of macrophages into lungs of CBDL rats. Altogether, these findings do not support a major role of estrogens in the impaired lung function in cirrhosis.

Liver diseases have been associated with an imbalance of serum estradiol and testosterone levels in men (Guechot et al., 1994), and also as observed in the present study of the testosterone level in CBDL rats. Although estradiol levels were similar in sham and CBDL rats, the values observed in male rats were close to the detection limit of the radioimmunoassay used and, thus, have to be analyzed with caution. Of interest, fulvestrant treatment markedly increased the estradiol level in CBDL rats without affecting that in sham rats. Thus, these findings indicate that the estrogen receptor antagonist revealed an enhanced estradiol level in CBDL rats presumably subsequent to its binding to estrogen receptors thereby preventing their interaction with estradiol.

Previous studies have suggested that an increased estradiol level in CBDL rats may contribute to pulmonary vasodilatation leading ultimately to HPS via an excessive formation of the potent vasodilator NO (Aller et al., 2001; Aller et al., 2002; Yol et al., 2005). The present findings are in agreement with these previous ones and indicate that an increased plasma level of nitrite and also of eNOS, iNOS and nitrotyrosine protein levels were observed in the lungs of CBDL rats. Fulvestrant treatment markedly reduced the stimulatory effect of CBDL on the lung protein expression level of both eNOS and nitrotyrosine and also reduced slightly but not significantly that of iNOS. Thus, these findings support the concept that

estradiol may contribute to some extent to the excessive vasodilatation in lungs of cirrhotic rats subsequent to the activation of estradiol receptors. Such a concept is also consistent with the fact that an increased plasma estradiol level and an increased lung ER-alpha expression level are observed in CBDL rats treated with fulvestrant.

An increased presence of macrophages especially in the intravascular field is observed in lungs of CBDL rats and is associated with an increased expression of iNOS (Ling et al., 2004; Nunes et al., 2001; Sztrymf et al., 2005; Sztrymf et al., 2004). Previous studies have indicated that 17β-estradiol increased iNOS expression in peritoneal macrophages in rats (You et al., 2003). Since fulvestrant treatment did not affect the accumulation of macrophages and reduced only slightly the expression of iNOS in lungs of CBDL rats, it is likely that estrogens contribute only to some extent to the increased expression of iNOS. Besides estrogens, oxidative stress as indicated by the increased expression of nitrotyrosine in the lung of CBDL rats (Dal-Ros et al., 2010) is also likely to contribute to iNOS expression as suggested previously by Kang et al (Kang et al., 2002).

The present findings also indicate an increased expression in HO-1 in the lung of CBDL rats which was significantly reduced by fulvestrant. Both endothelial cells and macrophages are likely to contribute to the increased HO-1 level since they have been shown previously to express HO-1 (Baruscotti et al., 2010; Zhang et al., 2003). Baruscotti et al (Baruscotti et al., 2010) have observed that estradiol stimulates HO-1 expression in endothelial progenitor cells. Such an effect may contribute to explain the inhibitory effect of fulvestrant on the CBDL-induced expression of HO-1 in lungs. In addition, Baruscotti et al (Baruscotti et al., 2010) further suggested that the stimulatory effect of estradiol involves an indirect mechanism via an increased expression of VEGF, which, in turn, activates Akt and HO-1. In the present study, the CBDL-induced upregulation of VEGF expression was not affected by fulvestrant indicating that estradiol has only a minor role. In addition, Carter et al (Carter et al., 2002) have observed an NO-dependent upregulation in HO-1 expression in the lungs of CBDL rats possibly via a cyclic GMP-independent mechanism as has been observed in vascular smooth muscle cells (Hartsfield et al., 1997). Thus, the inhibitory effect of fulvestrant on the CBDL-induced upregulation of HO-1 expression in lungs may possibly be due to a reduced NO formation.

An increased expression level of eNOS, iNOS and HO-1 was observed in lungs suggesting that these mechanisms may contribute to the occurrence of vasodilatation in the CBDL rat

lung by the formation of the potent vasodilators NO and CO. Indeed, plasma nitrite and carboxyhemoglobin levels were increased in CBDL rats. However, the level of p-VASP, an indirect marker of NO formation and action, was significantly reduced in the lungs of CBDL rats. These apparently contradictory findings suggest that NO may either act via a cyclic GMP-independent pathway or be biologically inactive. In a previous study (Dal-Ros et al., 2010), we have observed that the NO-mediated component of the relaxation in the CBDL rat mesenteric artery ring is not increased in comparison to sham rats. One possible explanation is that oxidative stress may promote to the degradation of NO by superoxide anions leading to an increased nitrotyrosine expression as observed in the CBDL lung. Thus, the increased formation of NO subsequently to the stimulation of eNOS by estradiol may not be biologically active but rather lead to an increased formation of peroxynitrite, a strong pro-oxidant. The inhibitory effect of fulvestrant on the CBDL-induced lung peroxynitrite formation most likely reflects the reduced formation of NO. Thus, the present findings taken in conjunction with previous ones (Chang et al., 2013; Dal-Ros et al., 2010; Roberts et al., 2010; Zhang et al., 2009) suggest that NO may have a minor role in the occurrence of HPS.

An increased expression of VEGF was observed in the lungs of CBDL rats and this effect was not affected by fulvestrant. Similarly, an increased expression of VEGF especially VEGF-A production by monocytes was also observed in the pulmonary vascular bed in CDBL rats (Zhang et al., 2009). These authors have highlighted the role of angiogenesis in the occurrence of the HPS in CBDL rats. Thus, vascular remodeling rather than acute vasodilatation may be the cornerstone of the occurrence of HPS. Such a concept is supported by previous findings indicating that acute inhibition of NO did not lead to increased oxygenation in patients as reported by Gomez et al (Gomez et al., 2006), and the resolution of the HPS after liver transplantation takes often several months (Schiffer et al., 2006; Swanson et al., 2005). Therefore, angiogenesis is likely to be an important event contributing to the human disease. Nevertheless, the mechanism leading to angiogenesis remains unclear. An increased activation of the Akt pathway associated to an increased expression of eNOS has been suggested to be one of the mechanisms potentially leading to angiogenesis (Dimmeler and Zeiher, 2000; Papapetropoulos et al., 1997). However, the contribution of Akt in the present study is not supported by the fact that p-Akt was not increased in the lungs of CBDL rats despite an increased expression of VEGF.

HPS is related to dilated blood vessels in the lung and the subsequent hypoxia to an impaired interaction of oxygen with haemoglobin in red blood cells, which are too far from

the alveolar membrane in dilated capillaries and precapillaries. In the present study, the diameter of small non-muscularized vessels was significantly increased in CBDL rats and this effect was not affected by fulvestrant. Moreover, fulvestrant treatment did not affect the infiltration of macrophages in the CBDL rat lung, which have been shown to express iNOS and VEGF and to play a critical role in the occurrence of the HPS (Thenappan et al., 2011).

The results of our study suggest that fulvestrant may not be an option to improve the HPS but there are some limitations. We chose to treat the rats with a bolus of 10 mg of fulvestrant (in castor oil) which has been shown to induce anti-oestrogenic activity for 1 month or more (Wakeling et al., 1991). Nevertheless we can question if a greater dose of fulvestrant would have had more effects on the lungs. A second limitation is that only male rats were studied, if the results could differ in female rats should be tested in the future.

Conclusions

In conclusion, the use of fulvestrant to prevent the stimulatory effect of estrogens on NO formation may not be an interesting option to improve the HPS since NO is mostly biologically inactive and appears to play only a minor role in the occurrence of the HPS. It is also not supported by the fact that neither the expression of iNOS and VEGF nor the increased infiltration of macrophages, which are key events contributing to the lung abnormalities observed in cirrhosis, are affected by fulvestrant.

Table I: Characteristics of CBDL rats and sham rats, results are given as mean±SEM or median (range)

	Initial body weight (g)	Final body weight (g)	Biliary density (% of total area)	Liver weight (% vs total weight)	Spleen weight (% vs total weight)
Sham	340.5±3.8	414.4±5.8	0.36 (0.06-1.01)	3.53 (3.14-4.39)	0.25 (0.21-0.32)
Sham+F	333.6±8.2	404.3±11.5	0.32 (0.12-0.64)	3.58 (3.10-3.69)	0.22 (0.17-0.26)
CBDL	336.5±6.1	398.1±7.9	20.12 (10.42-43.56)*	7.22 (6.31-8.41)*	0.67 (0.48-0.81)*
CBDL+F	344.5±5.4	398.9±8.7	19.08 (3.86-39.76) *	7.33 (4.20-8.87) *	0.63 (0.33-0.86) *

CBLD: chronic bile duct ligated rats, F: rats treated with fulvestrant

* P<0.001 CBDL vs sham

Table II: Plasma hormonal levels in sham and CBDL rats, results are given as mean±SEM or median (range)

	Estradiol ($ng.L^{-1}$)	Testosterone ($ng.mL^{-1}$)	Progesterone ($nmol.L^{-1}$)
Sham	4.0 (4.0-4.0) (n=6)	2.64±0.67 (n=7)	4.73 (2.52-6.25) (n=7)
Sham+F	4.0 (4.0-4.0) (n=5)	2.05±0.38 (n=8)	2.55 (1.06-11.24) (n=8)
CBDL	4.1 (4.0-15.5) (n=8)	1.00±0.21 (n=10)**	2.76 (1.68-25.39) (n=10)
CBDL+F	11.6 (6.0-17.9) (n=7)*	1.04±0.48 (n=6)[§]	2.94 (2.15-17.29) (n=8)

CBDL: chronic bile duct ligated rats, F: fulvestrant

* P=0.008 for CBDL vs CBDL+F; ** P=0.017 and [§] P=0.15 for sham vs CBDL rats

Table III : Infiltration of lung macrophages, small blood vessel diameter and plasma markers in sham and CBDL, results are given as mean±SEM or median (range)

	Nitrite ($\mu mol.L^{-1}$)	Lung macrophages (%)	Small vessel diameter (μm)	COHb (%)	Hematocrit (%)
Sham	22.7 (15.7-29.5) (n=8)	3.4±0.8 (n=5)	21.8±5.7 (n=5)	0.63±0.05 (n=8)	49.6±0.9 (n=8)
Sham+F	21.0 (18.7-25.7) (n=5)	2.3±0.1 (n=5)	22.7±6.2 (n=5)	0.56±0.05 (n=9)	50.6±0.8 (n=9)
CBDL	83.7 (51.3-171.9) * (n=10)	15.2±1.8** (n=9)	38.6±9.7* (n=8)	1.21±0.08* (n=8)	45.5±1.2[#] (n=8)
CBDL+F	71.1 (24.7-153.9) * (n=8)	14.2±0.9* (n=9)	39.8±9.7* (n=9)	1.23±0.06* (n=8)	46.3±1.8[§] (n=8)

CBDL: chronic bile duct ligated rats, F: rats treated with fulvestrant, COHb: carboxyhemoglobin

* P<0.001; ** P=0.007; [#] P=0.01; [§] P=0.04 for CBDL vs sham rats

Figure legends

Fig. 1 Western blot analyses for eNOS (endothelial nitric oxide synthase), iNOS (inducible nitric oxide synthase), nitrotyrosine, p-VASP (phosphorylated vasodilator-stimulated phosphoprotein) in the lungs

CBDL: chronic bile duct ligated rats, F: fulvestrant

* : P<0.05 for CBDL vs sham rats; ** : P<0.01 for CDL vs sham rats; # : P<0.01 for CBDL vs CBDL+F rats

Fig. 2 Western blot analyses for VEGF (vascular endothelial growth factor), ER-α (estrogen receptor alpha), p-Akt (phosphorylated serine/threonine kinase Akt) in the lungs

CBDL: chronic bile duct ligated rats, F: fulvestrant

* : P<0.05 for CBDL vs sham rats; ** : P<0.01 for CBDL vs CBDL+F rats

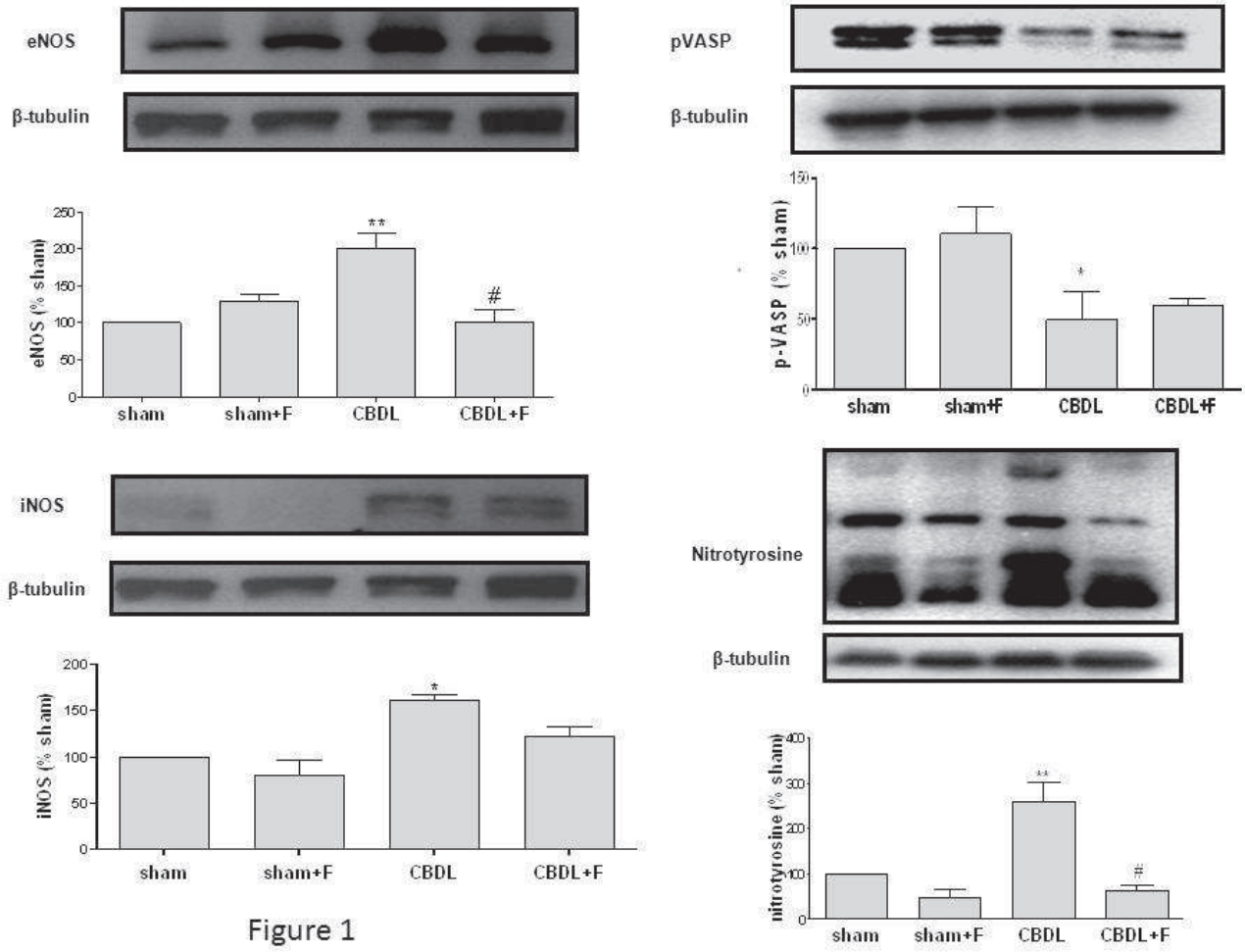
Fig. 3 Immunofluorescence studies for VEGF (vascular endothelial growth factor) and HO-1 (inducible heme-oxygenase) in lung sections

CBDL: chronic bile duct ligated rats, F: fulvestrant

** : P<0.05 for CBDL vs sham rats; # : P<0.05 for CBDL vs CBDL+F rats

Fig. 4 Immunohistochemical staining in paraffin lung sections

There is a clear increase in macrophages (mostly intravascular, see star) in CBDL rats (lower part, 2 different rats) in comparison with sham rats (upper part, 2 different rats). The diameter of the small muscular blood vessels is increased in CBDL rats (see arrow).


eNOS
β-tubulin
eNOS (% sham)
250
200
150
100
50
0
**
#
sham
sham+F
CBDL
CBDL+F
pVASP
β-tubulin
p-VASP (% sham)
150
100
50
0
*
sham
sham+F
CBDL
CBDL+F
iNOS
β-tubulin
iNOS (% sham)
200
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CBDL
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Nitrotyrosine
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nitrotyrosine (% sham)
400
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sham
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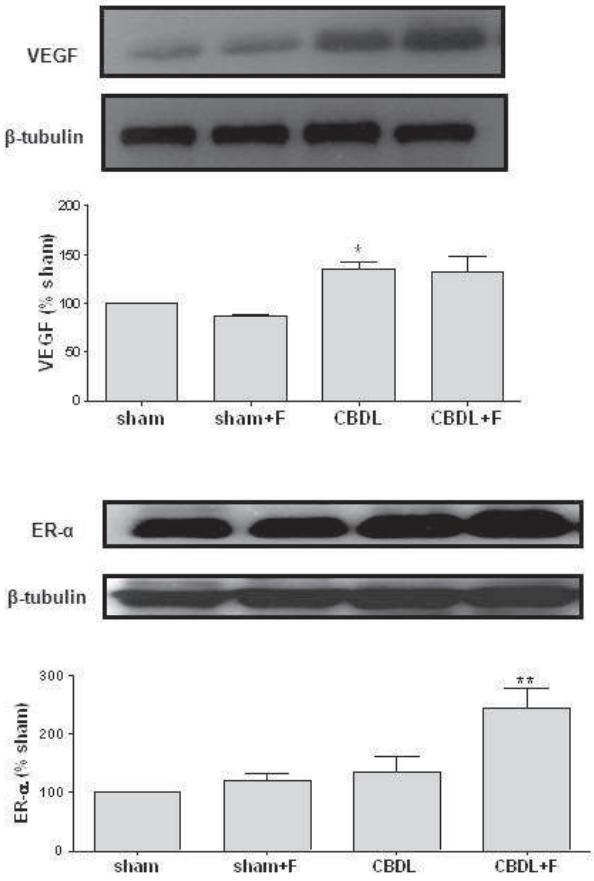
CBDL
CBDL+F

Figure 1


VEGF
β-tubulin
VEGF (% sham)
200
150
100
50
0
*
sham
sham+F
CBDL
CBDL+F
ER-α
β-tubulin
ER-α (% sham)
300
200
100
0
**
sham
sham+F
CBDL
CBDL+F

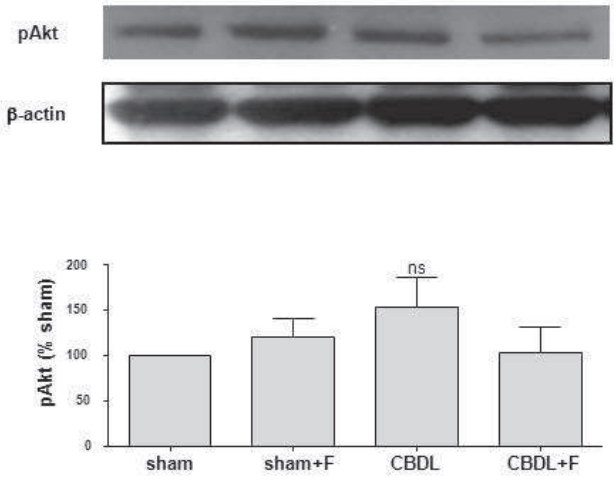

pAkt
β-actin
pAkt (% sham)
200
150
100
50
0
ns
sham
sham+F
CBDL
CBDL+F

Figure 2

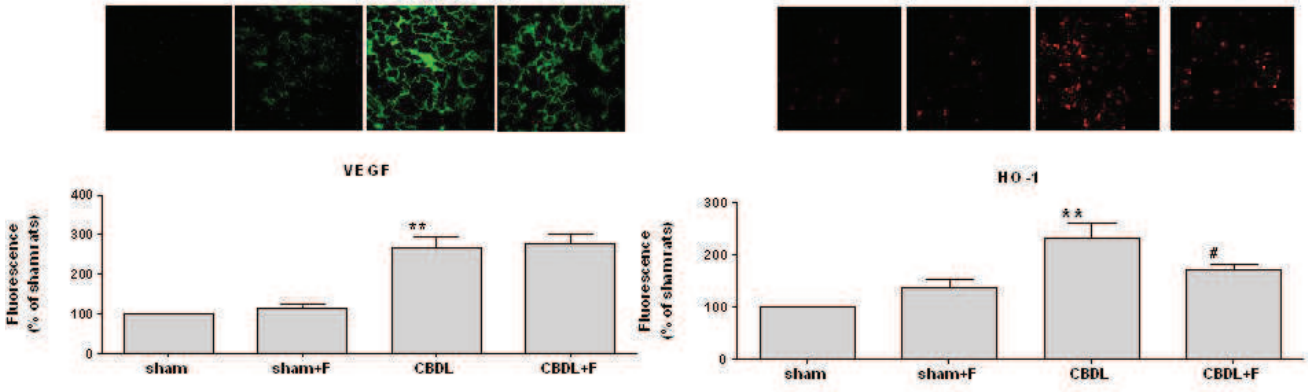

VEGF
Fluorescence (% of sham rats)
400
300
200
100
0
**
sham
sham+F
CBDL
CBDL+F
HO-1
Fluorescence (% of sham rats)
300
200
100
0
**
#
sham
sham+F
CBDL
CBDL+F

Figure 3

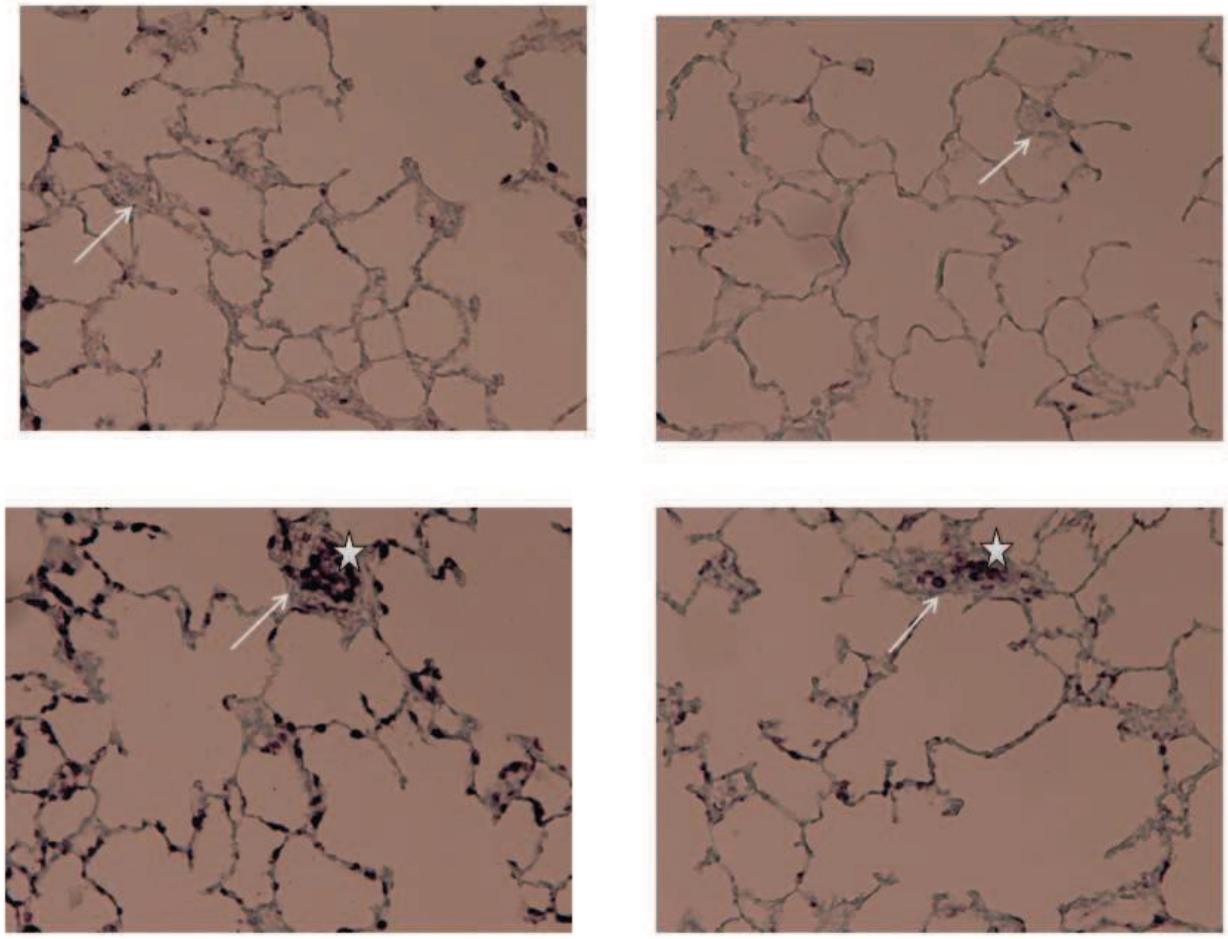


Figure 4

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Schematic presentation of the third article results:

After common bile duct ligation or sham operation, Rats were sacrificed 4 weeks after surgery. CBDL rats showed a significant increase in liver and spleen weight and a clear bile duct proliferation. These effects were not affected by fulvestrant treatment.

Hormone levels

Estradiol levels were similar in the control and CBDL group. Although fulvestrant did not affect estradiol levels in sham it significantly increased estradiol levels in the CBDL group.

Plasma nitrite and COHb levels were significantly increased in the CBDL group compared to the sham group, and these effects were not affected by fulvestrant treatment. The expression level of eNOS, iNOS, nitrotyrosine and VEGF was significantly increased in lungs of the CBDL group compared to the control group. Fulvestrant treatment prevented the CBDL-induced upregulation of both eNOS and nitrotyrosine expression but did not affect that of iNOS and VEGF. The expression level of ER-α was similar in the sham and the CBDL groups. Although fulvestrant did not affect the ER-α expression level in sham, it significantly increased that in the CBDL group.

There was an increased presence of macrophages in the lungs of CBDL rats compared to sham rats, an effect which was not affected by fulvestrant treatment. Macrophages in the lungs were mostly intravascular in CBDL rats. The HO-1 immunofluorescence signal was also significantly increased in lung sections of CBDL rats and this effect was prevented by fulvestrant treatment.

Finally, the mean diameter of small non-muscular blood vessels was 21.8±5.7 µm in sham and 38.6±9.7 µm in CBDL groups, which were not affected by fulvestrant treatment.

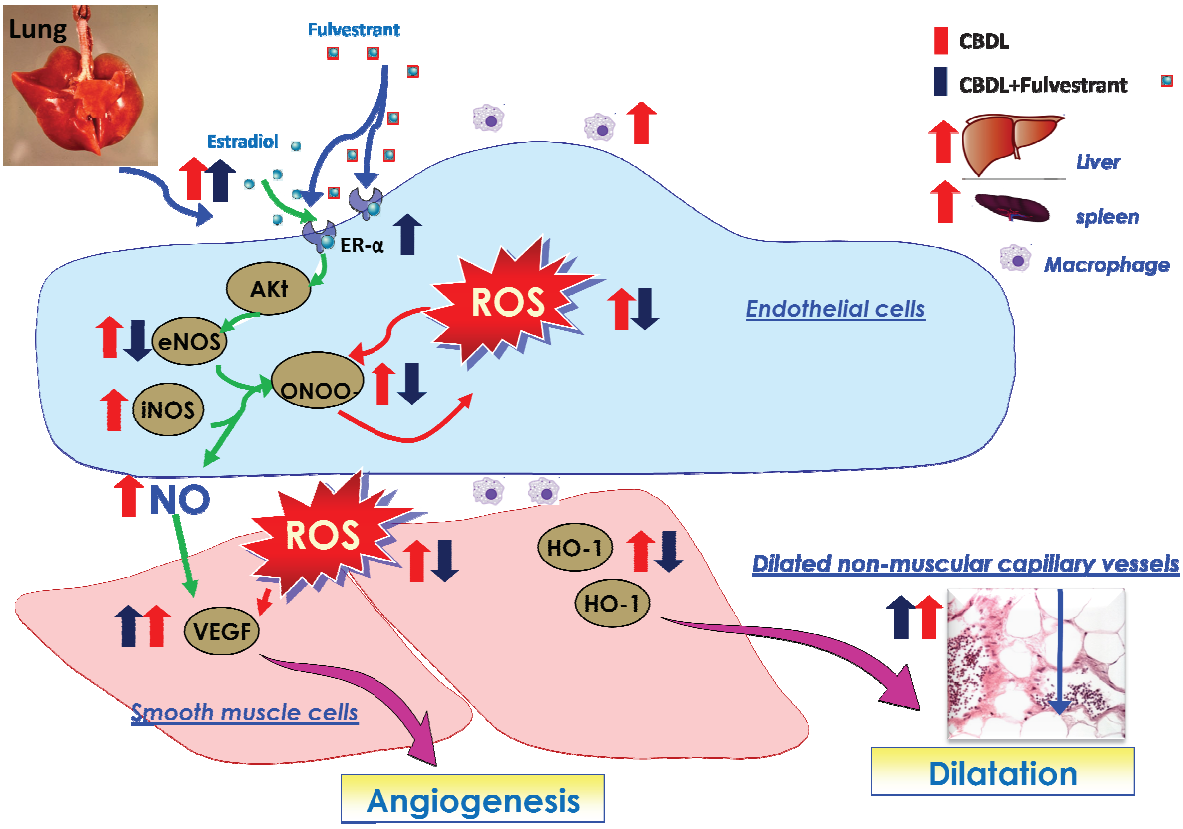

Figure 26. Schema presenting the effects of CBDL in rats' lung and the therapeutic preventive effects of fulvestrant: eNOS: endothelial NO synthase, iNOS: inducible NO synthase, ROS: reactive oxygen species, HO-1: hemooxygenase-1, NO: nitric oxide, ER-α: estrogen alpha receptor, VEGF: vascular endothelial growth factor, ROS: reactive oxygen species, $ONOO^-$: peroxynitrite ↑: effects of CBDL, ↑↓ : effects of CBDL+ Fulvestrant ↑↓, increase and decrease.

GENERAL DISCUSSION

GENERAL DISCUSSION

5. Discussion

5.1. Chronic liver disease models in animal

Chronic liver disease are frequent and potentially life threatening diseases for humans. A better understanding of the underlying mechanisms is mandatory for the design of new drugs to be used in clinic. Therefore, rodent models are being developed to mimic human liver disease. However, despite all efforts, no model to date can completely recapitulate the corresponding human disorders. Limiting factors are the time frame required in humans to establish a certain liver disease and the fact that rodents possess a distinct immune system compared with humans and have different metabolic rates affecting the homeostasis.

5.2. Classical animal models of experimental liver fibrosis

Liver fibrosis and its advanced form, cirrhosis, represent the common final pathway of most types of chronic liver disease. The fact that rats generally respond to fibrotic stimuli with development of a fibrotic reaction that is much more robust than that of other rodent (Bissell, 2011) makes it necessary to have a second animal models like mice taking in the advantage of the availability of genetic manipulation (Hillebrandt et al, 2002).

The available animal models of chemicaly induced liver fibrosis using hepatotoxins are:

Carbon tetrachloride (CCl_4)

Carbon tetrachloride is one of the most widely used hepatic toxins for experimental induction of liver injury in laboratory animals. The limitations of using this model are: a higher mortality rate and the degree of lesions achieved can vary depending on species, strain, dose, route and frequency of administration. In addition, the pathology is reversible upon its discontinuation (Bosma et al, 1988; Charbonneau et al, 1986).

Thioacetamide

Thioacetamide is not toxic by itself to the liver, but its metabolic intermediates, in particular, thioacetamide-S-oxide. Compared to CCl_4 the fibrotic lesions are more prominent in thioacetamide-induced cirrhosis in rats (Salguero et al, 2008), and they can persist for several weeks after discontinuation of thioacetamide. The limitations of using this model are a

relative long time needed to induce significant fibrosis and the increased risk of premature loss of test animals due to development of cholangiocarcinoma and hepatocellular carcinoma.

Dimethyl or diethyl nitrosamine

Dimethyl or diethyl nitrosamine is hydroxylated by CYP2E1 in liver cells yielding in the generation of diazonium ions, the bioactive intermediates. In this model, liver fibrosis and cirrhosis develop stably and progressively, even several weeks or months after withdrawal of the toxins. This model is useful for studying the progression of liver fibrosis to hepatocellular carcinoma (Jenkins et al, 1985; Schiffer et al, 2005).

Common bile duct ligation (CBDL)

Bile duct ligation is the classical experimental model for secondary biliary fibrosis. The technique has been mentioned previously in chapter four. Rats are especially adapted to the bile duct ligation model because they lack the gall bladder. In addition, CBDL is also widely used in mice. Relatively high mortality rates due to leakage of bile or rupture of biliary cyst is a major drawback when performing CBDL. Importantly, spontaneous resolution of biliary fibrosis is observed in rats following biliojejunal anastomosis (Issa et al, 2001; Popov et al, 2010). Therefore, CBDL has also been used to study reversibility of fibrosis. Using CBDL, we can obtain most pathological consequences of chronic liver diseases seen in humans like portal hypertension and hepatopulmonary syndrome. However, the presence of different factors like the long duration of chronic liver disease progression in human and the much faster metabolic rates of rodents may rule out the possibility of directly translate the results from animal experiments to the humans.

5.3. The factors implicated in the development of chronic liver diseases in experimental animals and humans and its consequences

5.3.1. Role of NO/NOS

Portal hypertension (PH) is a common clinical complication of chronic liver disease that is associated with high risk of morbidity and mortality. PH is classified as either; pre-hepatic, intra-hepatic or post-hepatic, with intra-hepatic being the most often form caused by cirrhosis irrespective of etiology (Buob et al, 2011). Many vasoactive substances contribute to the development of PH. Among these, NO is the key mediator that paradoxically regulates the intra-hepatic and systemic/splanchnic circulation.

A hyperdynamic splanchnic circulatory state is a major characteristic of portal hypertension. The mechanisms underlying this phenomenon are not fully understood, but overproduction of endogenous vasodilators and decreased vascular reactivity to vasoconstrictors have been involved (Rodriguez-Vilarrupla et al, 2007). There is evidence of eNOS up-regulation and increased NO release by the superior mesenteric artery endothelium that occurs before the development of hyperdynamic splanchnic circulation (Wiest et al, 1999). Furthermore, to support that NO has a role in portal hypertension, certain studies mentioned that overproduction of NO in the splanchnic and systemic circulation contributes to this phenomenon as NOS inhibition effectively improved splanchnic hyperemia (Martin et al, 1998;Thiesson et al, 2003). In contrast, an attenuated splanchnic blood flow was observed in eNOS knock-out mice injected by CCL4. However, this was associated with an increased intra-hepatic vascular resistance, presumably due to the reduced NO formation within the liver (Theodorakis et al, 2009). Taken together, these results indicate an up-regulation of eNOS expression during splanchnic hyperemia associated with relative eNOS deficiency in the liver. In addition, several other studies demonstrated the importance of iNOS in the hyperdynamic circulation of cirrhosis (Ferguson et al, 2006; Kajita et al, 2011; Theodorakis et al, 2003; Wei et al, 2005). In cirrhosis, endotoxins, cytokines and bacterial translocation promote iNOS expression and overproduction of NO (Ferguson et al, 2006; Bauer et al, 2002; Guarner et al, 1993; Albillos et al, 2003). Moreover, this concepts is supported by Ferguson et al, who observed that a selective iNOS inhibitor, N-[3-(aminomethyl) benzyl] acetamidine, caused peripheral vasoconstriction in patients with cirrhosis (Ferguson et al, 2006). It is of outmost importance to note that there exists also an interaction between eNOS and iNOS in the vasculature. For example, in cirrhosis, an increased expression of eNOS is observed in large arteries and this result in systemic hypotension and increased blood flow. Such effects can be blunted by activated iNOS in the small vessels of the splanchnic circulation since iNOS activation resulted in the inhibition of eNOS expression in the small vessels (Bhimani et al, 2003). However, this was not the case in our study using the mesenteric artery we observed a parallel up-regulation of both eNOS and iNOS in CBDL. Furthermore, previous studies suggest also that (neuronal NOS) nNOS may promote vasodilation of the splanchnic circulation, though its contribution is overall less significant (Kwon et al, 2007; Lores-Arnaiz et al, 2005).

5.3.2. Oxidative stress

Oxidative stress is a major event occurring during biological and pathological processes. Molecular oxygen (O_2) is fundamental for the surviving of all aerobic organisms. Aerobic energy metabolism relies on oxidative phosphorylation in mitochondria. However during this process, partially reduced and highly reactive O_2 metabolites, such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), can be found within the cells. These chemically reactive molecules containing oxygen are called reactive oxygen species. The generation of reactive oxygen species in a biological environment expose most living organisms to the so-called 'oxygen paradox': oxygen is necessary for life and reactive oxygen species have important roles in redox homeostasis and cell signaling but they are also potentially hazardous since reactive oxygen species may easily become a source of cell stress and tissue injury, like in most acute and chronic inflammatory processes and infections. However, most living organisms have mechanisms and strategies to overcome the excess generation of reactive oxygen species and oxidative stress, as well as to 'make use' of reactive oxygen species under physiological conditions (Novo and Parola 2008). The sources of reactive oxygen species can be of two types, exogenous or endogenous. The exogenous sources include atmospheric pollution, cigarette smoking, ionizing radiation, xenobiotic metabolism and alcohol. The endogenous sources of reactive oxygen species are mainly enzymatic in nature like NADPH oxidase, un-coupled eNOS, COXs, cytochromes P450, mono-oxygenases, xanthine oxidase, 5-lipo-oxygenase and the mitochondrial respiratory chain. NADPH oxidase and uncoupled eNOS are particularly important and well studied in the field of the cardiovascular system. The NADPH oxidase (Figure 27), is an important source of superoxide anions in human and animal blood vessels associated with vascular pathologies (Rajagopalan et al, 1996; Griendling et al, 2000). Furthermore, Guzik et al. highlighted that the production of superoxide anions by NADPH oxidase is correlated with the degree of endothelial dysfunction (Guzik et al, 2000).

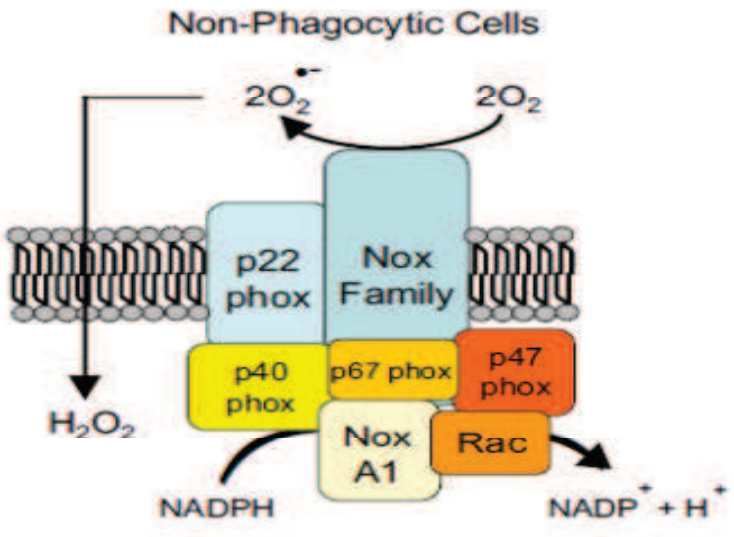

Figure 27. Structure of NADPH oxidase (Novo and Parola 2008).

It is worthy to mention that in every living organism, there are endogenous cellular defense mechanisms including both small molecular weight anti-oxidants (vitamins C and E etc.) and anti-oxidant enzymes (CuZn- and Mn-SOD, glutathione peroxidase, catalase, etc.). In normal physiological conditions, there is a balance response between oxidative stress and the endogenous cellular defense mechanisms. Recent data have shown that these anti-oxidant defense mechanisms, especially catalase, can be compromised under conditions of endothelial dysfunction (Sharma et al, 2007). Thus, the effect of H_2O_2 on eNOS can be separated into either a physiologic response (if catalase levels are maintained) or a pathologic response (when catalase levels are compromised). Thus, under physiological conditions of acute increases in oxidative stress, eNOS will be activated to produce NO, while under sustained oxidative stress like "inflammation", there can be a lack of the essential co-factor tetrahydrobiopterin (BH_4) due its transformation to dihydrobiopterin (BH_2) (by reactive oxygen species), eNOS dimerization will be compromised and finally the generation of NO signaling will be attenuated (Rafikov et al, 2011). In this pathologic case, the uncoupled eNOS subsequently generates superoxide anions rather than NO and, thus, increases the formation of peroxynitrite, which reduces the bioavailability of NO (Kawashima and Yokoyama, 2004). In our model of CBDL, we have shown the implication of reactive oxygen species in the development of the pathology associated with bile duct ligation manifested as augmentation of oxidative stress formation detected by the dihydroethidium redox-sensitive probe in mesenteric and aortic sections. In addition, there is an upregulation of both subunits of NADPH oxidase system the cytosolic p47phox, and membranous p22phox, further

suggesting the involvement of NADPH oxidase system in our model. Furthermore, in both studies of VSL#3 and polyphenol-rich blackcurrant juice (PRBJ), we have shown that there are beneficial effects in reducing oxidative stress, peroxynitrite and preventing the upregulation of NADPH oxidase subunits.

5.3.3. Role of renin angiotensin aldosterone system RAAS

It is well recognized that increased activation of the RAAS is being as important event leading to endothelial dysfunction, arterial stiffness and cardiovascular diseases (Yki-Jarvinen and Westerbacka 2007; Milan et al, 2011; Targosz-Korecka et al, 2013; Underwood and Adler 2013). Furthermore, The RAS (renin–angiotensin system) is now recognized as an important regulator of liver fibrosis and portal pressure in cirrhosis (Grace et al, 2012). Hepatic injury (ischemia or inflammation) induces the hepatic expression of components of the RAS, such as ACE and the AT1 receptors, which play an active role in promoting inflammation and deposition of extracellular matrix. In addition, the more recently recognized structural homologue of ACE, ACE2, is also up-regulated (Grace et al. 2012). ACE2 catalyses the conversion of Ang II into Ang (1–7), and there is accumulating evidence that this 'alternative axis' of the RAS has anti-fibrotic, vasodilatory and anti-proliferative effects, thus counter-balancing the effects of Ang II in the liver (Figure 28). Although the intra-hepatic circulation in cirrhosis is hypercontractile in response to Ang II, resulting in increased hepatic resistance; the splanchnic vasculature is hyporesponsive, promoting the development of the hyperdynamic circulation that, characterizes portal hypertension (Grace et al. 2012). The first approach in the management of portal hypertension is to reduce intra hepatic vascular tone induced by the activity of powerful vasocontrictors such as Ang II, endothelin-1 and the sympathetic nervous system, and mediated via contraction of peri-sinusoidal myofibroblasts and perivascular smooth muscle cells. The second approach is to reduce mesenteric and portal blood flow. In addition to the conventional circulating RAAS, the presence of RAAS components have been detected in tissues such as heart, kidney, vasculture, adipose tissue, immune cells, and the brain (Hayden et al, 2011; Nguyen Dinh Cat and Touyz 2011; Kumar et al, 2012; Underwood, 2013; Bader, 2010). Recent studies have shown that vascular smooth muscle cells, VSMCs, synthesize angiotensin II intracellularly. In vascular smooth muscle cells, the intracellular Ang II regulates the expression of angiotensinogen and renin, generating a feedback loop. The first reaction of intracellular Ang II synthesis (conversion of

angiotensinogen to Ang I) is catalysed by renin and/or cathepsin D, depending on the cell type, and chymase, not ACE, catalyzes the second step (conversion of Ang I to other forms) (Kumar et al. 2012; Bader 2010). In addition, to the classical Ang II system, the role of non-classical angiotensin peptides generated by tissue ACE2 comprising Ang (1-9) and Ang (1-7), which generally antagonize the actions of Ang II are increasingly recognized for their biological activity (Nguyen Dinh Cat and Touyz, 2011; Hayden et al, 2011; Bader 2010; Kumar et al. 2012). Ang (1-7) is also converted to Ang (1-5) by ACE. Ang III, Ang IV, Ang (3-7) are other peptides formed from Ang II (Kumar et al, 2012; Bader 2010). The role of these peptides in vascular tissue is not well understood. The role of cross talk between Ang II and aldosterone signaling is increasingly recognized in the development of endothelial dysfunction and arterial stiffness (Bender et al, 2013; Whaley-Connell et al, 2010; Rautureau et al, 2011; McGraw et al, 2013). Hence, aldosterone activates NADPH oxidase, thereby increasing oxidative stress and decreasing NO bioavailability as one of the mechanism implicated in endothelial dysfunction (Aroor et al, 2012; Whaley-Connell et al, 2010; Hwang et al, 2013).

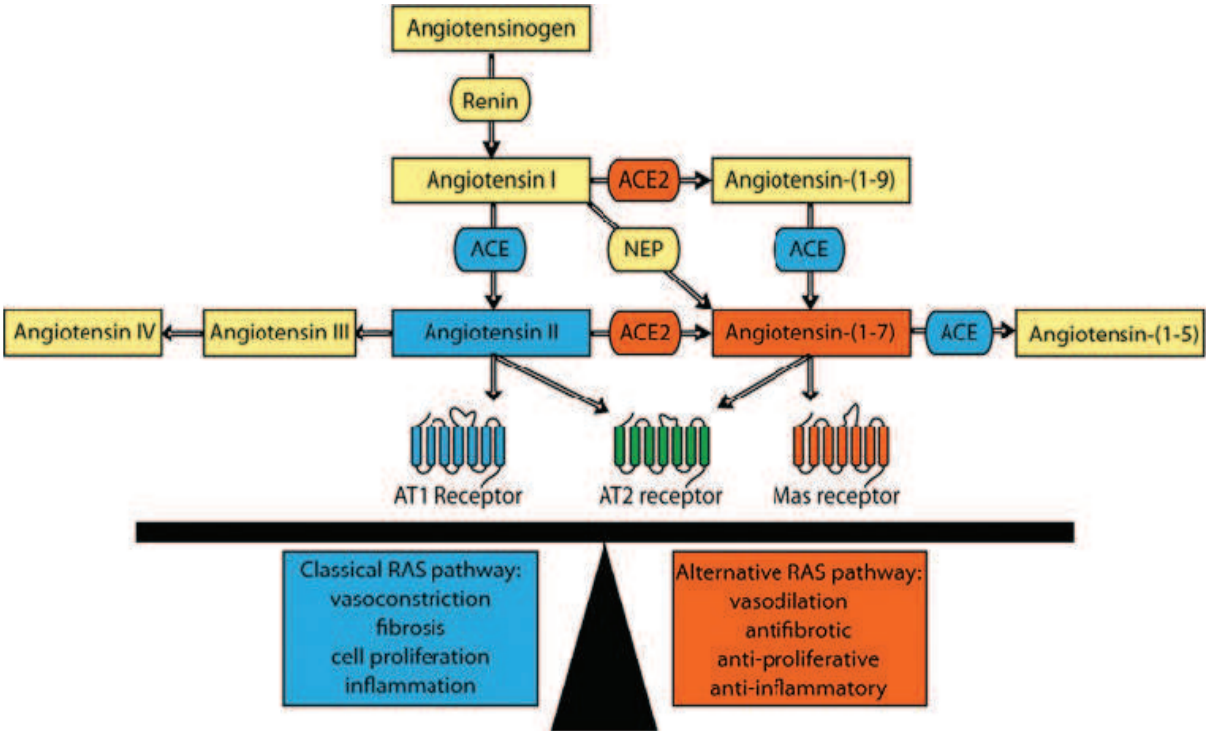

Figure 28. Schematic representation of different biological events depending on the pathway that angiotensinogen passes through (Grace et al, 2012).

5.3.4. Role of gut microbiota in liver diseases

The liver is well known as a detoxification center in almost all mammalians gastrointestinal systems. Furthermore, in normal conditions the liver encounters food-derived antigens and bacterial components such as lipopolysaccharide translocated from the gastrointestinal tract into the portal circulation. However, the liver has the unique capacity to induce immune tolerance. The Toll-like receptors (TLR) are responsible for immune-tolerance, which comprises a highly conserved family of receptors that recognize specific pathogen-associated molecular patterns (PAMPs), which play a key role in innate immunity by triggering inflammatory responses to the main ligands of TLR. On the other, a breakdown in TLR tolerance results in persistent inflammation and contributes to the development of chronic liver diseases (Miyake and Yamamoto 2013). The breakdown of TLR tolerance has been shown to occur in different inflammatory conditions affecting the intestinal integrity like inflammatory colitis, obstructive jaundice and ischemia reperfusion injury. However, previous observations have indicated that bacterial translocation occurs in both normal and diseased livers (Singh et al, 2011). It is the different TLR types and the pro-inflammatory cytokines released by monocytes like, IL-1β and IL-6 in response to bacterial components such as LPS, flagellin and others, that affect liver prognosis regarding inflammation and fibrosis (Figure 29, Miyake and Yamamoto 2013).

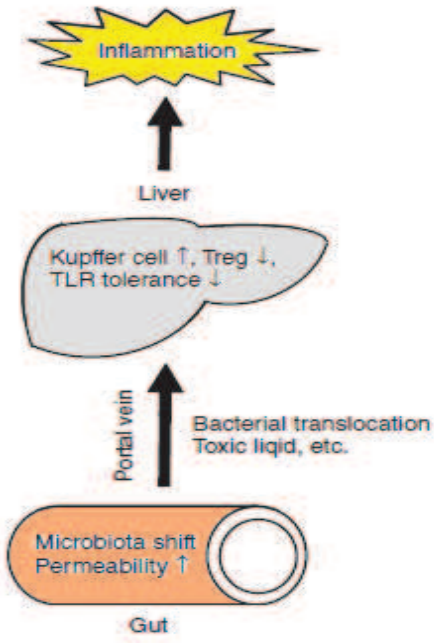

Figure 29. Role of TLR in liver pathology. Treg = T regulatory cell (Miyake and Yamamoto 2013).

The diversity in the bacterial population in the intestine wall reflects the local immune state. For example, Nardone et al., have shown that in a model of liver injury induced by ischemic reperfusion, there was a change in the gut flora characterized by increased intestinal *Enterococcus* spp. and *Enterobacteriaceae,* and decreased lactobacillus spp., *Bifidobactor* spp. and *Bacteroides* spp. The supplementation with *Lactobacillus paracasei* decreased *Enterococcus* spp. and *Enterobacteriaceae* and increased *Lactobacillus* spp., *Bifidobactor* spp. and *Bacteroides* spp., which results in reduced circulating levels of TNF-α, IL-1β and IL-6 associated with an improved inflammation in the liver (Nardone et al, 2010). Furthermore, Osman et al. and others showed that in liver injury induced by chemical substances or alcohol, the probiotic supplementation with species such as *Lactobacillus* spp., *Bifidobacterium* spp. decreased bacterial translocation by attenuating the number of aerobic bacteria such as *E.coli* as well as by increasing intestinal stability (i.e. reduced intestinal permeability), and reduced hepatic inflammation (Osman et al. 2007; Davis 1991; Kirpich et al. 2008). These data support our hypothesis that the beneficial effects of the VSL#3 treatment on endothelial dysfunction in rats with portal hypertension is related to a decreased bacterial translocation.

5.4. VSL#3 effect on local angiotensin system and portal hypertension

In our study, the findings indicate that ingestion of the probiotic VSL#3 formulation effectively prevented the CBDL-induced endothelial dysfunction, at least in part, by targeting the vascular angiotensin system. The results showed an increased expression of Ang II, ACE and AT1R in the aortic sections of CBDL rats associated with endothelial dysfunction. While, treating the CBDL rats with VSL#3 was able to prevent the activation of vascular angiotensin system. These results are consistent with the fact that losartan, an AT1 receptor antagonist, prevented the CBDL-induced endothelial dysfunction and oxidative stress in previous work done in our lab (Dal-Ros et al, 2010). However, we were not able to precise the exact underling mechanism through which VSL#3 affects the vascular angiotensin system. It is likely that bacterial translocation is a key event triggering a cascade of immune and inflammatory responses. The activation of local and systemic inflammatory responses manifested by increased plasma concentrations of pro-inflammatory cytokines such as IL-1α, MCP-1 and TNF-α leads to increased oxidative stress through different mechanisms among them involving the NADPH oxidase system. Previous studies have shown a link between NADPH oxidase activation and the activation of the local angiotensin system and vis versa

(Rajagopalan et al, 1996). Our findings suggest that VSL#3 treatment prevents bacterial translocation since the plasma concentrations of pro-inflammatory cytokines were reduced in CBDL rats treated with VSL#3, and that this effect was associated with a decreased oxidative stress and down-regulation of NADPH oxidase, and, ultimately, a normalized expression level of the local angiotensin system. Furthermore, some few other studies suggested the production of small peptides from probiotics, which have inhibitory effect on angiotensin converting enzyme. This could be additional mechanism for VSL#3 in our work.

In the recent study done by Gupta et al, treating patients with compensated liver cirrhosis with VSL#3 in adjuvant with propranolol was non-inferior to antibiotics plus propranolol (Gupta et al, 2013). They observed that VSL#3 plus propranolol and antibiotics plus propranolol reduced both the hepatic venous pressure gradient and plasma TNF-α level in comparison to propranolol alone (Gupta et al, 2013). In addition, during the course of the two month treatment, VSL#3 treatment was well tolerated by the treated patients. More recent clinical studies using VSL#3 probiotics showed a beneficial effect in patients with irritable bowel syndrome and childhood constipation (Sisson et al, 2014; Yoon et al, 2014). However, no data are available regarding the safety of using VSL#3 in severely immune-compromised patients. The possibility of VSL#3 probiotic, are translocating in immune-deficient patients needs to be addressed.

Uses of probiotics in clinical studies

In a double blind study probiotics vs. placebo, evaluating the effect of probiotics in improving the symptoms of irritable bowel syndrome, they observed that probiotics improved the symptoms such as abdominal colic, changes in bowel motions and dyspepsia (Sisson et al, 2014). They concluded that probiotics offers a potential new treatment for irritable bowel syndrome (Sisson et al, 2014). In a study evaluating the effect of probiotics on childhood constipation (a randomized controlled double blind clinical trial done using probiotics vs. Placebo), it was observed that probiotics have a positive role in increasing the frequency and improving the consistency of bowel motion at the end of the 4^{th} week of treatment (Yoon et al, 2014). In all these clinical studies, probiotics are well tolerated and no notable side effects were noticed, confirming the safety of using probiotics in humans. Finally, we can conclude that VSL#3 gives promising therapy for use in various inflammatory diseases in humans.

5.5. The protective effects of polyphenol-rich blackcurrant juice (PRBJ) in CBDL-induced endothelial dysfunction

In our work of polyphenol-rich blackcurrant juice (PRBJ) on CBDL rats, we have observed that chronic ingestion of PRBJ prevents endothelial dysfunction. This effect involving an improvement of EDH-mediated relaxation in the mesenteric artery associated with an improvement of factors implicated in the EDH-mediated pathway, the endothelial gap junction protein the connexin Cx37 and the expression of calcium-activated potassium channels, SK_{Ca} and the oxidative stress. These further support an important role of polyphenols in preventing cardiovascular diseases, inflammatory diseases and cancer. Polyphenols are well known not only to have antioxidant properties due to their structure but also to have the ability to modulate the activity of different kinds of enzymes and cell receptors. The fact that polyphenols are abundant in our diet makes it necessary to determine the nature and distribution of different kinds of polyphenols, so that we can identify which sources and key compounds are likely to provide an optimal protection especially in the cardiovascular system.

PRBJ and the CBDL-induced vascular oxidative stress

There is a lot of evidence supporting the anti-oxidant effect of polyphenols in vascular cells and, in particular, in smooth muscle cells. The anti-oxidant effect of polyphenols involves predominantly their ability to inhibit the expression and the activity of pro-oxidant enzymes such as NADPH oxidase and xanthine oxidase, and to increase that of anti-oxidant enzymes such as catalase (Lin et al, 2000; Ying et al, 2003). Our observations in the PRBJ study, provides informations regarding the effect of polyphenols in the prevention of the vascular upregulation of NADPH oxidase subunits p22phox and p47phox and of eNOS expression in an animal model of portal hypertension. Furthermore, this effect is associated with the reduction of vascular reactive oxygen species formation and ultimately, improvement of endothelial function. These observations are comparable with those of Sarr et al., (2006) who studies an animal model of hypertension induced by Ang II, where the chronic oral ingestion of red wine polyphenols prevented the development of hypertension and the associated endothelial dysfunction via reduction of vascular oxidative stress. The other interesting findings of the PRBJ study, is the anti-inflammatory properties as indicated by the fact that PRBJ in reduced the plasma concentration of pro-inflammatory cytokines including IL-1, TNF-α and MCP-1. It is known that the CBDL model is an aggressive inflammatory condition associated with systemic inflammatory response. The ability of PRBJ to reduce CBDL-induced inflammatory marker, supports their evaluation in human with other diseases. Our observations are comparable with those of Ergün et al (Ergun et al, 2007) who

highlighted the role of an increased iNOS in experimental necrotizing enterocolitis and the benefit of resveratrol treatment on inflammation and mucosal integrity. Some other studies observed the ability of polyphenols to reduce bacterial translocation in rat models. A decreased in bacterial translocation has been observed in mesenteric lymph nodes, liver and spleen with resveratrol in rats submitted to an intestinal ischemia/reperfusion injury (Ozkan, Yuzbasioglu et al. 2009). All together, these support the need to determine the active polyphenolic molecules and the characterization of the underlying molecular mechanisms.

Other biological effects of Blackcurrant

In addition to the antioxidant effect of the blackcurrant juice that we have observed in our 2nd study, other studies have observed anti-inflammatory and immune-modulatory effects of blackcurrant. It is known that in living tissues, the inflammatory response involves a cascade of eicosanoids, signalling molecules made from oxidation of essential fatty acids. One of the precursors of essential fatty acids is γ-linolenic acid which is abundant in blackcurrant seed oil. Moreover, eicosanoids derived from γ-linolenic acid, exerts anti-inflammatory responses (Barre, 2001). Furthermore, studies involving an administration of a blackcurrant extract on a human monocytic cell line derived from an acute monocytic leukemia patients, revealed a suppressive effect against TNF-α and its downstream molecules, especially NF-kB and IL-6 in response to lipopolysaccharide (Lyall et al, 2009).

The PRBJ and bioavailability of polyphenols

Although it is important to know the biological function of natural polyphenols and the underlying mechanism, the bioavailability of these compounds is also of importance. However, previous studies have indicated that anthocyanins and procyanidins have a low absorption and bioavailability (Tsang et al, 2005;Basu et al, 2010;Shoji et al, 2006). Despite their relatively poor apparent bioavailability, numerous studies have reported that intake of polyphenols-rich products has a beneficial effect on the endothelial function in experimental animals and humans (Schini-Kerth et al, 2010). In some feeding studies with human subjects and animal models, typically approximately <0.1% of the quantity ingested, has been detected in urine within 24 h of consumption of catechins from ready-to-drink tea (Del Rio et al, 2010). However, the structure of the anthocyanin aglycon and the attached glucoside moiety seem to have a strong effect on their absorption, metabolism and excretion (Manach et al,

2005). Despite, the fact that most of the polyphenols undergo hydrolysis by intestinal enzymes or the colonic microflora before absorption (Day et al, 1998; Nemeth et al, 2003) intact glycosides are the major circulating forms of anthocyanins (Manach et al, 2004) possibly due to the instability of the aglycone form, anthocyanidins, and to the existence of a specific mechanism of absorption or metabolism. Furthermore, some recent studies have shown that, in rat and mice, anthocyanins are absorbed from the stomach (Talavera et al, 2003; Passamonti et al, 2005; Matuschek et al, 2006). Other studies reported that, cyanidin-based anthocyanins can undergo cleavage of the sugar moiety followed by ring fission of the released cyanidin, which produces phenolic acids such as 3,4-dihydroxybenzoic acid (protocatechuic acid) by the microflora in the colon (Aura et al, 2005; Vitaglione et al, 2007). The possibility that these phenolic acids are absorbed and contribute to different biological function (anti-oxidant and anti-inflammatory) remains to be determined. Moreover, some authors have shown that metabolites of polyphenols affect growth of certain intestinal bacteria colonies. Hui Cheng et al observed in their study, that different strains of intestinal bacteria had varying degrees of growth inhibition to tea phenolic and metabolites. Growth of certain pathogenic bacteria such as *Clostridium perfringens, Clostridium difficile and Bacteriodes spp* was significantly suppressed by tea phenolics and their derivatives, while commensal anaerobes like *Bifidobacterium spp*. and probiotics such as *Lactobacillus sp.* were less severely affected (Lee et al, 2006).

5.6. Does the estrogen-mediated up-regulation of pulmonary eNOS is the sole events in hepatopulmonary syndrome

Estrogens have a regulatory effect on eNOS activity through genomic (transcriptional) and non-genomic (non-transcriptional) pathway. Experimental work in human endothelial cells showed that, estrogens enhance eNOS protein and mRNA abundances in the absence of changes in the stability of eNOS mRNA (Chambliss and Shaul 2002; Morales et al, 1995). In a model of vascular injury, estrogens affect vascular injury by promoting angiogenesis (Morales, McGowan et al. 1995). Estradiol-enhanced endothelial cell activation is important in neo-vascularization and suggests a promoting influence of estrogens on angiogenesis (Morales, McGowan et al. 1995). It is well known from previous studies that pulmonary dilatation in cirrhotic patients or animals is associated at least in part by an up-regulation of pulmonary eNOS (Nunes et al, 2001; Gomez et al, 2006). Moreover, several

clinical and animal studies have suggested that an increased level of estradiol may be implicated in the occurrence of the vasodilatation most likely subsequently to an increased NO formation (Aller et al, 2001; Aller et al, 2002; Yol et al, 2005). The generalized vasodilatation leads to complications and especially to a hepatopulmonary syndrome (HPS) in approximately 10%–15% of patients awaiting liver transplantation (Rodriguez-Roisin et al, 2004). The HPS, which is characterized by arterial deoxygenation because of intrapulmonary vascular dilatations, is of prognostic value for the survival before and also after liver transplantation (Rodriguez-Roisin et al, 2004; Arguedas et al, 2003). Estrogens have been shown to contribute positively in the evolution of different pathologies like pulmonary hypertension and trauma hemorrhage-induced lung injury (Resta et al, 2001; Kan et al, 2008) and certain developmental processes (MacRitchie et al, 1997). In the fulvestrant study, we have observed an attenuation of CBDL-induced pulmonary eNOS expression by fulvestrant as assessed using Western blot and immunofluorescence. Unfortunately, the lung pathology persisted as indicated by pulmonary capillary dilatation. These observations indicate that estrogens do not have a fundamental role and that other marker and factors contribute to the development of hepatopulmonary syndrome.

Conclusions and perspective

It is well known from previous works that probiotics are tolerable and have low toxicity. Our observations on VSL#3 study in accordance with what have been published, support potential benefit of VSL#3 in chronic inflammatory diseases including chronic liver diseases.

Perspective work on VSL#3 need to address the following, VSL#3-induced changes on the intestinal microbial composition are well established, the identification of the specific bacteria affected in the vast microbial community still remains unknown. The role of the VSL#3-induced changes in the intestinal microbiota and of specific bacteria in the improvement of the endothelial function in the CBDL rat model as observed in the first study remains unknown. We need to address this rather difficult challenge in a subsequent study in collaboration with microbiologists. The possibility whether or not ACE inhibitory peptides are generated by the VSL#3 treatment in the CBDL rat model has not yet been tested, it well be of high interest to test that in the future studis.

Our observations on polyphenols in general and on Blackcurrant juice, provide evidences suggesting that natural products might be of interest for improving liver diseases. In accordance with that, there are numerous reports that support an important role of polyphenols in preventing cardiovascular diseases, inflammatory diseases and cancer. In addition to the antioxidant properties of polyphenols, their ability to modulate the activity of different kinds of enzymes and cell receptors are also of interest. The fact that polyphenols are abundant in our diet makes it necessary to determine the nature and distribution of different kinds of polyphenols, so that we can identify which sources and key compounds are likely to provide the greatest protection for endothelial cells. Further works need to detect the exact active molecule of polyphenols and their metabolites plus more details about their bioavailability, before we can proceed to introduce them in human research.

Our results on fulvestrant, suggest that fulvestrant may not be an option to improve the hepatopulmonary syndrome. However, there are some limitations. We chose to treat the rats with a bolus of 10 mg of fulvestrant (in castor oil) which has been shown to induce anti-oestrogenic activity for 1 month or more. Nevertheless we can question if a greater dose of fulvestrant would have had more effects on the lungs. A second limitation is that only male rats were studied, if the results could differ in female rats should be tested in the future.

Overall further work needs to dentify the pathophysiological process of portal hypertension and its complications like hepatopulmonary syndrome. Furthermore, it will be interesting to test both VSL#3 and PRBJ to study other organs affected by portal hypertension like lung and kidneys. Our studies support that both VSL#3 and polyphenols are of potential interest for therapeutic use in liver diseases.

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