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REVIEW

# Current concepts on the role of nitric oxide in portal hypertension

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## Abstract

Portal hypertension (PHT) is defined as a pathological increase in portal venous pressure and frequently accompanies cirrhosis. Portal pressure can be increased by a rise in portal blood flow, an increase in vascular resistance, or the combination. In cirrhosis, the primary factor leading to PHT is an increase in intra-hepatic resistance to blood flow. Although much of this increase is a mechanical consequence of architectural disturbances, there is a dynamic and reversible component that represents up to a third of the increased vascular resistance in cirrhosis. Many vasoactive substances contribute to the development of PHT. Among these, nitric oxide (NO) is the key mediator that paradoxically regulates the sinusoidal (intra-hepatic) and systemic/splanchnic circulations. NO deficiency in the liver leads to increased intra-hepatic resistance while increased NO in the circulation contributes to the hyperdynamic systemic/splanchnic circulation. NO mediated-angiogenesis also plays a role in splanchnic vasodilation and collateral circulation formation. NO donors reduce PHT in animals models but the key clinical challenge is the development of an NO donor or drug delivery system that selectively targets the liver.



**Key words:** Nitric oxide; Portal hypertension; Hepatic stellate cell; Liver cirrhosis

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## INTRODUCTION

Portal hypertension (PHT) is a common clinical consequence of chronic liver disease that is associated with significant morbidity and mortality. PHT is classified as either pre-hepatic, intra-hepatic or post-hepatic, with intra-hepatic PHT being the form most often caused by cirrhosis, irrespective of etiology[1]. The extent of PHT is quantified in clinical practice by measuring the hepatic portal vein pressure gradient (HPVG)[2], representing the difference between the wedged hepatic vein pressure (a measure of pressure at the level of the hepatic sinusoid), and the free hepatic vein pressure. Thus, HPVG is often used to assess the effects of pharmacological therapy in reducing portal pressure[3].

Based on hydromechanics, fluid pressure in a hollow tube is determined by fluid resistance and flow. In PHT, therefore, the intra-hepatic vascular resistance (IHVR) and splanchnic blood flow are the two main contributors to portal pressure[4]. Under normal circumstances, postprandial increases in splanchnic blood flow is always associated with an autonomous down-regulation of IHVR, leading to no alteration in portal pressure. In contrast, IHVR is significantly up-regulated by mechanical and hemodynamic factors in the setting of cirrhosis, which is further aggravated by splanchnic vasodilation[5]. Clinically, this increase in portal pressure is the antecedent to

variceal bleeding with its associated morbidity and high mortality[6,7].

IHVR is influenced by both hepatic fibrotic architectural distortion in cirrhosis leading to obstruction to blood flow, as well as by dynamic hepatic stellate cell (HSC) contraction around sinusoidal blood vessels. Angiogenesis, or the formation of new blood vessels, is also an important component of the pathophysiology of PHT. The resulting alterations in vascular contractility and angiogenesis contribute to PHT in both the intrahepatic and splanchnic circulation.

Endothelin 1 (ET-1), angiotensin II, norepinephrine, prostaglandin F2, thromboxane A2, and thrombin can trigger liver sinusoidal contraction. In contrast, substances such as acetylcholine, vasointestinal peptides, nitric oxide (NO), carbon monoxide, prostaglandin E2, and adrenomedullin relax the sinusoidal vasculature[8,9]. Among these agents, ET-1 and NO are the most important regulators of the sinusoidal microcirculation[8,9]. In PHT, an insufficient release of vasodilators particularly NO from endothelial cells is critical to the genesis of the dynamic and modifiable component of increased vascular resistance[8,9]. Consistent with this, improvements in intrahepatic NO availability is beneficial for the treatment of PHT in animals and patients[10-14]. Hence, this review will focus on an update on the mechanisms whereby NO mediates PHT and on the potential to modulate this system to reduce portal pressure.

## SYNTHESIS AND FUNCTION OF NO

NO is synthesized by nitric oxide synthase (NOS) through a series of redox reactions involving L-arginine (the main substrate), oxygen and nicotinamide adenine dinucleotide phosphate. There are 4 major isoforms of NOS: endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and mitochondrial nitric oxide synthase[15]. Following synthesis by NOS, the half-life of endogenously generated NO is extremely short, about 1 s. Thus, endogenous NO production is intimately regulated by the activity of NOS.

The generated NO molecule has a large diffusion coefficient and can therefore freely penetrate cellular membranes in an autocrine or paracrine manner. Within the cell, NO stimulates the conversion of guanosine 5'-triphosphate (GTP) to cyclic guanosine 3'-5'-monophosphate (cGMP), thereby regulating calcium balance through the cGMP-dependent protein kinase pathway (Figure 1). This leads to vasodilatation[16]. The end products of NO metabolism *in vivo* are nitrate ($NO_3^-$) and nitrite ($NO_2^-$) that are an indirect measure of the total NO concentration[17].

NO is also highly reactive with other molecules including superoxide anion ($O_2^-$), oxygen ($O_2$) and hemoproteins such as hemoglobin and myoglobin. The intermediate products of these reactions are known as reactive nitrogen species, which promotes many pathophysiologically damaging reactions including lipid peroxidation, DNA strand breaks, and the generation of nitrosamines, nitrotyrosine and nitro guanosine.

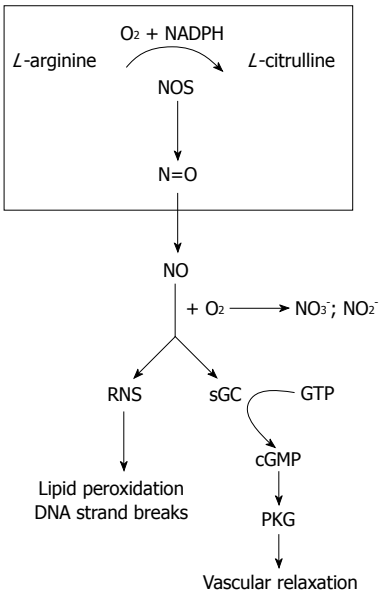


**Figure 1 Nitric oxide formation and function**. Nitric oxide synthase (NOS) catalyzes the biosynthesis of nitric oxide (NO) from *L*-arginine, nicotinamide adenine dinucleotide phosphate (NADPH) and $O_2$•NO freely diffuses into cells where it mediates vascular relaxation by stimulating the cyclic guanosine 3'-5'-monophosphate (cGMP)/cGMP-dependent protein kinase G (PKG) pathway. It also forms reactive nitrogen species (RNS) which leads to many damaging reactions including lipid peroxidation and DNA strand breaks. GTP: Guanosine 5'-triphosphate; sGC: Soluble guanylyl cyclase.

## MOLECULAR MECHANISMS REGULATING NOS

eNOS serves a key role in maintaining circulatory homeostasis and is expressed mainly in endothelial cells and to a lesser extent in cardiac myocytes and platelets[15]. The enzyme localizes to small invaginations of the plasma membrane named caveolae in quiescent cells. eNOS protein is constitutively expressed in the cell and activation mostly comprises post-translational regulation and modifications in its subcellular localization[18].

Within cells, eNOS closely associates with several proteins that impact on its function, including caveolin. Caveolin negatively regulates eNOS by directly abrogating the enzyme's activation and blocking the binding site for calmodulin[19]. In contrast, calmodulin acts as an indispensable protein competing with caveolin for binding with, and activating eNOS[20,21]. Other relevant proteins in relation to NO production include heat shock protein 90 and tetrahydrobiopterin (BH4) that are positive regulators of eNOS[22-24]. Finally, eNOS interacting protein and eNOS trafficking inducer protein participate in the subcellular trafficking of eNOS when eNOS translocates from caveolae into the cytoplasm[25-27].

Phosphorylation at key serine residues is the major post-translational modification that is required for eNOS

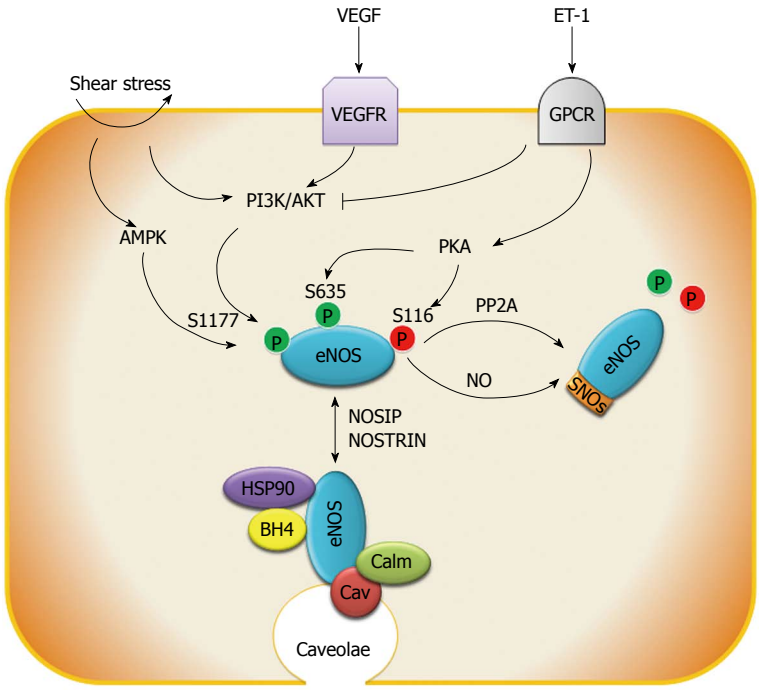


**Figure 2 The molecular regulation of endothelial nitric oxide synthase activity**. Endothelial nitric oxide synthase (eNOS) phosphorylation can be triggered by shear stress, vascular endothelial growth factor (VEGF), endothelin 1 (ET-1) and other factors though adenosine monophosphate-activated protein kinase (AMPK), protein kinase B (AKT) and protein kinase A (PKA) pathways, whereas protein phosphatase 2 (PP2A) de-phosphorylates eNOS. In addition, *S*-nitrosylation (SNOs) by eNOS-derived nitric oxide (NO) inhibits eNOS activity. Endothelial nitric oxide synthase interacting protein (NOSIP) and endothelial nitric oxide synthase trafficking inducer protein (NOSTRIN) regulate the sub-cellular location of eNOS protein between the caveolae and cytoplasm. The principal location of eNOS is in caveolae where its function is inhibited by binding to caveolin (Cav). HSP90, calmodulin (Calm) and tetrahydrobiopterin (BH4) are indispensable proteins and cofactors for catalyzing NO production. PI3K: Phosphatidylinositol-3-kinase; GPCR: G protein-coupled receptor.

function. Phosphorylation of Ser 1177, Ser 635 and Ser 617 activates eNOS whereas phosphorylation of Thr 495 and Ser 116 inhibits eNOS activity[28]. Phosphorylation at Ser 1177 can be initiated by activation of several intracellular pathways including phosphatidylinositol-3-kinase (PI3K/AKT), cAMP-dependent protein kinase A (PKA), adenosine monophosphate-activated protein kinase, cGMP-dependent protein kinase G (PKG) and *Calmodulin Kinase II*-dependent pathway (CaM kinase II)[29-32], while Ser 635 and Ser 116 is activated *via* a PKA-dependent pathway[33,34]. Additionally, shear stress, vascular endothelial growth factor (VEGF) and high-density fatty acids can phosphorylate and activate eNOS (Figure 2)[35]. In contrast, phosphatases like protein phosphatase 2 dephosphorylates and inactivates eNOS[36]. S-Nitrosylation inhibits eNOS activity by modifying its steric configuration, whereas de-nitrosylation is associated with an increase in eNOS activity[37,38].

Unlike eNOS, iNOS is more widely expressed, including in macrophages, vascular smooth muscle cells, HSCs and Kupffer cells after stimulated by lipopolysaccharide (LPS) or inflammatory cytokines. iNOS produces a relatively high level of NO compared to eNOS[39]. In contrast to eNOS, iNOS expression is principally modulated by transcriptional mechanisms. Many transcription factors regulate the expression of iNOS including nuclear factor-κB (NF-κB), activator protein, signal transduction and activation of transcription 1a, specificity protein 1, CCAAT/enhancer-binding protein (C/EBP), *cAMP* response element-binding, GATA binding transcription factor, hypoxia-inducible factor, interferon regulatory transcription factor, nuclear factor of activated T-cells, nuclear factor-interleukin 6, octamer-1 transcription factor, poly [ADP-ribose] polymerase 1, polyomavirus enhancer activator 3, tumor protein 53 and serum response factor[40]. Among these, NF-κB is considered the primary mediator for iNOS induction. In turn, NF-κB can be activated by a range of stimuli including LPS, interleukin-1β, tumor necrosis factor (TNF)-α and oxidative stress[41,42]. iNOS can also be post-transcriptionally regulated through mRNA stabilisation by RNA-binding proteins such as A+U rich RNA binding factor, human antigen R, K-homology splicing regulator protein, polypyrimidine tract-binding protein and tristetraprolin[40].

nNOS is principally expressed in neurons localized to the nervous system including the brain, the autonomic nervous system and neurons around interlobular arteries. The portal vein endothelial cells also expresses nNOS[43]. Like eNOS, nNOS is regulated by post-translational mechanisms and both Hsp90 and calmodulin are involved in the process of nNOS activation[44-46].

## MOLECULAR REGULATION OF NOS IN LIVER CIRRHOSIS AND PHT

### *Regulation of intra-hepatic eNOS*

In cirrhosis and PHT, there is reduced NO production

by hepatic endothelial cells that is attributed to dysfunction of the eNOS system[47-50]. Many factors contribute to intra-hepatic eNOS dysfunction/reduced eNOS activity. These include increases in oxidative stress, caveolin-1, RhoA, thromboxane $A_2$ ($TXA_2$), G-protein-coupled receptor kinase-2 (GRK2) and asymmetric dimethylarginine (ADMA) as well as decreased AKT and BH4 activity.

Reduced AKT activity and increased binding ability of caveolin-1 to eNOS in cirrhosis attenuates eNOS expression[51,52]. Liu *et al*[51], reported that ET-1 activates G-protein-coupled receptor kinase-2 (GRK2) which directly interacts with and inhibits AKT phosphorylation. They also noted that the IHVR was significantly reduced in bile duct ligation (BDL) mice genetically deficient in GRK2[52]. In another study of eNOS expression during BDL, Morvarid *et al*[53], noted that total eNOS protein was unchanged, but that functional, phosphorylated eNOS protein was decreased. Similarly, AKT expression was down-regulated in a time dependent manner. In contrast, caveolin-1 was increased[53].

Intrahepatic oxidative stress is a key mediator of sinusoidal endothelial dysfunction and impairment of eNOS/NO expression[54-57]. For example, Gracia *et al*[58], noted that increased intrahepatic oxidative stress (increased ROS and $O_2^-$) was associated with reduced NO production and NO bioavailability. The authors went on to demonstrate that cyclooxygenase (COX) attenuated eNOS activation by stimulating $TXA_2$ which inhibits AKT phosphorylation in endothelial cells[59]. A superoxide dismutase mimetic, Tempol significantly decreased superoxide, and increased NO in cultured hepatic endothelial cells. As expected, Tempol administration also resulted in a decline of portal pressure[60].

ADMA, an endogenous inhibitor of NOS, causes uncoupling of NOS leading to generation of RNS, such as peroxynitrite. In BDL rats, a higher serum ADMA level was observed[61]. Further, impaired endothelial cell-mediated relaxation in perfused livers of BDL rats was exacerbated by ADMA and was associated with a decreased rate of ADMA removal[61,62].

BH4, a cofactor of eNOS, has been reported to be associated with dysfunction of the NO system. BH4 expression is down-regulated in liver cirrhosis and can further be oxidized and inactivated by $O_2^-$. In the absence of BH4, eNOS cannot generate NO but instead produces $O_2^-$, thereby leading to further decreases in NO production[24,63]. In an *in vivo* study, Matei *et al*, observed that in rats rendered cirrhotic after the administration of carbon tetrachloride ($CCl_4$), exogenous BH4 resulted in hepatic NOS and cGMP activation and a reduction in portal pressure[64].

Rho-associated protein kinase (ROCK) is a kinase belonging to the AGC (PKA/PKG/PKC) family of serine-threonine kinases. It is mainly involved in regulating the shape and movement of cells by acting on the cytoskeleton. Rho-kinase is substantially involved in the contraction of activated HSCs[65,66]. In BDL rats, fasudil (a potent Rho-kinase inhibitor) significantly suppressed liver Rho-kinase activity and increased eNOS phosphorylation compared with controls[67]. Fasudil also reduced the binding of the serine/threonine AKT to Rho-kinase and increased the binding of AKT to eNOS[67].

### *Regulation of extra-hepatic vascular eNOS, iNOS and nNOS in cirrhosis*

In contrast to the hypoactive SECs in the intrahepatic microcirculation, hyperactive endothelial cells with increased NO production play a critical role in modulating the vascular changes observed in the splanchnic and systemic circulation. For example, increased activity of peripheral vascular AKT signaling is noted, while constitutive AKT inhibition by an inactive mutant decreases aortic eNOS and improves systemic hemodynamics, splanchnic perfusion pressure and renal excretory function without affecting portal pressure[68]. Other studies reported that VEGF induces NO production by activation of eNOS protein expression and activity[69,70]. Likewise, in portal hypertensive rats, NO production is increased in response to shear stress[71]. LPS detoxification is limited in liver with PHT thereby increasing plasma LPS. Resident macrophages in the splanchnic circulation respond to this circulating LPS with the production of proinflammatory cytokines, such as TNF-α[72] that then induces iNOS in extrahepatic vasculature[73-75]. Bacteria-derived TNF-α also triggers the expression and activity of the key enzyme involved in the regulation of BH4, GTP-cyclohydrolase I, thereby increasing eNOS-derived NO in the mesenteric vasculature[76,77]. Finally, nNOS expression is augmented in mesenteric nerves in portal hypertensive rats (portal vein ligation), an effect mediated by HSP-90[46,78,79].

## THE ROLE OF NO/NOS IN THE REGULATION OF IHVR

An increase in IHVR can be induced by reversible hemodynamic modifications to vascular tone which may represent 28%-40% of the increase in portal pressure in cirrhosis[80-82]. Anatomic structures leading to this change include vascular smooth muscle cells surrounding branches of the portal vein, and HSCs located in the space of Disse. Both cells types have contractile properties and thus modulate IHVR[82-84].

The role of NO in the modulation of IHVR has been well documented[85-87]. eNOS dysfunction in sinusoidal endothelial cells and consequent reduction in NO production (or bioavailability) plays an essential role[51]. This results in reduced vasodilation and a decreased capacity for antagonizing contractile factors such as ET-1, angiotensin II, norepinephrine, prostaglandin F2, and thromboxane A2[83,88].

Recently, gene delivery techniques have been used to increase NOS (eNOS or nNOS) delivery to the liver of $CCl_4$ treated mice. In one study, a plasmid eukaryotic expression vector (liposome-pcDNA3/eNOS) or control vector was injected into rat portal vein, leading to increased eNOS mRNA and protein in liver. Hepatic

NO production was enhanced and IHVR and portal vein pressure (PVP) reduced[89]. In another study, recombinant adenovirus carrying the nNOS gene (Ad.nNOS) or control vector was administered *via* the femoral vein to rats. Again, Ad.nNOS reduced IHVR and portal pressure[90]. These data indicate that NO deficiency in cirrhotic liver contributes to the elevation in IHVR and conversely that NO delivery may play a therapeutic role[89-92].

Activation and contraction of HSCs also contributes significantly to the dynamic and reversible component of IHVR. Indeed, activated HSCs are more susceptible to vasoconstrictor substances than quiescent cells[83,92,93]. Under physiological conditions, NO produced by hepatic endothelial cells inhibits the growth, migration and contraction of HSCs through paracrine pathways[94,95]. However, reduced NO production and/or impaired NO bioavailability in cirrhosis promotes HSCs activation and contraction, leading to sinusoidal remodeling and elevation of the IHVR.

iNOS has also been suggested to contribute to the hyperdynamic status seen in PHT. However, its role in mediating IHVR is unclear. In one study, liver iNOS was increased in BDL rats and reduction of portal pressure by ursodeoxycholic acid was associated with iNOS down-regulation[96,97].

## ROLE OF NO/NOS IN THE REGULATION OF SPLANCHNIC BLOOD FLOW

A hyperdynamic splanchnic circulatory state is a major accompaniment of PHT. The increase in splanchnic blood flow and the subsequent increase in portal venous inflow aggravates and perpetuates PHT. The mechanisms underlying this phenomenon are not fully understood, but overproduction of endogenous vasodilators and decreased vascular reactivity to vasoconstrictors has been suggested[98].

Overproduction of NO in the splanchnic and systemic circulation contributes to this phenomenon as NOS inhibition effectively ameliorates splanchnic hyperemia[99,100]. eNOS up-regulation and increased NO release by the superior mesenteric arteries endothelium occur before the development of the hyperdynamic splanchnic circulation[101]. Juan *et al*[70], noted increased eNOS expression in portal-hypertensive rats with even mild increases in portal pressure. In another study, phosphorylated eNOS protein was increased, whereas caveolin-1 was decreased in the aorta of BDL rats[52]. In contrast, in eNOS knockout mice injected with CCL4, attenuated splanchnic blood flow was observed. However, this was associated with an increase in IHVR, presumably due to the reduced NO within the liver[102]. Taken together, these results suggest up-regulated eNOS expression during splanchnic hyperemia, contrasts with the relative eNOS deficiency in liver.

There are also several studies demonstrating the importance of iNOS in the hyperdynamic circulation of cirrhosis[64,72,73,103,104]. In cirrhosis, endotoxins, cytokines and bacterial infection promote iNOS formation and overproduction of NO[64,105-107]. The increased splanchnic iNOS appears to reside in resident macrophages of the superior mesenteric artery[73,108]. Supporting this concept, Ferguson *et al*[64], observed that a selective iNOS inhibitor, *N*-[3-(aminomethyl) benzyl]acetamidine, caused peripheral vasoconstriction in patients with cirrhosis. It is interesting to note that there also exists an interaction between eNOS and iNOS in the vasculature. For example, in cirrhosis, increased and dominant expression of eNOS in large arteries results in systemic hypotension and increased blood flow. These effects could be abrogated by activated iNOS in the small vessels of the splanchnic circulation as iNOS activation inhibited eNOS expression in the small vessels[109]. nNOS may likewise promote vasodilation of the splanchnic circulation, though its contribution is overall less significant[110,111].

## NO AND ANGIOGENESIS IN PHT

It is now established that angiogenesis is associated with the progression of PHT[112,113]. Angiogenic factors stimulate collateral vessel formation both in the liver and in extrahepatic locations, manifesting as the reopening of pre-existing shunts[114,115]. This pathological angiogenesis may directly participate in the development of liver fibrosis[56,116,117].

Again, NO is an important mediator of intrahepatic microcirculatory remodeling[114,115]. Thus, NO inhibition prevents angiogenesis and diminishes mesenteric vascular proliferation in animals with PHT[118,119]. Shaki *et al*[120], found that NO-mediated angiogenesis was mediated by endothelial VEGF and VEGF receptor-1. Most recently, Huang *et al*[121], reported that through mesenteric eNOS and COX1 down-regulation, the cannabinoid receptor 2 agonist JWH 015, could alleviate mesenteric and intrahepatic angiogenesis, PHT, the severity of portosystemic collaterals and the extent of fibrosis in BDL cirrhotic rats.

## NO-BASED PHARMACOTHERAPY

As discussed, NO is paradoxically regulated in PHT. There is excessive production of NO in the splanchnic circulation (thereby leading to vasodilation), while in the intra-hepatic microcirculation, a deficit of NO production is associated with increased IHVR. These paradoxical roles of NO initially raised concerns about the use of NO inhibitors or donors as therapy for PHT. However, inhibition of NO release has been shown in animals and humans to attenuate the hyperdynamic circulation of cirrhosis[122-125]. No significant reduction in portal pressure was achieved[122-125]. This is likely a consequence of reductions in portal venous inflow induced by the NO inhibitors being offset by an increase in intra-hepatic resistance.

In recent years, many animal and clinical studies have

demonstrated that NO donors result in a substantial reduction in portal pressure[10-14]. These agents could theoretically aggravate the cirrhotic vasodilatory syndrome leading to harmful effects such as systemic hypotension and renal dysfunction[126,127]. For these reasons, the ideal NO drug for the treatment of PHT should act to decrease IHVR without worsening splanchnic/systemic vasodilatation[128].

NCX-1000 is a drug synthesized by adding an NO-releasing moiety to ursodeoxycholic acid. The compound is selectively metabolized by hepatocytes to release NO in the liver[129,130]. Animal studies demonstrate that this drug alleviates IHVR and portal pressure without changes in systemic hemodynamics[129-131]. However, human clinic trials were disappointing as NCX-1000 failed to decrease HVPG, there were postprandial increases in portal pressure and systolic blood pressure was reduced in a dose-dependent manner[132].

O2-vinyl1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO) was designed as a liver-selective NO-producing pro-drug activated by hepatic P450s[133]. The drug has a short half-life and may additionally alleviate liver injury by NO-mediated protection of hepatocytes[134-136]. Continuous administration of V-PYRRO/NO to BDL rats was shown to improve liver fibrosis and splanchnic hemodynamics without adverse systemic effects[137]. However, in another study in mice using the $CCl_4$ model, V-PYRRO/NO significantly lowered mean arterial pressure making it less suitable for use in humans[138].

AVE-9488(4-fluoro-*N*-indan-2-yl-benzamide) is a novel agent that up-regulates eNOS expression[139]. Biecker *et al*[139], reported that oral application of AVE 9488 ameliorated portal pressure by 24% in BDL rats, without any impact on the mean arterial pressure. Additional experiments confirmed that AVE 9488 increased hepatic eNOS protein synthesis, but not in the aortic and superior mesenteric artery[139]. However, following 3-d use, AVE 9488 increased blood flow in the collateral circulation[139].

Recently, an inorganic gold and silica nanoparticle mediated drug delivery system using SNAP (*S*-nitroso-*N*-acetyl-DL-penicillamine), an NO donor was reported[140]. This system inhibited HSC proliferation and HSC tube formation, though the relevance of the latter to the situation *in vivo* is unclear. The methodology described however, does provide a novel approach to deliver NO into specific liver cell types. Whether this drug modulates PHT *in vivo* is unclear. Taken together, the data presented indicates that there are no liver-selective NO donors/drugs with demonstrated efficacy for the treatment of PHT.

## CONCLUSION

NO plays a pivotal role in the pathogenesis of PHT. NO levels are differentially altered in cirrhosis, with reduced production in the intrahepatic circulation and increased NO production in the splanchnic bed. Ideally, a NO donor or drug delivery system that selectively targets liver cells (HSCs or SECs) without actions on the systemic circulation is required to reduce PHT without adverse systemic effects.

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