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REVIEW | *Cardiovascular Actions of Hydrogen Sulfide and Other Gasotransmitters*

Decoding the vasoregulatory activities of bile acid-activated receptors in systemic and portal circulation: role of gaseous mediators

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Fiorucci S, Zampella A, Cirino G, Bucci M, Distrutti E. Decoding the vasoregulatory activities of bile acid-activated receptors in systemic and portal circulation: role of gaseous mediators. *Am J Physiol Heart Circ Physiol* 312: H21–H32, 2017. First published October 7, 2016; doi:10.1152/ajpheart.00577.2016.—Bile acids are end products of cholesterol metabolism generated in the liver and released in the intestine. Primary and secondary bile acids are the result of the symbiotic relation between the host and intestinal microbiota. In addition to their role in nutrient absorption, bile acids are increasingly recognized as regulatory signals that exert their function beyond the intestine by activating a network of membrane and nuclear receptors. The best characterized of these bile acid-activated receptors, GPBAR1 (also known as TGR5) and the farnesoid-X-receptor (FXR), have also been detected in the vascular system and their activation mediates the vasodilatory effects of bile acids in the systemic and splanchnic circulation. GPBAR1, is a G protein-coupled receptor, that is preferentially activated by lithocholic acid (LCA) a secondary bile acid. GPBAR1 is expressed in endothelial cells and liver sinusoidal cells (LSECs) and responds to LCA by regulating the expression of both endothelial nitric oxide synthase (eNOS) and cystathionine-γ-lyase (CSE), an enzyme involved in generation of hydrogen sulfide (H_2S). Activation of CSE by GPBAR1 ligands in LSECs is due to genomic and nongenomic effects, involves protein phosphorylation, and leads to release of H_2S. Despite that species-specific effects have been described, vasodilation caused by GPBAR1 ligands in the liver microcirculation and aortic rings is abrogated by inhibition of CSE but not by eNOS inhibitor. Vasodilation caused by GPBAR1 (and FXR) ligands also involves large conductance calcium-activated potassium channels likely acting downstream to H_2S. The identification of GPBAR1 as a vasodilatory receptor is of relevance in the treatment of complex disorders including metabolic syndrome-associated diseases, liver steatohepatitis, and portal hypertension.

FXR; GPBAR1; hydrogen sulfide; nitric oxide; TGR5

BILE ACIDS ARE amphipathic molecules essential for lipid- and fat-soluble vitamins solubilization, absorption, and metabolism (14, 24, 27). The principal human bile acids are the primary bile acids, cholic acid (CA) and chenodexycholic acid (CDCA); their glycine (GCA and GCDCA) and taurine (TCA and TCDCA) conjugates; and the secondary bile acids, deoxycholic acid (DCA) and lithocolic acid (LCA), and their glycine (GDCA and GLCA) and taurine (TDCA and TLCA) conjugates. Bile acids are synthesized in the liver (Fig. 1) from cholesterol by a pathway consisting of 15 enzymatic reactions. The main bile acid biosynthetic pathway (known as *neutral or classic*) is initiated by 7α-hydroxylase cytochrome P-450 (CYP7A1) (14). The alternative (or *acidic*) pathway is initiated by the sterol 27-hydroxylase (CYP27A1). In humans, the classical pathway produces CA and CDCA in roughly equal amounts, whereas the acidic pathway produces mainly CDCA. In the classical pathway, the sterol 12α-hydroxylase is involved in the synthesis of CA and controls the ratio of CA to CDCA (14, 24, 27). In the small intestine bile acids are subjected to deamidation and 7α-dehydroxylation by the intestinal microbiota, yielding secondary bile acids (Fig. 1) that are then absorbed in the distal ileum completing a cycle of "the entero-hepatic circulation." In each cycle a portion of bile acids reach the systemic circulation and is partially excreted by the kidney (14). The hydrophilic/hydrophobic properties of bile acids account for a large part for their role in nutrient absorption. Indeed, the bile acid molecule has a convex face that is hydrophobic because of methyl groups and a hydrophilic face with hydroxyl groups. The index of hydrophilicity depends on the number and position of OH groups and whether amidation of the lateral chain is with glycine or taurine. Bile acids conjugated with taurine are more hydrophilic than those con-

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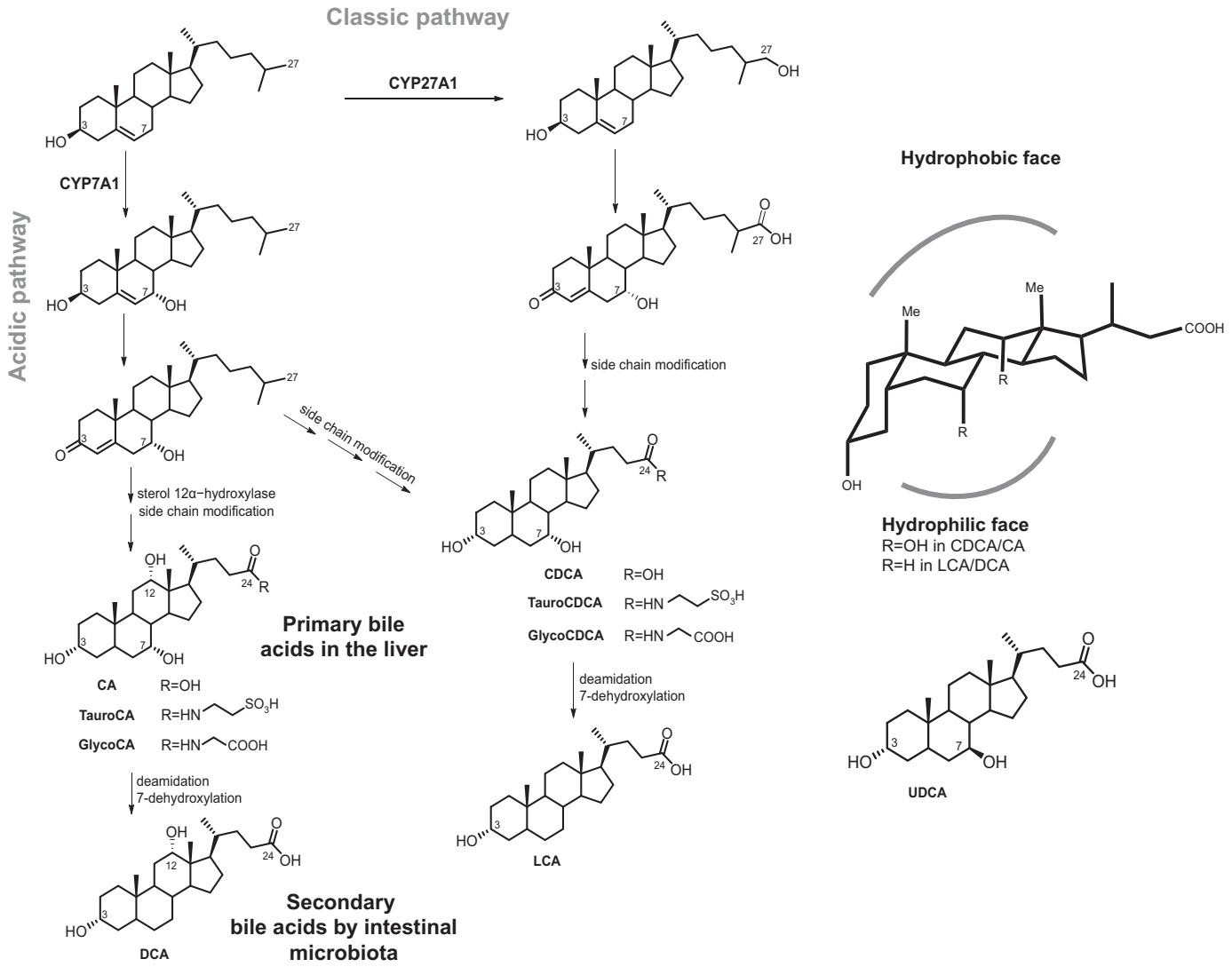

Fig. 1. Chemical structure of primary and secondary bile acids. *A*: bile acid species in human. Bile acids are synthesized in the liver from cholesterol. The primary bile acids are CA and CDCA, and the secondary bile acids are DCA and LCA. In the liver, the rate-limiting enzyme for CDCA synthesis is the cholesterol CYP7A1 (in the classic pathway) and CYP27A1 (in the alternative pathway), while CYP8B1 is required for CA synthesis. In the small intestine, primary bile acids are converted into secondary bile acids by the action of intestinal microbiota. Primary and secondary bile acids are then reabsorbed in the distal ileum and transported back to the liver to complete a cycle in their entero-hepatic circulation. *B*: the equatorial location of hydroxyl groups confers polarity to the steroid nucleus. The hydroxyl groups that are in α-orientation are located below the tetracyclic steroid nucleus, are axial to the plane of the steroid nucleus, and define an hydrophylic face. The hydroxyl groups that are in β-orientation are located above the steroid nucleus, are equatorial to the plane of the steroid nucleus, and define an hydrophobic face. CA, cholic acid; CDCA, chenodeoxycholic acid; CYP7A1, 7α-hydroxylase; CYP27A1, sterol 27-hydroxylase; CYP8B1, sterol 12α-hydroxylase; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodexycholic acid.

jugated with glycine, and trihydroxylated bile acids are more hydrophilic than dihydroxylated. Bile acids with a high index of hydrophilicity [ursodeoxycholic acid (UDCA) and CA] increase bile flow and are less toxic to cells, whereas hydrophobic bile acids (CDCA, DCA, and LCA) are generally cytotoxic at concentrations >200 μM.

Bile Acid-Activated Receptors: G Protein-Coupled Receptors

While the hydrophilic/hydrophobic properties of bile acids account for their role in nutrient absorption, bile acids, similarly to other cholesterol metabolites, act as signaling molecules activating a networks of cell membrane and nuclear receptors, whose expression is prevalent in, but not limited to, entero-hepatic tissues (21, 24, 27). Secondary bile acids, LCA and DCA, are the endogenous ligands for several G protein-coupled receptors (GPCRs), including GPBAR1 (also known as M-BAR, TGR5, or BG37) (33), the sphingosine 1 receptor 2 (SP1R2) (79), and the subtype 2 and 3 (M_2R and M_3R) of muscarinic receptors (35, 36). CDCA antagonizes the effect of the fMLP receptor (13). Additionally, bile acids interact with tyrosine kinase-coupled receptors causing the transactivation of the EGF receptor (58) (Table 1).

GPBAR1 is a G protein-coupled receptor that is preferentially activated by conjugated and unconjugated secondary bile acids, DCA and LCA (33). The receptor, highly expressed in the gallbladder, small intestine, colon, spleen, adipose tissues (brown and white), and immune cells (macrophages and T cells), is a $G_{\alpha s}$-coupled receptor and its activation increases

Table 1. *Bile acids activated receptors, tissue distribution, and ligands*

Receptors	Tissue Distribution	Vascular Expression	Natural Bile Acid Ligands	Synthetic Bile Acid Derivatives with Demonstrated Activity on Vascular System	Synthetic Bile Aacid Derivatives (References)
Cell membrane receptors					
GPCRs					
GPBAR1 (TGR5)	Ileum, macrophages, gallbladder, adipose tissues	Endothelial cells, liver sinusoidal cells, cardiomyocytes,	LCA>DCA>CDCA>UDCA>CA	BAR501, BAR502	(15, 20, 33, 66, 70, 71)
Sphingosine-1-phosphate receptor 2 (S1PR2)	Hepatocytes	Endothelium	LCA		(79)
Muscarinic receptors: M2 and M3	CNS, smooth muscle cells	Endothelial cells, cardiomyocytes (M2)	DCA-LCA		(35, 36)
fMPL	Macrophages		CDCA		(13)
Tyrosine kinases					
VEGF-R	Gastric and colon cancer cell lines	Endothelial cells	CDCA		(58)
Nuclear receptors					
FXR (NR1H4)	Hepatocytes, small intestine	Vascular smooth muscle cells and cardiomyocytes, endothelial cells;	CDCA>CA>LCA-DCA	6-ECDCA (OCA), BAR502	(16, 55, 68)
LXR	Hepatocytes	Endothelial cells	HyoDCA		
CAR (NR1H3)	Hepatocytes				
VDR (NR1H1)	Various	Cardiomycytes	LCA		
PXR (NR1H2	Hepatocytes	Mesenteric artery	CDCA-LCA		
K^+ Channels					
$BKCa^{2+}$	Smooth muscles	Vascular smooth muscle cells	LCA>DCA>CDCA>CA>UDCA	5B-Cholanic 3a-ol derivatives	(9, 10, 19)

CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; TCA, taurocholic acid; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; GDCA, glycodeoxycholic acid; TDCA, taurodeoxycholic acid; GLCA, glycolithocholic acid; OCA, obeticholic acid; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; CNS, central nervous system.

cAMP synthesis and, via PKA, activates a cAMP-response element binding protein (CREB) that induces genomic effects by binding to a cAMP-responsive element (CRE) in the promoter of target genes (12, 75). The physiological function of GPBAR1 has been demonstrated in a limited number of systems. In the small intestine GPBAR1 is highly expressed by endocrine L cells and regulates the secretion of glucagon-like peptide 1 (GLP-1), a hormone that stimulates insulin secretion and suppresses appetite. GPBAR1 is also a likely mediator of itching (52). Stimulation of GPBAR1 by DCA causes the release of GLP-1 from the murine enteroendocrine cell line STC-1 (32). This effect is mediated by an increased ATP/ADP ratio, which causes the closure of ATP-dependent potassium channels (K_{ATP}) and a subsequent calcium (Ca^{2+}) influx, leading to GLP-1 release (32). In thermogenic tissues (brown adipose tissues and muscle), GPBAR1 activation increases energy expenditure (86). These antidiabetic and antiobesity effects have sparked interest in the role of GPBAR1-based therapies for the treatment of these highly prevalent diseases (24, 27).

GPBAR1 also regulates intestinal transit and secretions (1): GPBAR1 mediates the prokinetic actions of bile acids in the colon and is required for normal defecation. GPBAR1 immunoreactivity in the mouse colon colocalizes with two transmitters of the afferent limb of the peristaltic reflex: 5-hydroxytryptamine (5-HT) in enterochromaffin cells and calcitonin gene-related peptide (CGRP) in intrinsic primary afferent neurons and GPBAR1 deletion and antagonism to 5-HT_4 and CGRP receptors and abrogates GPBAR1-evoked peristalsis (2). Genetic variations in GPBAR1, particularly single nucleotide polymorphism (SNP; rs11554825), associates with diarrhea-predominant variant of irritable bowel syndrome (11).

GPBAR1 is expressed in monocytes and macrophages and its activation reduces macrophage's effector functions. GPBAR1 ligands protect against colonic inflammation in mice and the expression of the receptor is essential to maintaining the integrity of the colonic epithelial barrier (15). GPBAR1-deficient mice ($Gpbar1^{-/-}$) have an altered architecture of the colonic mucosa that is characterized by disruption of epithelial tight junctions and a redistribution of zonulin-1 from tight junctions, which result in increased mucosal permeability. Following challenge with dextran sodium sulfate (DSS) or trinitrobenzene sulphonic acid (TNBS), two rodent models that mimic human colitis, $Gpbar1^{-/-}$ mice exhibit an increased susceptibility to develop inflammation and immune dysregulation (15, 24, 27). Genetic variations of GPBAR1 have been linked to development of primary sclerosing cholangitis, a autoimmune disease of the liver (20, 27).

Bile Acid-Activated Receptors: Nuclear Receptors

In addition to GCPRs, bile acids are ligands for at least five members of the nuclear receptors superfamily (Table 1), including the farnesoid-X-receptor (FXR), the constitutive androstane receptor (CAR), the pregnane-X- receptor (PXR), the vitamin D receptor (VDR), and the liver-X-receptor (LXR) (24, 27, 81). The best characterized of these bile acid-activated nuclear receptors is FXR. FXR is a bile acid sensor highly expressed in the liver and intestine (54, 62, 84). Gene transcription by FXR requires the formation of a heterodimer complex of FXR with the retinoid-X-receptor (RXR). Then, the FXR/RXR heterodimer binds to specific DNA sequences. The prototype FXR-responsive element is a sequence composed of two inverted repeats (IR) separated by one nucleotide, IR-1 (30), and can be activated by ligands for both receptors. Upon ligand binding, FXR undergoes a series of conformational changes in the helix 12 to release corepressors and recruits coactivators (69).

In hepatocytes and small intestinal epithelial cells, FXR functions as a bile acid sensor: a rise in intracellular bile acid concentrations causes the transcriptional activation of FXR. In hepatocytes, one FXR target gene is the small heterodimer partner (SHP), which binds with and inactivates LRH1, decreasing the expression of CYP7A1. This event results in a profound inhibition of synthesis of endogenous bile acids (14, 54, 62, 83). In addition, FXR negatively regulates basolateral bile acid uptake by hepatocytes via repression of NTCP and OATP-1 and -4, while it stimulates the expression of both canalicular (MRP3 and BSEP) and alternative basolateral efflux transporters (MRP3, MRP4, and OSTα and -β). These coordinated changes result in reduced bile acid synthesis and uptake by hepatocytes, while excretion is increased. Additionally, in hepatocytes, FXR activation increases the expression of genes encoding for proteins involved in bile acids detoxification (14). In the intestine, FXR modulates the expression of specific transporters by repressing the human apical sodium bile acids transporter (ASBT) and inducing the basolateral organic solute transporters (OSTα and OSTβ). Activation of intestinal FXR causes the release of fibroblast growth factor (FGF)-15, FGF-19 in humans (45). FGF-15, by binding to the type-4 of FGF receptor (FGF-R4), inhibits bile acid synthesis by repressing the activity of both ASBT in enterocytes and CYP7A1 in hepatocytes. In addition, FXR regulates the expression/activity of sterol regulatory element-binding protein 1C (SREBP1C) and fatty acid synthase (FAS) genes (24, 27) and its activation reduces liver accumulation of triacyglycerols and cholesterol (57). Finally, similarly to GPBAR1, FXR increases insulin secretion and sensitivity (57, 67) and regulates intestinal and liver immunity (57, 87).

Bile Acids and K^+ Channels

In the vascular system bile acids activate large conductance Ca^{2+}-activated K^+ channels (BK_{Ca} or $K_{Ca1.1}$), a class of Ca^{2+} channels that regulate arterial tone (Table 1). Patch-clamp studies have shown (19) that bile acids reversibly activate BK_{Ca} channels in vascular smooth muscle cells (VSMCs) raising the possibility that a direct activation of these channels by bile acids occurs in the vascular system. Monohydroxylated bile acids (LCAs) appear to be more effective than di (CDCA and DCA)- or trihydroxylated (CA, GCA, or TCA) bile acids (9, 10). Bile acids activate BK_{Ca2+} channels also in endothelial cells. The activation of the BK_{Ca} channels might explain the observation that in several systems vasodilation caused by bile acid is endothelium independent and reversed by treatment with iberiotoxin, a BK_{Ca} channel blocker (10).

Bile Acids Are Vasodilatory Agents

The fact that bile acids are vasodilatory agents has been documented extensively in the last three decades (7) but has gained increased attention in recent years (65). In large part this renewed interest stems from the identification of bile

acid-activated receptors in cardiovascular tissues and recognition of the therapeutic potential of these vascular activities in the treatment of metabolic disorders.

In 1983, Lautt and Daniels (48) reported that intravenous administration of TCA caused a vasodilation of mesenteric and hepatic arteries in cats. These findings were then confirmed by several other observations made in vivo and in vitro. Bomzon et al. (6) demonstrated in 1984 that the reduced total peripheral resistance and hypotension observed in patients with obstructive jaundice can be explained, at least in part, by the vasodilatory effect exerted by some of the bile salts on smooth muscles. Using a model of cholestasis, induced by the ligation of common bile duct in rats, these authors demonstrated that cholestasis attenuates response to norepinephrine (NE) of smooth muscle cells (including VSMCs). The exposure of aortic rings obtained from cholestatic animals to endogenous bile acids, DCA, CA, and TCA, also attenuated the contractile response to NE, with DCA being more potent than either CA or TCA (6). Bile acids regulate the vascular tone in the mesenteric circulation as demonstrated by the fact that intravenous infusion of TCDCA and TDCA increased mesenteric arterial blood flow and reduced arterial pressure in rats (61). In isolated preparations of mesenteric and carotid arteries and portal vein, TCDCA and TDCA induced a dose-dependent (1 μM to 10 mM) vasodilation, mimicking in vivo experiments. Vasodilation caused by TUDCA, TCDCA, and TDCA is dose dependent and the relative vasorelaxant potency (measured using the isolated and perfused rat mesentery precontracted with the selective A_1-adrenoceptor agonist cirazoline) was TDCA>TCDCA>TUDCA (61). In rat aortic rings precontracted by NE (53), the valorelaxant activity of various bile acids correlates with the relative lipid solubility, since lipophilic bile acids (DCA and CDCA) exhibit the greatest vasorelaxant activity compared with more hydrophilic bile acids (CA and TDCA).

There is substantial evidence that vasodilation caused by bile acids is endothelium independent. Pak and Lee (61) reported that endothelium denudation did not alter vasodilation caused TDCA in rat mesenteric arteries (61). Similarly, endothelial denudation fails to reduce vasodilation caused by DCA in the rat aortas (53). Further confirming these views, both studies observed that incubation of endothelium-intact vessels with N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase (NOS) inhibitor, had no effect on vasodilation caused by TDCA and DCA in rat mesenteric arteries and abdominal aorta (53, 61).

In addition to these effects on the vascular tone, bile acids have been reported to modulate the cardiac function. In vitro studies have shown that exposure of neonatal rat cardiomyocytes to TCA, CA, or TDCA attenuates cell contraction (31, 76) by activation of M_2R. However, the biochemistry of these interactions is poorly defined. FXR and GPBAR1 are expressed in rat neonatal cardiomyocytes but since pharmacological inhibition and siRNA-knockdown of M_2R completely abolished the effects of TCA on contraction, it appears that neither FXR nor GPBAR1 are involved in these effects (76). On the other hand, the expression of FXR and GPBAR1 on the heart became undetectable in adulthood (55). The administration of GPBAR1 ligands increases the heart rate and the cardiac output in intact animals (28), but these effects are indirect and related to the reduction of systemic blood pressure. Thus whether these studies have physiological relevance is still to be defined.

Mediators of Bile Acid-Induced Vasodilation

The vasodilation caused by bile acids is the result of their interaction with multiple targets and involves the activation of bile acid-activated receptors (Figs. 2 and 3).

GPBAR1. Keithel et al. in 2007 (34) were the first to demonstrate that GPBAR1 (TGR5) is expressed in liver sinusoidal cells (LSECs), a subset of nonparenchymal cells, that represent the liver counterpart of endothelial cells in the systemic circulation. Exposure of LSECs to bile acids increases cAMP concentration and induces mRNA expression of endothelial NO synthase (eNOS), a known cAMP-dependent gene (34). Additionally, in LSECs, bile acids activated eNOS by inducing the phosphorylation of Ser^{1177}. However, this observation was not substantiated by a genetic or pharmacological evidence of GPBAR1 activation. These findings have been confirmed later by others (37–39) in endothelial cells: indeed, in bovine aortic endothelial cells and human umbilical vein endothelial cell (HUVEC), exposure to TLCA causes the Ser^{1177} phosphorylation of eNOS and increases NO production and intracellular cGMP concentration in a GPBAR1-dependent manner (39). This response associates with Akt phosphorylation on Ser^{473} and intracellular Ca^{2+} mobilization. Inhibition of these signals decreased NO production caused by TLCA. TLCA attenuates TNF-α-induced adhesion of HUVECs to monocytes, as well expression of NF-κB and VCAM-1 and monocyte adhesion to mesenteric venules in vivo (39). These anti-inflammatory effects were abrogated by NO synthase inhibition. Using the endothelial monolayer, Kida et al. (38) have also shown that exposure to TLCA attenuates the thrombin-induced dextran infiltration. These activities were abrogated by protein kinase A (PKA) or Rac1 inhibitors, suggesting that stimulation of GPBAR1 enhances endothelial barrier function by cAMP/PKA/Rac1-dependent cytoskeletal rearrangement (38). Overall these studies support the view that GPBAR1 is expressed by endothelial cells and its activation leads to eNOS phosphorylation and NO release (39).

Renga and Bucci (65) have further characterized the role of GPBAR1 in regulating the vascular function by investigating $Gpbar1^{-/-}$ mice (82). These studies have confirmed that GPBAR1 is expressed in the vascular system. However, $Gpbar1^{-/-}$ mice do not develop overt alterations in their vascular phenotype and have a normal arterial pressure and vasodilation caused by primary bile acids is maintained. Only vasodilation caused by LCA, a natural GPBAR1 ligand, was partially reduced by the ablation of the receptor (65). As shown previously in rats (53, 62), Renga and Bucci et al. (65) have confirmed that vasodilation caused by primary and secondary bile acids is endothelium independent. In addition, in contrast to rats, LCA-induced vasodilation in mice appears to be BK_{Ca} independent. As opposed to LCA, vasodilation caused by CDCA in mice is, similarly to what is observed in dogs, reversed by iberiotoxin, supporting a role for BK_{Ca} channels in these two species (28).

In vivo studies have confirmed that in dogs, but not in rats, systemic administration of GPBAR1 ligands bluntd the vascular tone and produces a reflex tachycardia and a positive inotropic response (28), thus enhancing the cardiac output. A

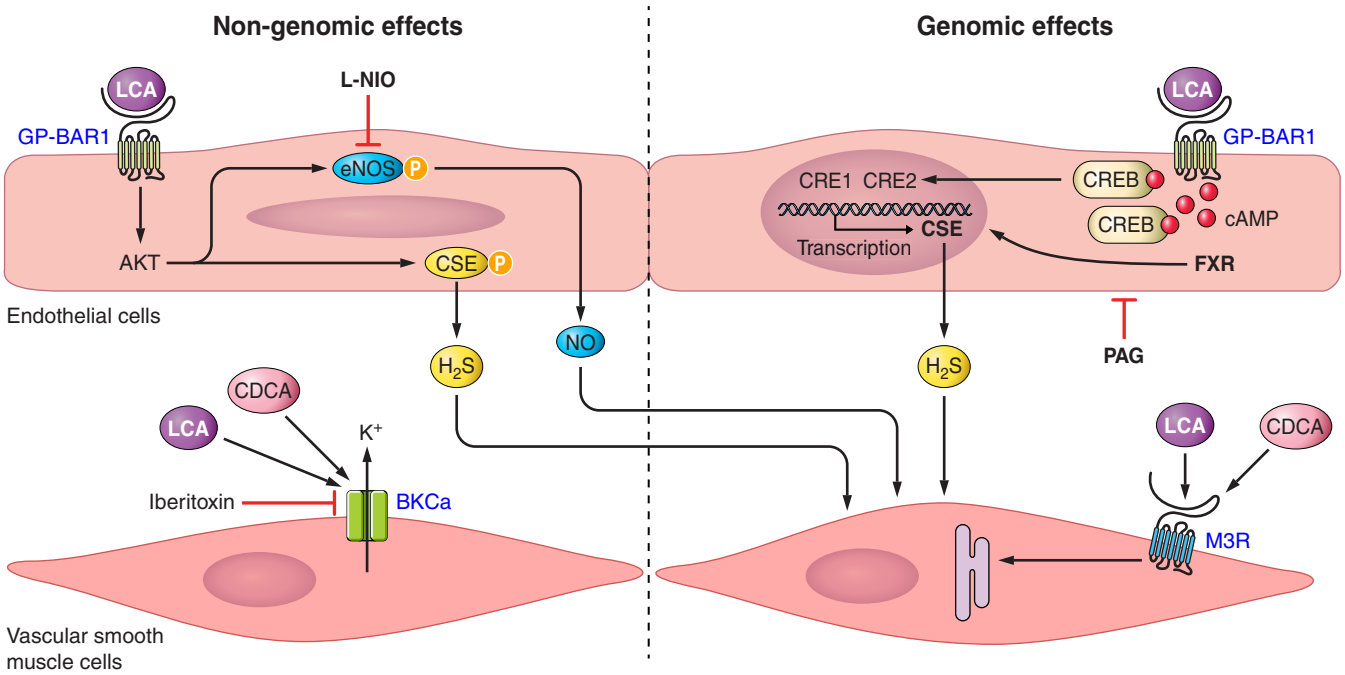

Fig. 2. Mechanism of bile acids induced vasodilation. Schematic representation of vasorelaxant effect primary and secondary bile acids and bile acid-activated receptors: GPBAR1 and farnesosid-X-receptor (FXR). In liver sinusoidal cells (LSECs) and systemic human umbilical vascular endothelial cells (HUVECs) the interaction LCA with GPBAR1 regulates the expression and activity of both endothelial nitric oxide synthase (eNOS) and cystathionine-γ-lyase (CSE), an enzyme required for conversion of cysteine into H_2S. Binding of LCA to GPBAR1 increases cAMP concentrations and induce H_2S generation by genomic and nongenomic effects. The genomic effects involves the phosphorylation of AKT which in turn causes an activatory phosphorylation of eNOS on Ser^{1177} and CSE on Ser^{377}. Phosphorylation of the two proteins results in generation of NO and H_2S. The later in turn might activate BK_{Ca} channels on the vascular smooth muscle cells (VSMCs). Genomic effects involve and AKT-dependent phosphorylation of activation of a cAMP response element-binding protein (CREB), which translocates into the nucleus and activates the transcription of CSE by binding to two specific cAMP-responsive elements (CREs). Production of H_2S resulting from CSE is blocked by propargyl-glycine (PAG) a CSE inhibitor. LCA can also activate M3R (type 3 muscarinic receptors) and BK_{Ca} channels in VSMCs. The effects of LCA on BK_{Ca} are blocked by iberiotoxin. The primary bile acid CDCA activates FXR. FXR might increase NO production by exerting genomic and nongenomic effects on eNOS. FXR is also able to induce the transcription of CSE by activating a specific responsive element in the promoter of CSE. CDCA also directly activates BK_{Ca} on VSMCs. Finally, a cross talk between H_2S and NO downstream to receptor activation might also lead to reciprocal regulation of the production of the two gaseous mediators.

direct effect on vascular tone was confirmed in dog-isolated vascular rings, where concentration-dependent decreases in tension were detected. In this study (28), the vasodilatory effects exerted by GPBAR1 ligands were blocked, at least in part, by iberiotoxin indicating that GPBAR1 stimulation in vivo involves the activation of BK_{Ca} channels (28).

In addition to modulation of eNOS (phosporylation) and activation of BK_{Ca} channels, GPBAR1 ligands modulate the activity of enzymes involved in generation of hydrogen sulfide (H_2S), a potent vasodilatory agent (85). Indeed, vasodilation caused by exposure of aortic rings to LCA in vitro is completely reversed by pretreatment with propargylglycine (PAG) (3, 8), an agent that inhibits the activity of cystathionine γ-lyase (CSE), an enzyme that is essential for generation of H_2S (65). LCA increases the expression of CSE mRNA in HUVECs (65) by a mechanism that is GPBAR1 dependent. Renga and Bucci et al. (64, 65) have shown that the CSE promoter contains two CREs (64, 65). The two CREs are conserved across species, and under GPBAR1 activation, CREB elements are recruited to the region of human CSE promoter that contains the two CREs (64, 65) (Fig. 2). In addition, activation of GPBAR1 by TLCA increases Akt phosphorylation and CSE phosphorylation on serine residues (likely Ser^{377}). Importantly, GPBAR1 antagonism by 5β-cholanic acid abrogates vasodilation caused by TLCA and reverses CSE phosphorylation (65).

As mentioned above, bile acids might be toxic at concentrations that exceed the 200 μM for most cell systems. Bile acid concentrations in the plasma are usually <10 μmol/l (14) but can reach 40–60 μmol/l in cholestatic liver disorders (26). Thus, even in the later conditions, bile acids never reach the concentrations required to exert direct cytotoxic effects on endothelial cells or circulating leukocytes, suggesting that modulation of cells signaling described above represents a physiologic activity.

FXR. In 2004, Bishop-Bailey et al. (4) demonstrated by immunohistochemistry that VSMCs obtained from normal and atherosclerotic vessels (aorta and coronary artery) expresses the FXR protein. Ex vivo treatment of VSMCs with a range of FXR ligands led to apoptosis in a manner that correlates with the ability of these ligands to activate FXR. Later, however, He et al. (41) have shown that FXR is also expressed by human and rat pulmonary endothelial cells and that activation of FXR in these cells inhibits the release of endothelin-1 (ET-1). Furthermore, exposure of HUVECs and bovine aortic endothelial cells to DCA, CDCA, and TDCA caused a concentration-dependent increase in cytoplasmic Ca^{2+} and NO production (59, 88). The regulatory activity exerted by bile acids on the eNOS is at least in part dependent on FXR. Li et al. (50) have demonstrated that treatment of HUVEC with GW4064 (a nonsteroidal FXR ligand) upregulates the expression of eNOS (mRNA and protein) and increases NO production. FXR ap-

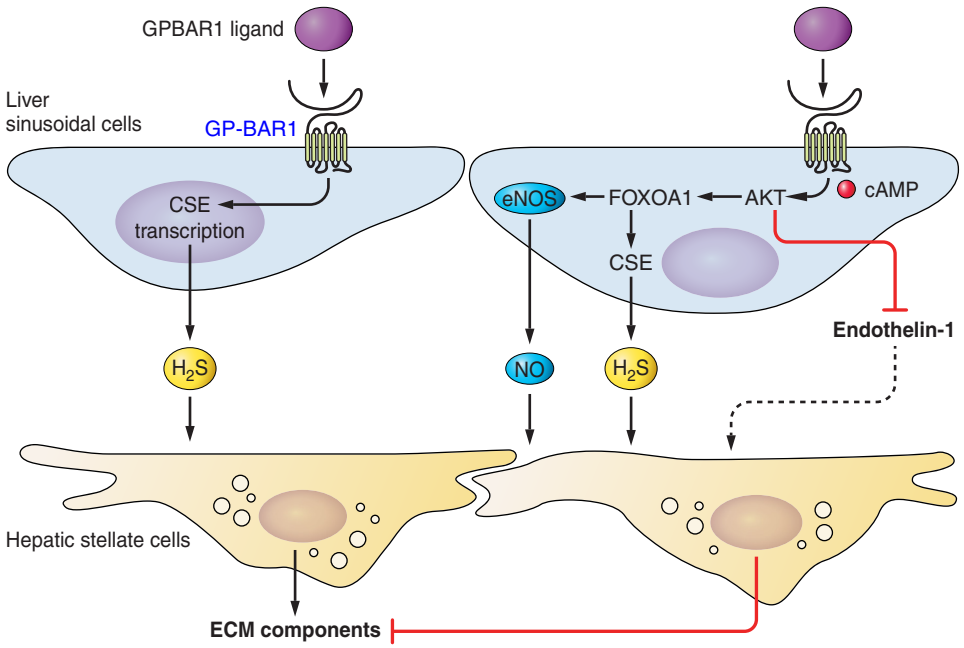

Fig. 3. GPBAR1 ligands might reverse endothelial dysfunction in the cirrhotic livers. In liver cirrhosis, excessive extracellular matrix (ECM) deposition by hepatic stellate cells (HSCs) and defective generation of NO by liver sinusoidal cells (LSECs) lead to increased intravascular resistance. GPBAR1 is expressed by LSEC and its activation causes eNOS and CSE activation (see Fig. 2), resulting in H_2S and NO generation. H_2S, in turns act on HSC to reduce their tendency toward contraction and increasing blood flow. GPBAR1 activation in LSECs reduces endothelin-1 production.

pears to regulate eNOS expression at thetranscriptional level because the ligand-mediated increase in the expression of eNOS mRNA was abrogated by actinomycin D and eNOS promoter activity was significantly increased by pharmacological or genetic activation of FXR. Supporting this view, Li et al. (50) have identified an IR2 (a noncanonical FXRE) in the eNOS promoter and suggested that this regulatory element might be involved in the GW4064-mediated upregulation of eNOS. However, a functional characterization of this IR2 was not shown.

In addition to this genomic effect, there is evidence that FXR activation might cause eNOS phosphorylation. Indeed, other than at a transcriptional level, eNOS activity can be regulated at posttranscriptional levels via phosphorylation, *S*-nitrosylation, and interaction of eNOS with other proteins such as caveolins (75). Particularly, phosphorylation of eNOS at Ser^{1177} has been shown to be an important nongenomic mechanism by which nuclear receptors such as estrogen activate eNOS. However, GW4064 fails to induce eNOS phosphorylation (50) despite the fact that it reduces blood pressure (51). In short, these data suggest that activation of FXR might increase the expression of eNOS in endothelial cells, but the mechanism (genomic vs. nongenomic) is unclear.

A role for FXR in regulating endothelial cells has also been demonstrated by Cipriani et al. (16) and Das et al. (17). These authors have shown that CDCA significantly increases endothelial cells motility and angiogenesis. These effects were associated with increase in focal adhesion plaques and were inhibited by FXR or MMP-9 small interference RNAs (siRNA). Mechanistically, incubation of HUVECs with CDCA causes the phosphorylation of focal adhesion kinase (FAK). Studies using a site-specific phosphorylation mutant of FAK revealed that FAK phosphorylation at tyrosine residue-397 was required for CDCA-induced activation of the downstream FA assembly protein, paxillin (17).

FXR activation in endothelial cells has been linked to several biochemical effects (44, 56) including regulation of dimethylarginine dimethylaminohydrolase-1 (DDAH1). *DDAH1* degrades asymmetrical dimethylarginine, which is a potent eNOS inhibitor. Activation of FXR also resulted in a repression of ET-1 mRNA while it increased trombomodulin and decorin (42, 43).

In contrast, others have reported that activation of FXR might cause vasoconstriction via increased expression of the angiotensin type II receptor (AT_2R) (37).

The vascular phenotype of FXR-deficient mice (78) does not provide a clear response to whether or not FXR is an essential component of the vasodilatory machinery activated by bile acids. Indeed, we have found (Fiorucci S, Bucci M, unpublished observations) that aortic rings isolated from $FXR^{-/-}$ mice have a reduced constricting response to NE and 5HT, while the vasodilatory response to Ach is maintained. The mean arterial pressure of these mice at the age of 8 mo is similar to that of wild-type mice. However, one conundrum of this model is that $FXR^{-/-}$ mice develop very high levels of circulating bile acids.

While the above-mentioned mechanisms are elicited by the independent activation of FXR and GPBAR1, it is increasingly appreciated that some of the pathways activated by the two receptors appear to be overlapping. In addition, both NO and H_2S generated downstream to the two receptors activate their own networks, further increasing the complexity of these signaling systems. In addition, in endothelial cells a cross talk between NO and H_2S occurs at different checkpoints. Thus exposure of HUVECs to L-cysteine and NaHS (a H_2S donor) induces the eNOS phosphorylation and enhances NO production, and inhibition of CSE by PAG reverts the effects of L-cysteine (a CSE substrate). Furthermore, CSE siRNA inhibited, while CSE overexpression increased, NO generation (2). This cross talk appears to converge on Akt, which is also phosphorylated in response to GPBAR1 and FXR activation. There is evidence that Akt/PI3K inhibition abrogated the effects of H_2S on eNOS phosphorylation/activation. Because H_2S directly causes a Akt-dependent phosphorylation of

eNOS, there is substantial possibility that regulation of NO observed in response of exposure of endothelial cells to FXR and GPBAR1 ligands could be due, at least in part, to downstream convergence of these signaling pathways on CSE (2). Because the cooperation of NO and H_2S is a widespread phenomenon, further investigations are need to understand the functional relevance of this cooperation in the context of FXR and GPBAR1 signaling.

Muscarinic receptors. In rat and mouse aortas, vasodilation caused by TDCA is attenuated by exposure to a synthetic ACh:bile acid hybrid that acts as an M_3R antagonist and by M_3R gene ablation (35, 36), which seems to support the notion that activation of M_3R could be involved in the vasodilation caused by TDCA.

BK_{Ca} channels. The role of K channels as potential mediators of vasodilation caused by bile acids has been documented in several studies and is mostly supported by the fact that iberiotoxin reduces the vasodilation caused by primary or secondary bile acids. In vascular smooth muscles, activation of BK_{Ca} channels by steroids (including estrogen and bile acids) elicit a relaxation resulting in a vasodilatory response (19). Vasodilation induced by LCA is blunted after genetic ablation of KCNMB1, a BK channel accessory subunit of the β_1 type, which is particularly abundant in smooth muscle and is necessary for LCA to increase the activity of BK channel complexes. Additionally structural analyses have identified two regions in the β_1-transmembrane domain 2 that meet unique requirements for binding with LCA (10). In dogs (but not in rats and mice), peripheral vasodilation due to GPBAR1 agonism in vivo is blocked by iberitoxin, suggesting that GPBAR1 activation by hydrophobic bile acids may activate ATP-sensitive K^+ channels via a cAMP-protein kinase A-dependent mechanism (49). Since vasodilation caused by LCA is iberiotoxin resistant in mice (65), there is a species-specific activity for these receptors/channels. Finally, since BK_{Ca} channels could be activated by H_2S (77), the demonstration that iberiotoxin attenuates vasodilation caused by GPBAR1 ligands is consistent with the hypothesis that BK_{Ca} channels are activated downstream of GPBAR1 by H_2S.

Exploiting Bile Acid-Activated Receptors in the Treatment of Portal Hypertension

Cirrhosis, the end stage of any chronic liver disease, is characterized histologically by a diffuse nodular regeneration and fibrosis leading to parenchymal extinction, hepatic synthetic decline, and distortion of hepatic vascular architecture (81). The later feature results in increased intrahepatic resistance to liver blood inflow: a condition known as portal hypertension, a severe clinical entity that is associated with increased risk of mortality (5, 29). Development of portal hypertension in cirrhotic patients is associated with appearance of a hepatic endothelial dysfunction (74), which manifests itself by a reduced ability of liver microcirculation to adapt to change to portal blood flow by vasodilation. This mechanism is largely due to the inability of cirrhotic LSECs to generate NO (73, 74). In the cirrhotic livers, an increased expression of caveolin-1 in LSECs leads to sequestration of eNOS protein and impaired enzyme activity causing a defective generation of NO in response to shear stress (60, 73, 74). So far it has been proven impossible to restore the production of NO by modulating the activity of eNOS in the liver and alternative modalities of NO delivery to liver have been attempted (25).

In addition to this impaired ability to generate NO, a reduction in the expression/activity of a family of genes encoding for protein involved in generation of H_2S (transsulfuration pathway) occurs in the cirrhotic livers (18, 22, 25, 26, 43, 72, 85). Cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MST) acting in concert with cysteine aminotransferase (CAT), are the enzymes responsible for the synthesis of endogenous H_2S in the liver (85). These enzymes use cysteine and/or homocysteine or their derivatives as the substrates. The transsulfuration pathway has a major role in the disposal of homocysteine, a toxic intermediate in the methionine cycle, and represents the main source of endogenous H_2S (72). A deficit in the transsulfuration pathway by causing the accumulation of homocysteine promotes endothelial dysfunction (47) and impairs endothelial-dependent vasodilation by interfering with ability of LSECs to generate NO (18, 25). On the other end, H_2S regulates hepatic blood flow (22, 46, 80), and an impaired generation of this gaseous mediator will contribute to increase intrahepatic resistance. In addition to this detrimental effects on the hepatic microcirculation, a deficit in H_2S contributes to HSC activation and contraction, a factor that further contributes to reduce sinusoid blood flow and to increased intrahepatic resistance (68). Both GPBAR1 and FXR have been shown effective in modulating the expression of enzymes involved in transsulfuration pathway.

GPBAR1. GPBAR1 is expressed only by subsets of nonparenchymal liver cells: resident macrophages (Kupffer cells) and LSECs. Activation of GPBAR1 in LSECs causes the phosphorylation of eNOS increasing the output of NO (34). However, a major limitation of targeting eNOS in LSECs, as described above, is the fact that impaired NO generation and endothelial hyporeactivity that occur in cirrhosis is largely related to the excessive sequestration of the eNOS protein by caveolin 1 (73, 74), while the expression of the gene and the protein is generally conserved.

In the cirrhotic livers, treatment with 6β-ethyl-3α,7β-dihydroxy-5β-cholan-24-ol (BAR501), a UDCA derivative (20, 71) endowed with a potent and selective agonistic activity toward GPBAR1, protects against development of portal hypertension in mice administered CCL_4 (a model of liver cirrhosis) and counteracts the vasoconstriction caused by NE and methoxamine by a mechanism that is completely reversed by PAG implying the activation of an H_2S-mediated mechanism (66). In vivo administration of BAR501 increases the liver expression of CSE mRNA despite the parenchymal cell loss that takes place in the model. In contrast, the GPBAR1 ligand failed to increase the expression of eNOS and inducible NOS (iNOS) and had no effect on liver concentration of nitrites/nitrates. As described previously the fact that BAR501 had no effect on NO production could be due to the binding of eNOS to caveolin-1 (73, 74).

GPBAR1 agonism also protects against development of portal hypertension in a model of liver endothelial injury caused by feeding mice with a diet enriched in methionine (18, 66). In this model, the LSEC dysfunction caused by hyperhomocysteinemia can be rescued by GPBAR1 activation, since treating mice with BAR501 rescues them from development of defective generation of NO by LSECs (66). Using $Gpbar1^{+/+}$

mice fed methionine, Renga et al. (66) have shown that administration of BAR501 increases the expression of CSE, eNOS, and iNOS, while these effects were not observed in mice harboring a disrupted Gpbar1. These results are consistent with the concept that activation of GPBAR1 effectively regulates CSE and eNOS by a transcription mechanism. In addition, exposure to BAR501 causes an Akt-dependent phosphorylation of both CSE and eNOS (Fig. 3) providing a molecular explanation for the robust vasodilatory activity exerted by this GPBAR1 agonist in the liver microcirculation.

In vitro data demonstrate that GPBAR1 agonism in LSEC negatively regulates ET-1 expression by a mechanism that involves a Akt-dependent phosphorylation of FoxO1. The unphosphorylated form of FoxO1 functions as a coactivator of ET-1 gene transcription and is constitutively recruited to the ET-1 promoter (66). In contrast, FoxO1 phosphorylation disrupts the coactivator complex (61) repressing the transcription of the gene.

In aggregate, these findings suggest that GPBAR1 activation in LSECs might protect from development of endothelial dysfunction by modulating the expression/activity of CSE and H_2S production. Of relevance H_2S has been demonstrated effective in reducing liver fibrosis and portal hypertension in the CCl_4 model (80).

FXR. In addition to eNOS, FXR regulates the expression of CSE. The expression of CSE (mRNA and protein) in HepG2 cells (a hepatocarcinoma cell line) is induced by treatment with CDCA and CA (23, 68) and 6-ethylchenodeoxycholic acid (6-ECDCA, also known as INT-747 or obeticholic acid), a semisynthetic FXR ligand (63, 68). Supporting a direct regulatory effect of FXR on CSE, Renga et al. (68) have identified a sequence in the 5′-flanking region of the CSE gene, containing a noncanonical IR1 binding site (AGTTCAgTGTACCT). Results from in vitro and in vivo studies demonstrated that FXR activation enhances CSE mRNA expression and increases the generation of H_2S from hepatocytes and HSCs. These data suggest that FXR directly regulates the transsulfulration pathway in the liver. In vivo, oral feeding with obeticholic acid (6-ECDCA) increases the expression of CSE in the liver and this effect is partially lost in $FXR^{-/-}$ mice. Additionally, mice lacking FXR (64, 68) display a reduced levels of CSE mRNA in the liver along with reduced levels of H_2S in the systemic circulation.

Long-term administration of 6-ECDCA to mice rendered cirrhotic by administration of CCL_4 also effectively reduces liver fibrosis (23) and intrahepatic resistance to flow (68) and increases the expression of CSE (68). In summary, these data add to the concept that the transsulfuration pathway could be rescued in the cirrhotic livers and exploited by using GPBAR1 and FXR ligands in the treatment of portal hypertension.

Vascular Disorders: Atherosclerosis

FXR and GPBAR1 agonists are currently developed for metabolic indications, including nonalcoholic steatohepatitis (NASH) and associated conditions such as obesity, diabetes, and dislipidemia. These applications have been the subjects of several recent reviews (22, 24, 25, 27) to which the readers are redirected. In the present context, however, it worthy to mention that FXR and GPBAR1 ligands might have relevance in the treatment of vascular component of complex metabolic diseases such as atherosclerosis. The deposition of plasma lipids in the arterial intima induces a local inflammatory response and extensive vascular remodeling, resulting in the formation of atherosclerotic plaques, a major risk factor for myocardial infarction and stroke (24). Risk factors for formation of atherosclerotic plaques are elevated plasma triacylglycerol levels and low HDL cholesterol in conjunction with features of metabolic syndrome. Preclinical studies have provided evidence that treatment with FXR and GPBAR1 protects from development of atherosclerotic plaques in a variety of genetic and dietary models of dislipidemia. In mice, FXR deficiency results in a proatherogenic serum lipoprotein profile characterized by increased plasma triacylglycerols, free fatty acids, VLDL, HDL, and LDL along with glucose intolerance and insulin resistance (78). Additionally, FXR deficiency associates with impaired adipocytes differentiation (69) and hyperabsorption of cholesterol and triglycerides from the intestine (78). Despite these changes, FXR-deficient mice fail to develop aortic plaques spontaneously and, surprisingly, even when fed a Western diet. However, FXR gene ablation seems to exacerbates the development of atherosclerotic lesions in other genetic phenotypes such as ApoE- and LDLr-deficient mice (40). Thus, in comparison with single ApoE knockout mice, double FXR/ApoE knockout male mice feed a high-fat diet show a severe deterioration of metabolic response and exacerbation of aortic lesions (40). Importantly a strong gender effect was observed in these studies (40), but the relevance of this observation to human pathologies remains unclear. In genetic models of dyslipidemia, ApoE (56)- and LDLr (40)-deficient mice, as well as in rats with insulin resistance and liver steatosis, Zucker rats (16), exposure to a FXR ligand ameliorates the proatherogenic lipid profile and attenuates the tendency toward atherosclerotic plaques formation. In addition to these beneficial effects on lipid and glucose metabolism, as described in this review, FXR is expressed by cells of innate immunity and both natural and synthetic FXR ligands counterregulate macrophage activation induced by Toll-like receptor-4 ligands (55–57, 87) suggesting a role for these anti-inflammatory activities in protection toward atherosclerosis in these models. In rodent models of atherosclerosis, the regulatory effects of FXR on macrophage involve the downregulation of CD36 mRNA along with the induction of genes mediating cholesterol efflux from macrophages such as ABCG5 (55–57). Similar to FXR, GPBAR1 agonism attenuates monocyte adhesion to vascular endothelial cells (39) and exerts favorable impact on glucose metabolism and energy expenditure (86) and might have utility in treating metabolic disorders and atherosclerosis (70). The beneficial effects exerted by FXR and GPBAR1 ligands on atherosclerosis might involve the release of gaseous mediators as outlined previously in this review. Activation of FXR seems to also exert negative effects including reduction of circulating levels of HDL (16).

Conclusions

FXR and GPBAR1 are expressed in the vascular system and activation of these receptors leads to a vascular response that is vasodilatory in nature. These observations might have a clinical readout and could be exploited in the treatment of human diseases. Indeed, ligands for FXR and GPBAR1 effectively reduce portal pressure in rodent models of liver cirrhosis and

atherosclerotic plaques formation in rodent models of dyslipidemia (55, 56). Exploitation of the vasodilatory effects of bile acid-activated receptors by selective or dual ligands for GPBAR1 and FXR might have a utility in the treatment of vascular disorders associated with lipid disorders and insulin resistance. The recognition that FXR and GPBAR1 ligands exert vasoregulatory activities expands their therapeutic potential in the treatment of highly prevalent human diseases including obesity, diabetes, and metabolic syndrome-related disorders. However, due to the overlap of the activities exerted by different bile acids, identification of cell targets and specific signal transduction pathways is essential to define the physiological role and therapeutic opportunities of each receptor. In addition, the specific role that NO and H_2S exert downstream to GPBAR1 activation needs to be addressed.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.F., A.Z., G.C., and E.D. conception and design of research; S.F., M.B., and E.D. drafted manuscript; S.F., A.Z., G.C., M.B., and E.D. edited and revised manuscript; S.F., A.Z., G.C., M.B., and E.D. approved final version of manuscript.

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