Mas-related G protein-coupled receptor type D antagonism improves portal hypertension in cirrhotic rats

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ORIGINAL ARTICLE

Mas-related G protein-coupled receptor type D antagonism improves portal hypertension in cirrhotic rats

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Abstract

Splanchnic vasodilatation contributes to the development and aggravation of portal hypertension (PHT). We previously demonstrated that in cirrhosis, angiotensin- mediates splanchnic vasodilatation through the Mas receptor (MasR). In this study, we investigated whether the recently characterized second receptor for angiotensin-(1–7), Mas-related G protein-coupled receptor type D (MrgD), contributes to splanchnic vasodilatation in cirrhotic and noncirrhotic PHT. Splanchnic vascular hemodynamic and portal pressure were determined in two rat models of cirrhotic PHT and a rat model with noncirrhotic PHT, treated with either MrgD blocker D-Pro^7-Ang-(1-7) (D-Pro) or MasR blocker A779. Gene and protein expression of MrgD and MasR were measured in splanchnic vessels and livers of cirrhotic and healthy rats and in patients with cirrhosis and healthy subjects. Mesenteric resistance vessels isolated from cirrhotic rats were used in myographs to study their vasodilatory properties. MrgD was up-regulated in cirrhotic splanchnic vessels but not in the liver. In cirrhotic rats, treatment with D-Pro but not A779 completely restored splanchnic vascular resistance to a healthy level, resulting in a 33% reduction in portal pressure. Mesenteric vessels pretreated with D-Pro but not with A779 failed to relax in response to acetylcholine. There was no splanchnic vascular MrgD or MasR up-regulation in noncirrhotic PHT; thus, receptor blockers had no effect on splanchnic hemodynamics. *Conclusion*: MrgD plays a major role in the development of cirrhotic PHT and is a promising target for the development of novel therapies to treat PHT in cirrhosis. Moreover, neither MrgD nor MasR contributes to noncirrhotic PHT.

INTRODUCTION

Portal hypertension and its complications are a major cause of morbidity and mortality in patients with cirrhosis.[1] There are two main mechanisms that drive the development of portal hypertension in cirrhosis. First is increased intrahepatic vascular resistance due to fixed obstruction of the portal vascular bed resulting from tissue fibrosis and the activity of vasocontractile cells.[1] The second is a hyperdynamic circulatory state

characterized by a high cardiac output, increased total blood volume, and splanchnic vasodilatation, resulting in increased mesenteric blood flow (MBF) and portal pressure.[1,2] There is considerable evidence that the renin angiotensin system (RAS) plays an important role in the pathogenesis of these changes.[1] The powerful vasoconstrictor and profibrotic angiotensin II (Ang II), the effector peptide of the so-called classical axis of the RAS, contributes to portal resistance by activating hepatic stellate cell (HSC)-driven fibrogenesis in the liver and by increasing sinusoidal tone.[3,4] In addition, the alternate axis of the RAS, comprising angiotensin converting enzyme 2, the vasodilatory peptide angiotensin-(1–7) (Ang-(1–7)), and the Mas receptor (MasR), is up-regulated in patients and animals with cirrhosis, and this axis is a key mediator of splanchnic vasodilatation in cirrhosis.[5–7] In patients with advanced cirrhosis, the circulating Ang-(1–7)/Ang II ratio is increased and correlates with the degree of vasodilatation,[7] and Ang-(1–7) levels have been shown to correlate with liver disease severity and clinical surrogates of vasodilatation, including increased cardiac output.[8] Mechanistic support for these findings comes from evidence that mesenteric Ang-(1–7) production is increased in cirrhotic rats and mediates splanchnic vascular hypocontractility and that the specific MasR blocker A779 increases splanchnic vascular resistance (SPVR), thus lowering portal pressure.[5,6]

Recent work has shown that the vasodilatory effects of Ang-(1–7) may also be mediated through a receptor named Mas-related G protein-coupled receptor type D (MrgD),[9] and we recently demonstrated that blockade of this receptor acutely can reduce portal pressure in cirrhotic rat models of portal hypertension.[6] In the current study, we further explored the role of MrgD in experimental portal hypertension and demonstrate that a continuous infusion of the MrgD blocker D-Pro^7-Ang-(1–7) (D-Pro) completely restores SPVR, leading to a reduction in portal pressure by a clinically significant magnitude without affecting the systemic circulation in two different cirrhotic rat models. We also show that in animal models of cirrhosis and patients with cirrhosis, the expression of MrgD is markedly up-regulated in splanchnic resistance vessels but remains unchanged in the liver. These findings suggest that MrgD is a potential target for the design and development of novel therapies to reduce splanchnic vasodilatation and thus lower portal pressure in patients with cirrhosis.

MATERIALS AND METHODS

Human subjects

Human samples were obtained with informed consent of patients and as approved by Austin Hospital human ethics committee. Omental arteries were isolated from patients with cirrhosis with primary sclerosing cholangitis (PSC) (n = 6) undergoing liver transplantation and compared with arteries obtained from noncirrhotic organ donors (n = 3). Cirrhotic livers obtained from patients with PSC (n = 6) and patients with alcoholic cirrhosis (ALC) (n = 8) undergoing liver transplantation were compared with noncirrhotic livers obtained from patients with cancer resection (n = 5).

Animal models of cirrhosis and portal hypertension

Experimental procedures were approved by Austin Hospital animal ethics committee and performed according to the National Health and Medical Research Council Australia guidelines for animal experimentation. To induce cirrhosis and portal hypertension, 6-week-old male Sprague Dawley rats underwent twice weekly carbon tetrachloride (CCl_4) injections over 10 weeks or bile duct ligation (BDL) surgery for 4 weeks, as described.[5,6,10] Partial portal vein ligation (PPVL) surgery was performed to induce noncirrhotic portal hypertension, as described.[6] Rats were housed in a controlled environment with a 12:12-hour light to dark cycle with controlled temperature (22°C to 24°C) and fed standard rat chow (Norco, Australia) and water *ad libitum*.

In vivo treatment with receptor blockers

Two weeks after BDL and 8 weeks after CCl_4, portal hypertension was established. At these time points, continuous infusions of receptor blocker treatments were commenced and continued for 2 weeks. In the PPVL model, receptor blocker treatment was commenced 1 week after PPVL surgery and continued for 1 week. Animals in each model were divided into three groups (n = 15 per group). The MasR blocker D-Ala^7-Ang-(1–7) (A779) (Mimotopes, Australia) (28 μg/kg/hour), MrgD blocker D-Pro^7-Ang-(1–7) (D-Pro) (Mimotopes) (28 μg/kg/hour) or saline were infused through a subcutaneously implanted osmotic minipump, as described.[11,12] Sham-operated or olive oil-injected healthy rats receiving saline served as controls.

In vivo hemodynamic experiments

In vivo hemodynamic studies to measure portal pressure, mean arterial pressure (MAP), vascular resistance, and regional blood flows were performed in anesthetized (ketamine/xylazine; 75/10 mg/kg body weight) rats at 4 weeks after BDL and 10 weeks after CCl_4 (i.e., 2 weeks posttreatment in cirrhotic models)

or 2 weeks after PPVL (i.e., 1 week posttreatment in the noncirrhotic model), as described in the Supporting Materials and as previously described.[5,6] For blood flow measurement experiments, fluorescent-labeled microsphere beads of two colors (purple high and yellow high) were used (IMT, Staton Pharma, USA).

Wire myograph experiments

Separate groups of cirrhotic CCl_4 and control rats were used to isolate proximal (approximately 200–300 μm in diameter) and distal (approximately 50–200 μm in diameter) mesenteric resistance vessels and abdominal aorta for vascular myograph studies. Isolated vessels were mounted in the organ bath (Zultek Engineering, Australia). After 20 minutes of equilibration, mesenteric resistance vessels were preconstricted with methoxamine (3×10^{-7} M) and abdominal aorta with phenylephrine (3×10^{-7} M). After stabilization, the vessels were pretreated with D-Pro or A779 or saline for 10 minutes, and vasodilatory responses of the vessels were then recorded by adding increasing doses of acetylcholine (1×10^{-9} to 1×10^{-4} M.)

MrgD and MasR gene expression in rat and human samples

Total RNA was extracted from mesenteric resistance vessels and livers of the cirrhotic, noncirrhotic, and control rats and from livers and omental vessels of human patients, using Trizol reagent (Sigma Aldrich, Australia). Gene expression analysis of *MasR* and *MrgD* was carried out using real-time quantitative polymerase chain reaction (RT-qPCR), as described[5,11] Gene expression values were normalized to 18S, and healthy controls were given a value of 1. Sequence details of probes and primers for rat/human *MasR* and *MrgD* (Thermo Fisher, Australia) are provided in Table S1.

Assessment of liver biochemistry and fibrosis

Liver biochemistry was assessed by measuring plasma enzymes, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). We also measured plasma albumin, creatinine, and bilirubin levels. Liver fibrosis was quantified by staining liver sections for picrosirius red. Gene expression of HSC activation marker alpha-smooth muscle actin (*α-SMA*) and fibrosis marker collagen type 1 alpha 1 (*COL1A1*) was carried out using RT-qPCR. Sequence details of probes and primers for rat *α-SMA* and *COL1A1* are provided in Table S1.

Western blotting and immunohistochemistry

Liver and mesenteric resistance vessels collected from BDL, CCl_4, PPVL, and control rats and human liver samples collected from control subjects and patients with PSC and ALC were used for western blot analysis, as described.[5,13] Western blot analysis of MasR and MrgD in rat and human samples and of total endothelial nitric oxide synthase (eNOS) and phosphorylated eNOS (p-eNOS) in rat samples was performed. β-actin was used as the loading control. Band intensities were detected and quantified using Gel-Doc (BioRad, Australia). Immunostaining of MasR and MrgD was performed in 4-μm sections obtained from 4% paraformaldehyde-fixed paraffin-embedded vessels and liver tissues of rats and human samples. Positive signals were detected by incubation of tissue sections with primary and secondary antibodies and 3,3′-diaminobenzidine chromogen. Sections were counterstained with hematoxylin and visualized under the microscope at magnification ×200. Immunostaining quantification of liver samples was performed using Fiji ImageJ. Details of the antibodies used for western blotting and immunohistochemistry are provided in Table S2.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) with Tukey post hoc test or repeated measures ANOVA, where appropriate. Results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 9.0. $p < 0.05$ was considered as statistically significant.

RESULTS

MrgD blockade profoundly improves SPVR and reduces MBF, leading to a large reduction of portal pressure in cirrhotic rats

The cirrhotic CCl_4 and BDL rat models had significantly reduced ($p < 0.005$) SPVR compared to olive oil-injected or sham-operated healthy controls (Figure 1A). Reduced SPVR was accompanied by a significant increase ($p < 0.01$) in MBF (Figure 1B). Hepatic vascular resistance (HVR) was also increased ($p < 0.05$) in both cirrhotic rat models compared to healthy controls (Figure 1C). Consistent with reduced SPVR, increased MBF, and elevated HVR, portal pressure was significantly increased ($p < 0.001$) in both cirrhotic rat models compared to controls (Figure 1D).

Treatment with the MrgD blocker D-Pro significantly increased SPVR in both CCl_4 ($p < 0.01$) and BDL ($p < 0.05$) rats (Figure 1A), restoring it to the level of their

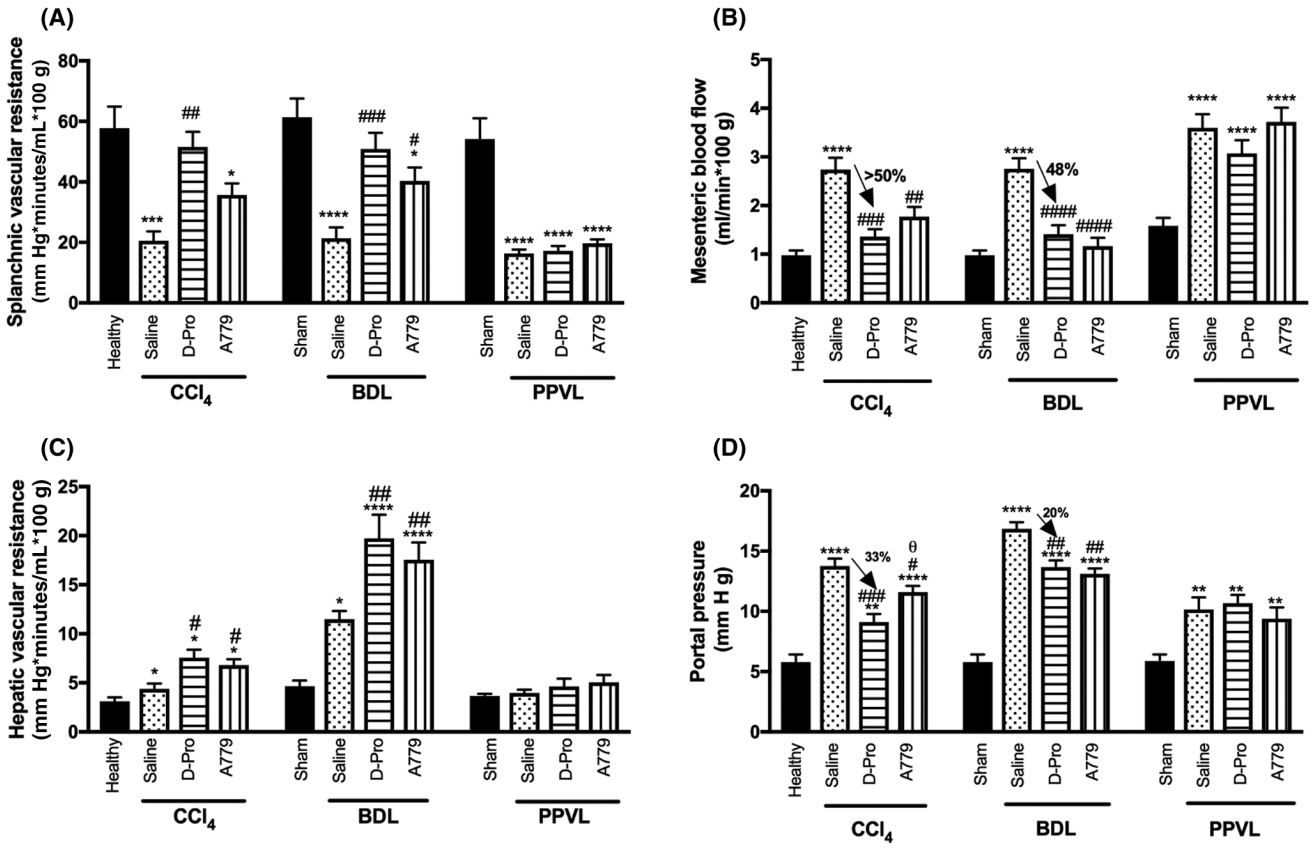

FIGURE 1 *In vivo* hemodynamic changes in rats with cirrhotic portal hypertension induced by carbon tetrachloride intoxication or bile duct ligation surgery and noncirrhotic portal hypertension induced by partial portal vein ligation surgery compared with respective controls. Rats were given 2 weeks (CCl_4 and BDL) or 1 week (PPVL) continuous infusion of MrgD blocker D-pro or MasR blocker A779. (A) Splanchnic vascular resistance, (B) mesenteric blood flow, (C) hepatic vascular resistance, and (D) portal pressure in three models are shown. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.005$, $^{****}p<0.001$, diseased (CCl_4 or BDL or PPVL) versus olive oil-injected/sham-operated healthy controls. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.005$, $^{\#\#\#\#}p<0.001$, saline-infused diseased controls versus diseased rats treated with D-pro or A779. $^{\theta}p<0.05$, D-pro versus A779-treated diseased rats. Each bar represents the mean ± SEM profile from 10 to 15 rats per treatment or control group. A779, D-Ala^7-Ang-(1–7); BDL, bile duct ligation; CCl_4, carbon tetrachloride; D-Pro, D-Pro^7-Ang-(1–7); MasR, Mas receptor; MrgD, Mas-related G protein-coupled receptor type D; PPVL, partial portal vein ligation

respective healthy controls. Increased SPVR led to more than 50% and 48% reduction ($p<0.005$) in MBF in the CCl_4 and BDL models, respectively (Figure 1B). The MasR blocker A779 also increased SPVR in both cirrhotic rat models. In contrast to D-Pro, A779 did not completely restore the SPVR to the level of healthy control rats, and the increase was only significant ($p<0.05$) in the BDL model (Figure 1A). However, A779 significantly reduced ($p<0.01$) the MBF by 50% in the BDL rats but by only 38% in the CCl_4 model (Figure 1B). Thus, in the CCl_4 model, the reduction of MBF was approximately 30% greater with D-Pro compared to A779.

Treatment with D-Pro and A779 also increased HVR in both CCl_4 ($p<0.05$) and BDL ($p<0.01$) models (Figure 1C). Despite this, D-Pro and A779 significantly reduced portal pressure in both CCl_4 ($p<0.005$) and BDL ($p<0.05$) rat models (Figure 1D). Importantly, in the CCl_4 model, D-Pro reduced portal pressure by 33%, almost twice the reduction with A779 treatment (17%). The significant difference in portal pressure between D-Pro- and A779-treated groups ($p<0.05$) in this model was consistent with the differences in the reduction of MBF mediated by the two treatments (Figure 1B). However, in the BDL model, the reduction in portal pressure was 20% and 22% with D-Pro and A779, respectively, consistent with similar reductions of MBF achieved with the two drugs. In addition, A779 but not D-Pro significantly increased ($p<0.05$) renal vascular resistance and thus decreased renal arterial blood flow in the CCl_4 model compared to healthy controls (Table S3). Moreover, we found that A779 but not D-Pro significantly increased ($p<0.05$) mean arterial pressure in the BDL model compared to saline-injected cirrhotic controls (Table S3).

D-Pro treatment was associated with a slight but nonsignificant reduction in p-eNOS levels and the ratio of p-eNOS/total eNOS; however, A779 treatment significantly ($p<0.05$) reduced p-eNOS levels and the p-eNOS/total eNOS ratio in cirrhotic splanchnic vessels compared to diseased controls. In marked contrast, eNOS phosphorylation in the liver was unchanged in cirrhosis, and neither of the drugs had any major effect

on eNOS phosphorylation. These results are described in detail in the Supporting Materials and presented in Figure S4.

Noncirrhotic portal hypertension created by PPVL was associated with a reduction ($p < 0.001$) in SPVR compared to sham-operated healthy controls (Figure 1A). Consistent with reduced SPVR, MBF was significantly ($p < 0.001$) increased in PPVL rats (Figure 1B). Induction of noncirrhotic portal hypertension did not affect the HVR (Figure 1C). As expected, the portal pressure was significantly ($p < 0.01$) increased in PPVL rats compared to healthy controls (Figure 1D). However, in marked contrast to the findings in cirrhotic rats, treatment with either D-Pro or A779 did not increase SPVR or reduce MBF and thus had no effect on portal pressure.

MasR blockade with A779 altered liver biochemistry and increased liver fibrosis

Elevated liver enzyme levels in cirrhotic CCl_4 and BDL rats compared to controls were further increased after the treatment with the MasR blocker A779 ($p < 0.05$); however, MrgD blockade with D-Pro did not affect plasma ALT or AST levels. While cirrhosis or drugs did not affect serum albumin, creatinine, and bilirubin levels in CCl_4 rats, both creatinine and bilirubin levels were significantly ($p < 0.05$) elevated in BDL rats compared to controls, and A779 but not D-Pro caused further elevation ($p < 0.05$) of plasma bilirubin levels. These results are described in detail in the Supporting Materials and are presented in Figure S2.

Gene expression of α-*SMA* (Figure S3A), a marker of activated HSCs, was significantly elevated in the livers of CCl_4 and BDL ($p < 0.001$) rats compared to controls. The expression of α-SMA was significantly ($p < 0.05$) elevated after A779 treatment in the BDL but not in the CCl_4 model. Gene expression of *COL1A1* was significantly ($p < 0.001$) increased in the cirrhotic livers of both models compared to controls and was further elevated ($p < 0.05$) by A779 treatment (Figure S3B). In keeping with the above findings, quantification of hepatic collagen protein deposition using picrosirius red-stained liver sections showed that hepatic collagen content was significantly increased in both CCl_4 and BDL ($p < 0.01$) rats compared to controls and was further elevated after A779 treatment ($p < 0.05$) (Figure S3C,D). D-Pro did not significantly increase these markers of fibrosis.

MrgD blockade inhibits acetylcholine-induced vasodilatory responses in distal mesenteric resistance vessels in cirrhosis

A779 treatment had no effect on acetylcholine-induced vasodilatory responses of the proximal (Figure 2A) mesenteric resistance vessels isolated from healthy rats or the proximal and distal vessels isolated from cirrhotic rats (Figure 2C,D). In marked contrast, D-Pro treatment markedly ($p < 0.05$) inhibited the vasodilatory response in distal mesenteric resistance vessels (Figure 2D). At the highest doses of acetylcholine, D-Pro-treated vessels relaxed by only 13% while the cirrhotic vessels treated with A779 or saline relaxed by more than 75%. Moreover, both drugs failed to inhibit the vasodilatory responses of the abdominal aorta to acetylcholine (data not shown). This implies that *ex vivo* blockade of MrgD increases SPVR in cirrhosis by inhibiting the MrgD-activated downstream signaling pathway in distal mesenteric resistance arteries. Moreover, it appears that acetylcholine-induced vasorelaxation in these vessels may involve signaling molecules that are common to downstream pathways of MrgD and acetylcholine.

MrgD and MasR gene and protein expression is increased in cirrhotic rat mesenteric resistance vessels

MrgD and *MasR* gene (Figure 3A,B) and protein (Figure 3C–F) expressions were markedly ($p < 0.001$) increased in the mesenteric resistance vessels of saline-infused diseased control rats of both CCl_4 and BDL models compared to healthy or sham-operated controls. Although treatment with D-Pro or A779 differentially affected ($p < 0.05$) gene expression of *MrgD* and *MasR* in CCl_4 and BDL rat mesenteric vessels compared to untreated diseased controls (Figure 3A,B), western blot analysis (Figure 3C,D) and immunohistochemical staining (Figure 3E,F) showed that the drugs had no effect on MasR or MrgD protein levels.

MasR is up-regulated but MrgD expression is unchanged in cirrhotic rat livers

In both cirrhotic models, hepatic *MasR* gene ($p < 0.005$) (Figure 4B) and protein ($p < 0.05$) (Figure 4C–E) expressions were markedly increased. Interestingly, *MrgD* gene expression, which was at a low level in healthy control livers, was unchanged in cirrhotic livers (Figure 4A), and protein expression of MrgD was undetectable in both control and cirrhotic CCl_4 and BDL livers (Figure 4F). Moreover, none of the receptor blockers affected hepatic MrgD or MasR expression in either model. The increased MasR expression in cirrhotic livers suggests that it could be an important receptor mediating HVR in cirrhosis. In contrast, the lack of detectable MrgD protein makes it unlikely that this receptor plays a significant role in modulating hepatic vascular tone.

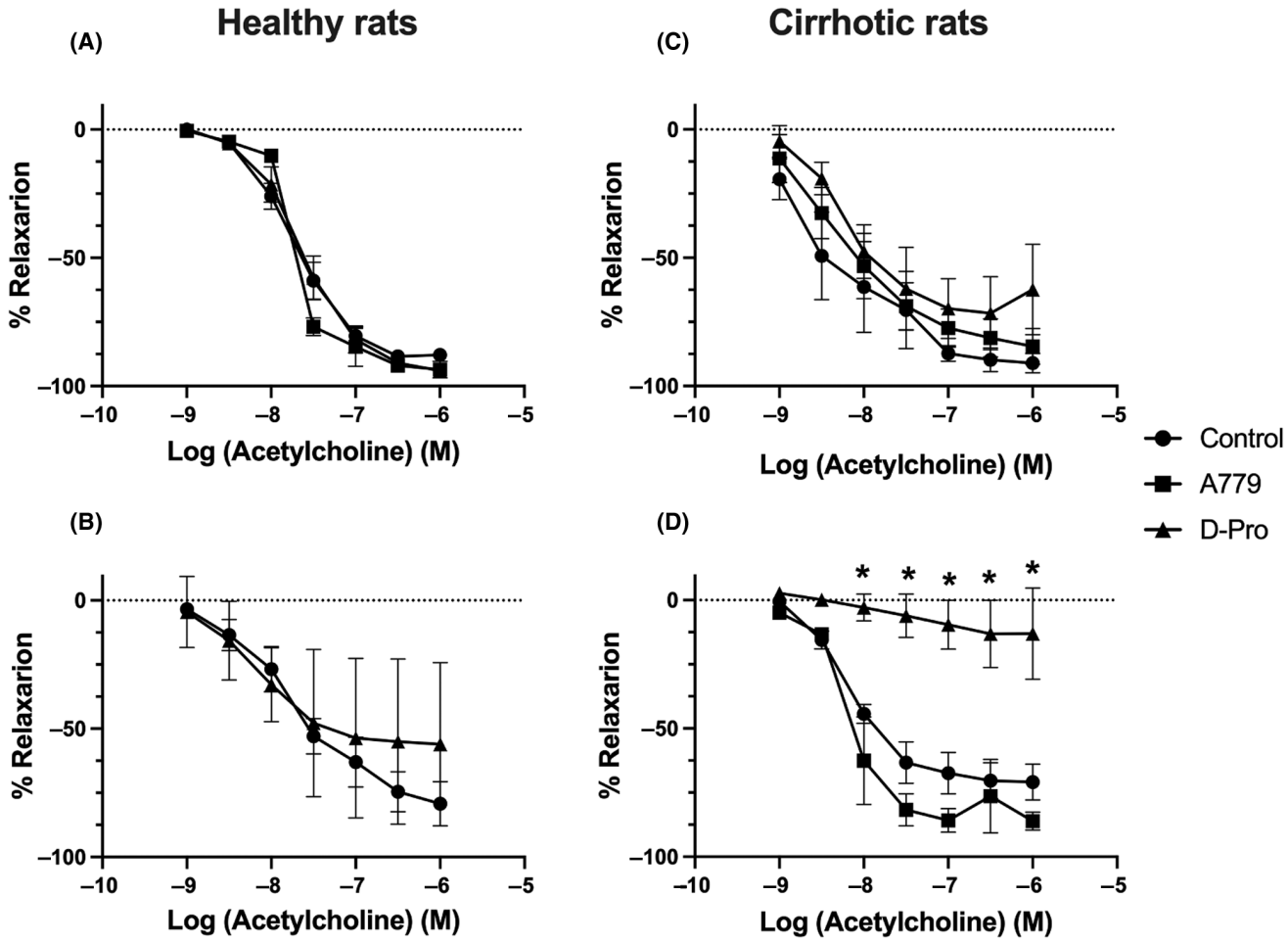

FIGURE 2 Effects of MrgD blocker D-pro and MasR blocker A779 on acetylcholine-induced vasodilatory responses. (A,B) Healthy and (C,D) carbon tetrachloride-intoxicated cirrhotic rats were analyzed for the effects of MrgD blocker D-pro and MasR blocker A779 on acetylcholine-induced vasodilatory responses in (A,C) proximal and (B,D) distal mesenteric resistance vessels. The A779 response curve in panel B is not shown due to a technical reason. Each data point represents mean ± SEM profile of vascular responses from n = 4–6 rats per group. *$p < 0.05$, D-pro versus saline or A779 groups. A779, D-Ala^7-Ang-(1–7); D-Pro, D-Pro^7-Ang-(1–7); MasR, Mas receptor; MrgD, Mas-related G protein-coupled receptor type D

Expression of both MrgD and MasR is increased in human cirrhotic omental arteries, and MasR but not MrgD is up-regulated in human cirrhotic livers

As in our cirrhotic animal models, gene and protein expressions of both *MrgD* (Figure 5A,C) and *MasR* (Figure 5B,C) were significantly ($p < 0.05$) up-regulated in the omental arteries isolated from patients with cirrhosis compared to the vessels isolated from subjects without cirrhossis, indicating that both of these receptors may play an important role in regulating SPVR in cirrhosis.

Also consistent with our findings in cirrhotic rats, we found that the *MasR* gene ($p < 0.001$) (Figure 5D) and protein ($p < 0.05$) (Figure 5E,F) expression were up-regulated in the cirrhotic livers of patients with PSC and ALC. However, gene (data not shown) and protein (Figure 5G) expression of *MrgD* was not detectable in liver of either control or patients with cirrhosis, which suggests that, as in our animal models, this receptor is unlikely to have a significant role in regulating HVR in human cirrhosis.

MrgD and MasR are not up-regulated in mesenteric resistance vessels of PPVL rats

In marked contrast to the up-regulated MrgD and MasR expression in the mesenteric resistance vessels of cirrhotic BDL and CCl_4 rats (see Figure 3), gene (Figure 6A,B) and protein (Figure 6C–E) expressions of both receptors were not altered in the mesenteric resistance vessels of noncirrhotic PPVL rats. This is consistent with our functional data showing that MasR and MrgD blockade had no effect on SPVR in this model (see Figure 1A). Importantly, unlike the cirrhotic models (see Figure 3), treatment with D-Pro or A779 did not alter receptor expression in mesenteric vessels in

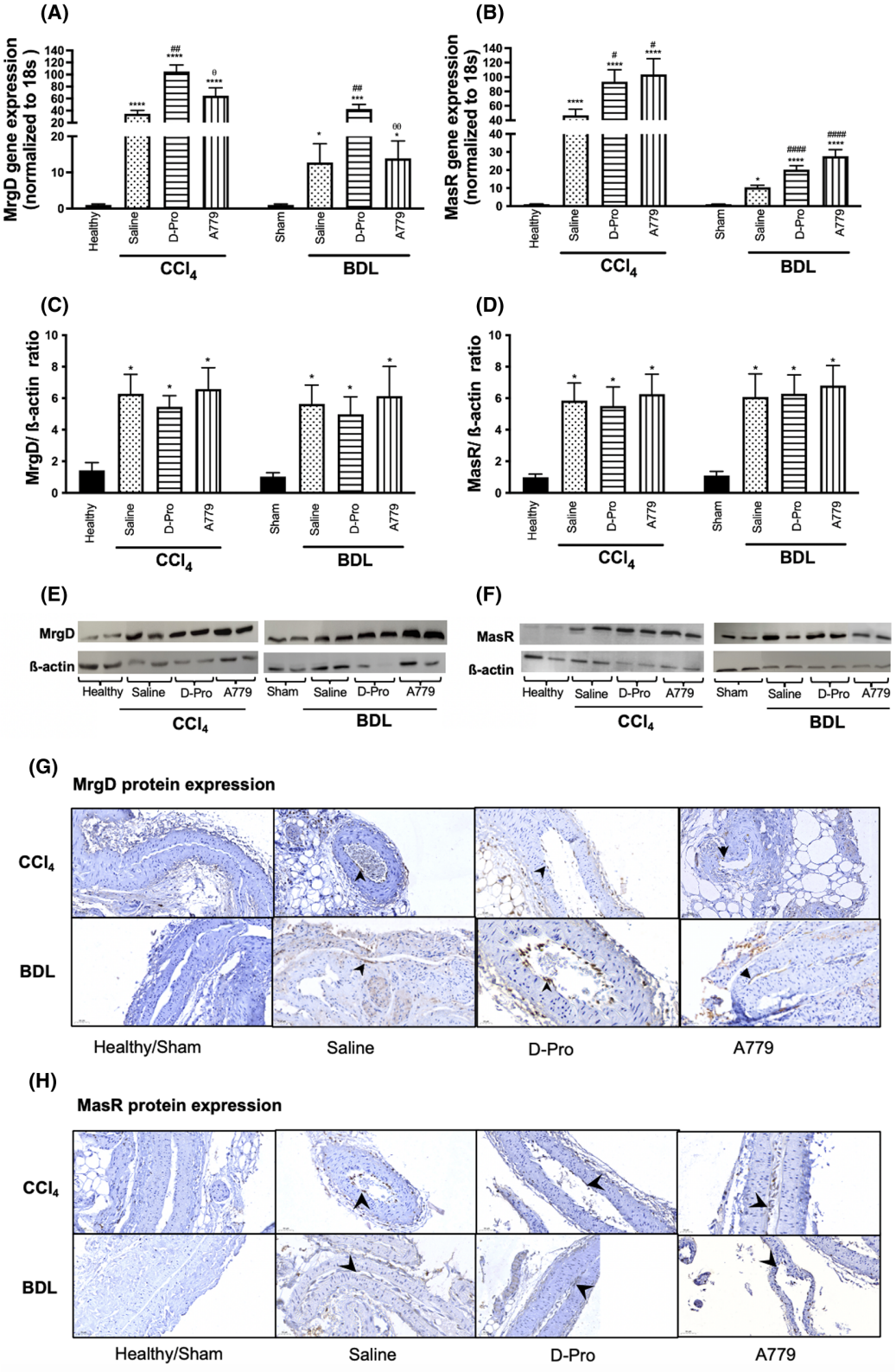

FIGURE 3 Gene expression of *MrgD* and *MasR*. (A,B) *MrgD* and *MasR* were analyzed in cirrhotic mesenteric arteries isolated from carbon tetrachloride-intoxicated and bile duct-ligated rats, cirrhotic rats treated with the MrgD blocker D-pro or MasR blocker A779, and healthy controls. (C–H) Protein expression of MrgD and MasR quantified by western blot and immunohistochemistry (magnification ×200), respectively, is shown. Each bar represents the mean ± SEM profile from 10 to 15 rats per group. Arrowheads show positive endothelial staining. $^{*}p<0.05$, $^{***}p<0.005$, $^{****}p<0.001$, diseased (CCl_4 or BDL) versus olive oil-injected or sham-operated healthy controls. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#\#}p<0.001$, diseased saline versus D-pro or A779-treated diseased rats. $^{\theta}p<0.05$, $^{\theta\theta}p<0.01$, D-pro versus A779-treated diseased rats. A779, D-Ala^7-Ang-(1–7); BDL, bile duct ligation; CCl_4, carbon tetrachloride; D-Pro, D-Pro^7- Ang-(1–7); MasR, Mas receptor; MrgD, Mas-related G protein-coupled receptor type D

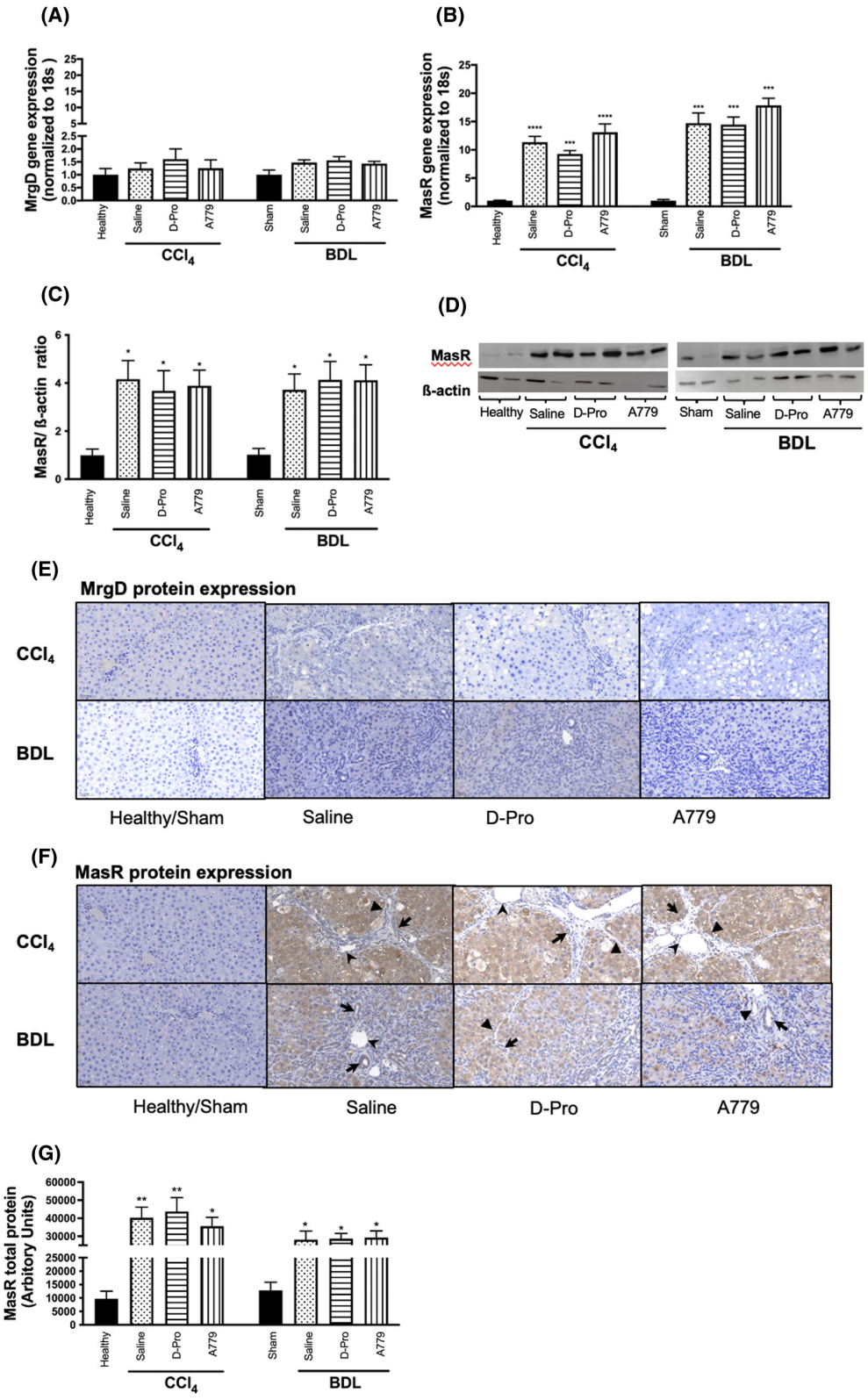

FIGURE 4 Gene expression of *MrgD* and *MasR*. (A,B) *MrgD* and *MasR* were analyzed in cirrhotic livers isolated from carbon tetrachloride-intoxicated and bile duct-ligated rats, cirrhotic rats treated with the MrgD blocker D-pro or MasR blocker A779, and healthy controls. Up-regulation of MasR protein expression as quantified by western blot (C,D) and immunohistochemistry (F,G) (magnification ×200) is shown. Positive staining of MasR in endothelium (large arrowhead), bile duct epithelial cells (arrow), and hepatic arterioles (small arrowhead) is shown. MrgD protein was not detectable by western blot or by immunohistochemistry (E) (magnification ×200) in cirrhotic or control livers. Each bar represents the mean ± SEM profile from 10 to 15 rats per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$, diseased (CCl_4 or BDL) versus olive oil-injected/sham-operated healthy controls. A779, D-Ala^7-Ang-(1–7); BDL, bile duct ligation; CCl_4, carbon tetrachloride; D-Pro, D-Pro^7-Ang-(1–7); MasR, Mas receptor; MrgD, Mas-related G protein-coupled receptor type D

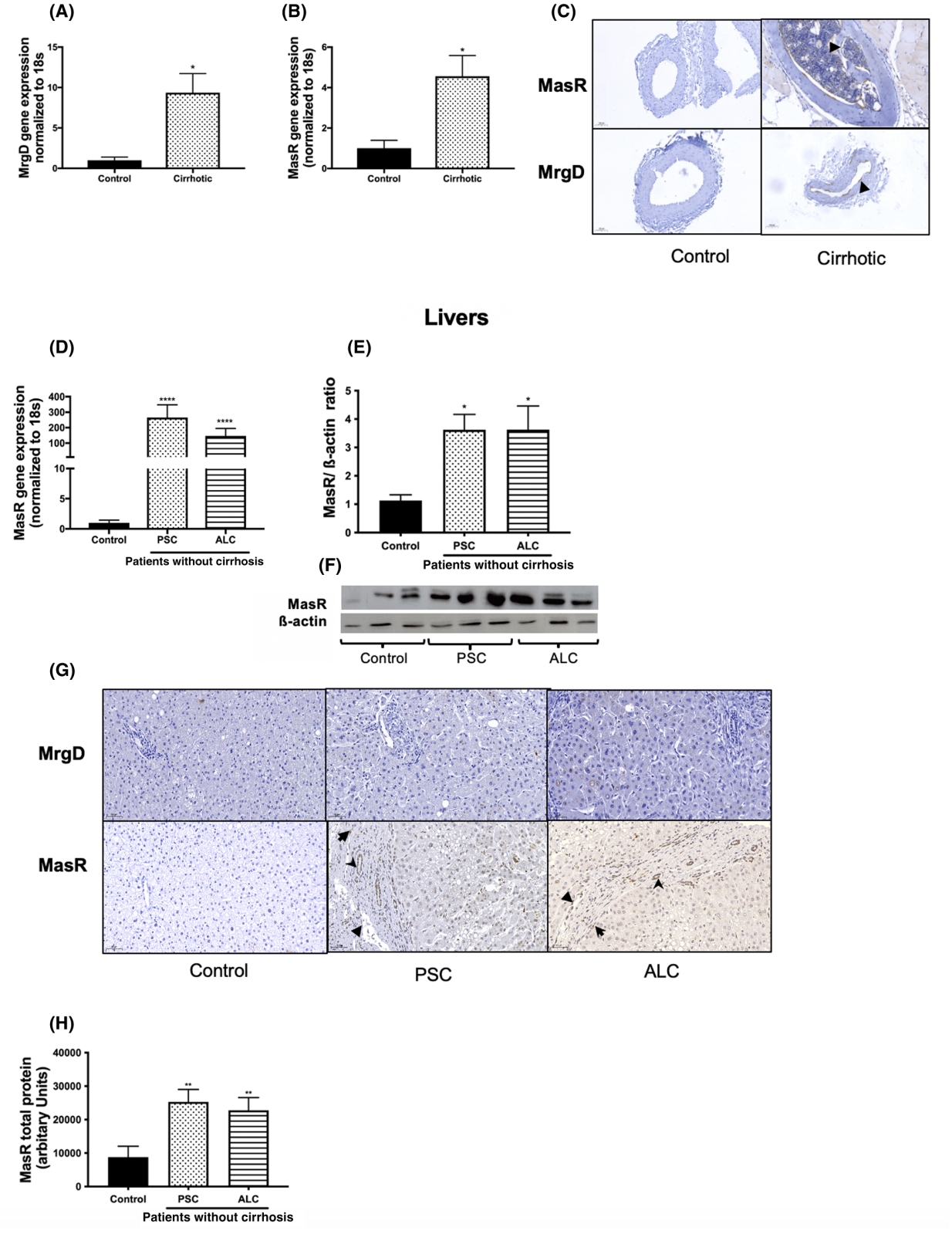

FIGURE 5 Gene expression in omental vessels of patients with cirrhosis with PSC (n = 6) and subjects without cirrhosis (n = 3). (A) *MrgD*. (B) *MasR*. (C) Immunohistochemical staining (magnification ×200) of MasR and MrgD protein in omental vessels are indicated by arrowheads. (D) Liver gene and (E,F) protein expression of MasR by real-time quantitative polymerase chain reaction and western blot, respectively, in PSC (n = 6) and ALC (n = 8) and subjects without cirrhosis (n = 5). (G,H) Positive immunostaining of MasR protein was detected in endothelium (large arrowhead), bile duct epithelial cells (arrow), and hepatic arterioles (small arrowhead) (magnification ×200). However, liver MrgD protein was not detected by western blot (data not shown) or (G) immunohistochemistry (magnification ×200). Each bar represents the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.001$. ALC, alcoholic cirrhosis; MasR, Mas receptor; MrgD, Mas-related G protein-coupled receptor type D; PSC, primary sclerosing cholangitis

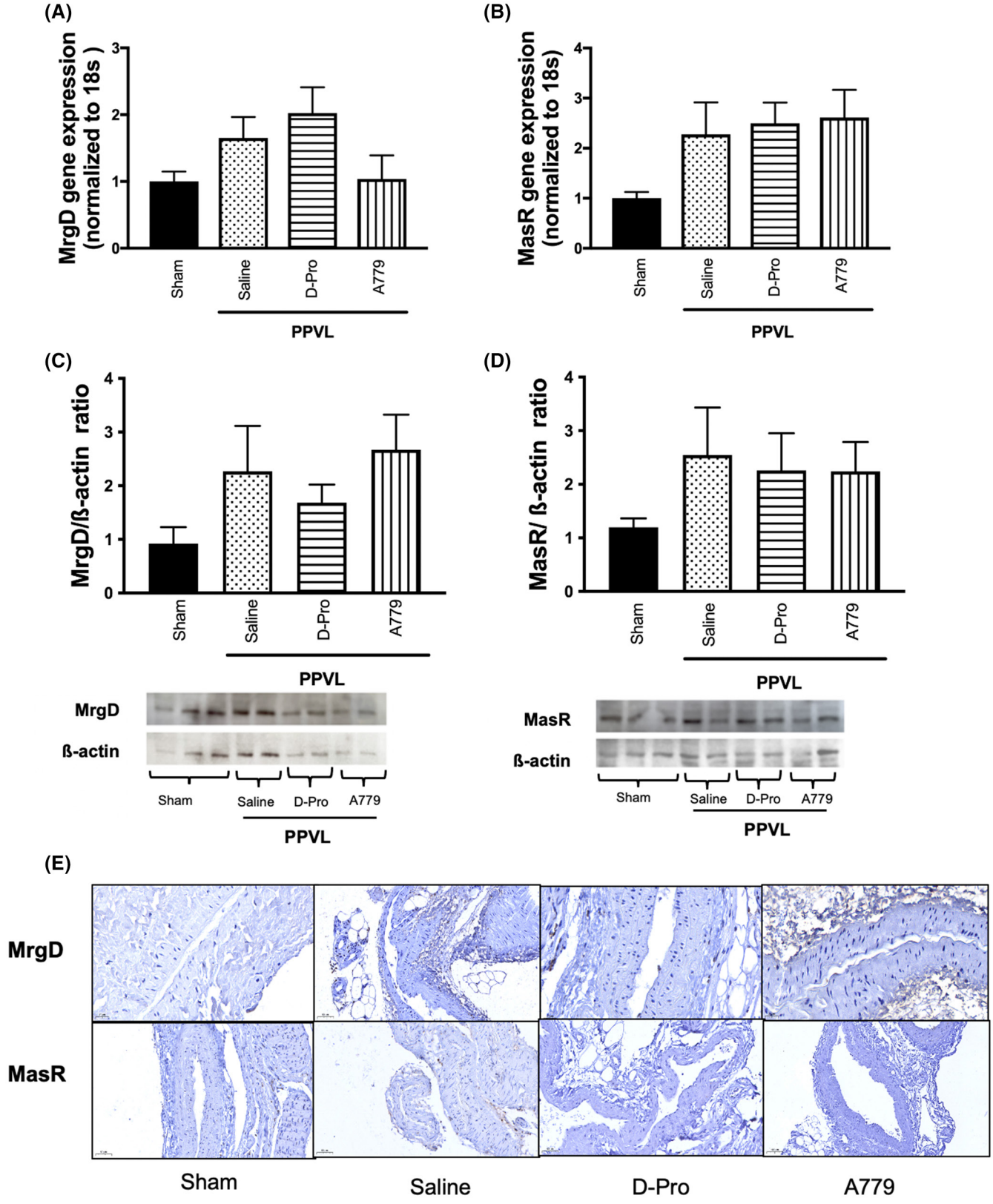

FIGURE 6 Gene expression of *MrgD* and *MasR* in mesenteric arteries isolated from PPVL rats with noncirrhotic portal hypertension, PPVL rats treated with the MrgD blocker D-pro or MasR blocker A779, and sham-operated controls. (A) *MrgD*. (B) *MasR*. (C,D) Western blot analysis of mesenteric arterial protein expression of (C) MrgD and (D) MasR showed no difference between the four groups. (E) MrgD and MasR protein was not detected by immunohistochemical staining, likely due to very low expression levels. Each bar represents the mean ± SEM profile from 10 to 15 rats per group. A779, D-Ala^7-Ang-(1–7); D-Pro, D-Pro^7-Ang-(1–7); MasR, Mas receptor; MrgD, Mas-related G protein-coupled receptor type D; PPVL, partial portal vein ligation

PPVL rats (Figure 6), suggesting that MrgD and MasR have no role in regulating splanchnic vasodilatation in this model of noncirrhotic portal hypertension.

MrgD and MasR are differentially regulated in different vascular beds

Although *MasR* was up-regulated in mesenteric resistance vessels (see Figure 3), liver (see Figure 4), and kidneys of cirrhotic rats (Figure 7; Figure S5), the up-regulated expression of *MrgD* was confined to mesenteric resistance vessels (Figure 3; Figure S5). Interestingly, we found that vascular gene expression profiles of MasR and MrgD were closely related to hemodynamics of the vascular bed under consideration; thus, we have summarized the relationship between gene expression profiles and hemodynamics of different vascular beds in Figure 7.

DISCUSSION

The major aim of the current study was to investigate the contribution of MrgD to pathologic mesenteric vasodilatation in both cirrhotic and noncirrhotic portal hypertension and to investigate a potential new avenue for the manipulation of the alternate RAS in the treatment of portal hypertension in cirrhosis. This is the first report to demonstrate that long-term infusion of a peptide-derived receptor blocker for MrgD increases SPVR, leading to a large reduction in MBF and reducing portal pressure in cirrhotic rats. In CCl_4-treated rats in particular, the reduction in portal pressure (33%) was greater than what has previously been reported with other pharmacotherapies in animal models of cirrhotic portal hypertension.[2] We also demonstrated that the effects of MrgD blockade may be superior to those of MasR blockade. In the splanchnic vascular bed of patients and animals with cirrhosis, expression and activity of MrgD was up-regulated, supporting the possible role for this receptor in pathologic mesenteric vasodilation in cirrhosis. However, in contrast to the findings in splanchnic resistance vessels, hepatic MrgD expression in healthy rat livers was minimal and not up-regulated in cirrhosis while receptor expression was undetectable in the livers of control subjects and patients with cirrhosis. Moreover, although MasR blockade increased renal resistance to blood flow and mean arterial pressure, MrgD blockade did not have these off-target effects. These findings collectively imply that MrgD blockers may inhibit splanchnic vasodilatation without having effects in other vascular beds and offer a potentially important new approach to the treatment of portal hypertension. However, MrgD or MasR blockers were not effective in reducing portal pressure in noncirrhotic PPVL rats with portal hypertension, suggesting that the alternate RAS may not contribute to pathologic splanchnic vasodilatation in this condition.

Ang-(1–7), the effector peptide of the alternate RAS, possesses potent vasodilatory properties and is elevated in the liver and circulation in experimental and human cirrhosis.[7,11,12,14,15] It is well known that Ang-(1–7) acts through its receptor MasR.[16] Our previous findings showed that Ang-(1–7) produces a MasR-mediated increase in mesenteric vasodilatation and MBF, thereby contributing to elevation of portal pressure in cirrhosis.[5] This was supported by the finding that blockade of MasR with an acute bolus injection of A779 reduced splanchnic vasodilatation and improved portal hypertension in cirrhotic rats.[5,6] In keeping with our published work, which used bolus doses of receptor blockers,[5,6] our current study found that long-term infusion of A779 produced a clinically significant (22%) reduction in portal pressure in experimental cirrhosis. However, it also increased HVR, presumably as a result of its effects on up-regulated MasR in the cirrhotic liver.

Although MasR was initially considered as the specific receptor for Ang-(1–7),[16] several studies raised the possibility of the existence of a second receptor for Ang-(1–7). The initial evidence for this was provided by a study showing that vasodilatory effects of Ang-(1–7) in rat aorta were unaffected by the blockade of MasR with A779 but were completely abolished by D-Pro.[17] D-Pro is another Ang-(1–7) analogue with a proline residue at the C-terminal substituted with D-proline, which was later known to block MrgD activity.[18] In 2008, Gembardt and colleagues[19] showed that, in response to stimulation with Ang-(1–7), COS cells transfected with MrgD released arachidonic acid, a precursor of vasodilatory epoxyeicosatrienoic acids (EETs). EETs are known to act as endothelium-derived hyperpolarizing factors (EDHFs),[20] suggesting that MrgD has a possible role in regulating Ang-(1–7)-mediated vasodilatation. Subsequently, Lautner and colleagues[18] showed that MrgD is activated following binding to the vasodilatory RAS peptide alamandine and that the effects of alamandine were only blocked by D-Pro but not by A779. A more recent study strongly supported the concept that MrgD is a second vasodilatory receptor for Ang-(1–7) by showing that Ang-(1–7) reduced mean arterial pressure in wild-type mice but failed to elicit a vasodilatory response in mice lacking MrgD, confirming the functional role of MrgD in regulating vasodilatory effects of Ang-(1–7).[9]

There are some potentially important differences between the effects of MrgD blocker D-Pro and those of the MasR blocker A779 in our study. In particular, D-Pro had greater effects on SPVR in both cirrhotic models, and in the CCl_4 model, it had a larger effect on MBF and produced almost twice the reduction in portal pressure achieved with A779. However, we also found that both A779 and D-Pro significantly increased

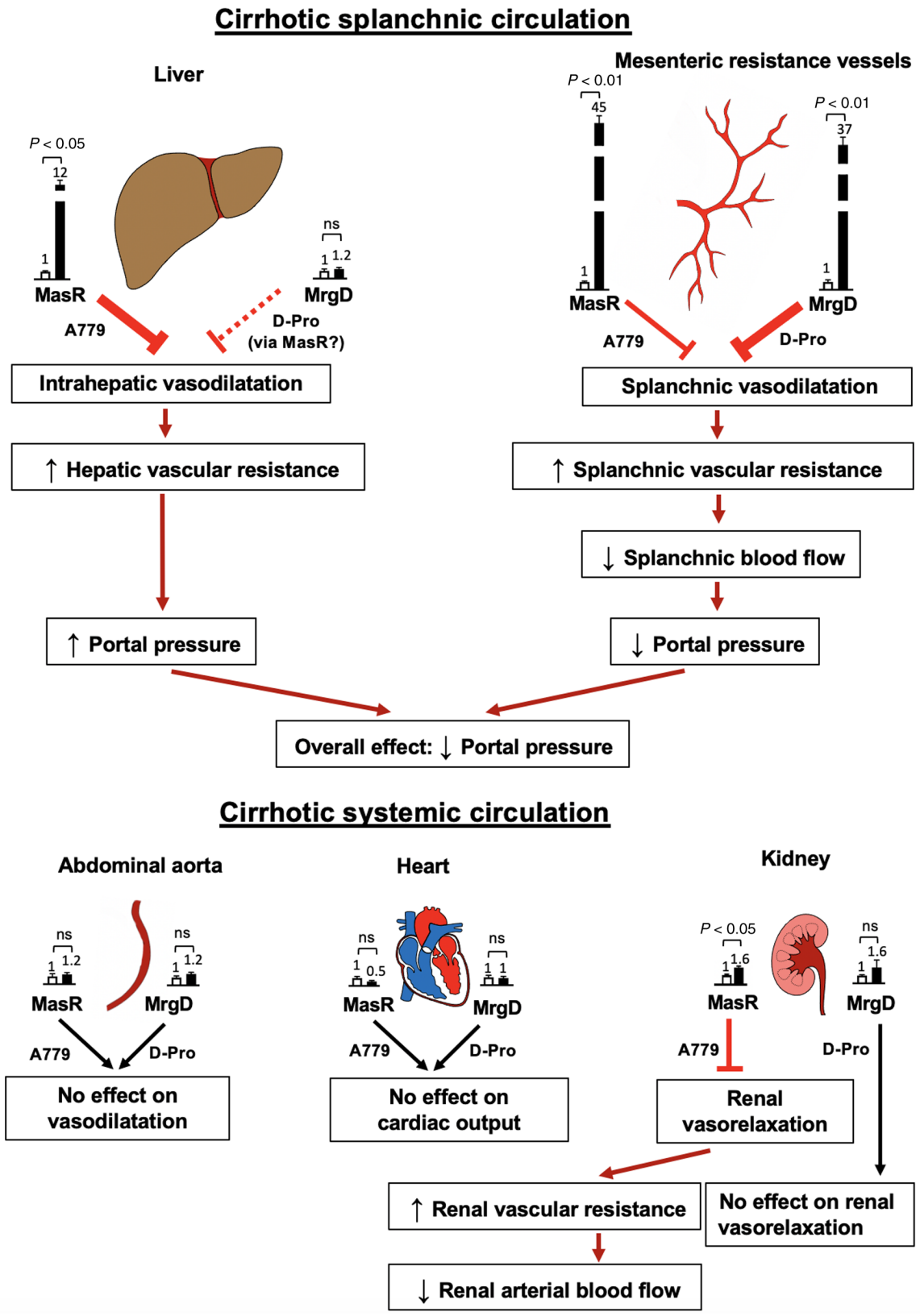

FIGURE 7 Relationship between hemodynamic changes and the expression of *MasR* and *MrgD* in different vascular beds of healthy and cirrhotic carbon tetrachloride-intoxicated rats. Each bar represents the mean ± SEM profile of 10–15 rats. Fold changes are shown on top of each bar of control (open bars) and cirrhotic (closed bars) rats. Thickness of receptor blocking lines indicates relative contribution by the receptor. Broken line denoting a receptor-blocking step represents a possible pathway. A779, D-Ala^7-Ang-(1–7); D-Pro, D-Pro^7-Ang-(1–7); MasR, Mas receptor; MrgD, Mas-related G protein-coupled receptor type D; ns, nonsignificant

intrahepatic vascular resistance despite the finding that, unlike MasR expression, MrgD expression in the liver of healthy and cirrhotic animals of both models was minimal. A possible explanation for this is that although D-Pro is known to block MrgD[18] it may also nonspecifically bind to MasR to inhibit the vasodilatory effects of Ang-(1–7) on MasR signaling, as suggested in a recent study.[9] However, our findings also suggest that, unlike A779, this nonspecific interaction between D-Pro and MasR did not have a major influence on the antifibrotic effects of MasR and did not appear to influence renal or systemic hemodynamics.

Although the vasodilatory downstream signaling pathway(s) activated by MrgD is yet to be elucidated, we and others have shown that the vasodilatory effects of Ang-(1–7)/MasR activation are mediated by the Gs protein and protein kinase A and the activation of protein kinase B (Akt)-dependent pathways that generate nitric oxide (NO) by eNOS phosphorylation.[5,21,22] Consistent with published studies, we found that treatment with A779 significantly reduced eNOS phosphorylation in cirrhotic mesenteric vessels. However, the magnitude of the effect of D-Pro on eNOS inhibition appears less than that of A779 in mesenteric vessels. This suggests that although eNOS/NO is a major downstream pathway of MasR activation, an eNOS/NO-independent mechanism may also contribute to MrgD-activated vasodilatation in cirrhotic splanchnic vessels. Further evidence of a specific MasR-independent mechanism through which D-Pro affects splanchnic vasodilatation in cirrhosis comes from our studies in the vascular myograph. D-Pro inhibited the vasodilatory response to acetylcholine in the distal mesenteric resistance vessels by more than 60%, with a maximum relaxation of 13% compared to more than 75% relaxation in healthy as well as cirrhotic vessels treated with A779 or saline but did not affect responses in larger vessels. Our findings are in keeping with the notion that different regions of the mesenteric vascular bed have different vasodilatory mechanisms[23] and suggest that the downstream signaling pathways activated by MrgD in cirrhosis may have a major role in controlling vasodilatory responses in the distal end of the mesenteric vascular tree. It has been reported that EDHFs are the major modulators of vasodilation in distal mesenteric arteries in cirrhosis and smaller peripheral vessels are highly sensitive and produce higher amounts of vasodilatory EETs,[20,24,25] a class of EDHFs derived from arachidonic acid, while NO activity is lowest in these vessels. Thus, our findings suggest that, in cirrhosis, D-pro specifically blocks up-regulated MrgD in distal mesenteric vessels, which stimulates the production of EDHFs involved in acetylcholine-stimulated vasorelaxation.

The absence of MrgD- or MasR-mediated effects on acetylcholine-induced vasorelaxation in large conduit vessels, such as the abdominal aorta, of cirrhotic animals may be attributable to unchanged receptor expression in these vessels. On the other hand, although MrgD up-regulation was confined to the mesenteric resistance vessels in cirrhotic animals, MasR was up-regulated in several tissues. This suggests that MrgD-mediated effects in cirrhosis may be confined to the mesenteric vasculature and possibly to the distal mesenteric vessels whereas MasR-mediated effects might be expected in other vascular beds. This is supported by our findings that MasR but not MrgD blockade significantly increased mean arterial pressure in the BDL model. Furthermore, MasR but not MrgD blockade significantly increased renal vascular resistance, resulting in a reduced renal blood flow in the CCl4 rats. This is consistent with studies demonstrating that deletion of the MasR gene increased vascular resistance in coronary arteries[26,27] and renal vasculature.[16,28] These findings therefore suggest a mesenteric vasculature-specific role of MrgD in cirrhosis, and in contrast to MasR or beta blockade,[2] inhibition of this receptor may not produce off-target systemic effects. The relationship between hemodynamic changes and the expression of MasR and MrgD in different vascular beds of healthy and cirrhotic rats is depicted in Figure 7.

The clinical translatability of our findings from cirrhotic animal models is strongly supported by comparable data obtained from human specimens demonstrating that MrgD is up-regulated in the splanchnic vessels but not in the livers of patients with cirrhosis. Furthermore, as in our animal models, MasR expression was up-regulated in both splanchnic vessels and the liver of patients with cirrhosis, in agreement with our previous reports.[5,12,29] These findings support the concept that although the peptide MrgD blocker D-Pro increased intrahepatic resistance, likely due to its nonspecific binding to MasR,[9] nonpeptide drugs specifically targeting MrgD would not be expected to have this unwanted effect. Furthermore, MasR blockade has been shown to increase liver collagen deposition in the BDL model,[30] and we saw evidence of this effect in both our models. This may have contributed to the increase in hepatic resistance with this compound in our study and its lesser effects on portal pressure compared with that of D-Pro-treated animals in the CCl_4 model. This was further supported by liver biochemistry profiles that showed elevated liver enzyme levels with MasR blockade but not with MrgD blockade. In agreement with this, we found the liver collagen level was also unchanged with D-Pro treatment. These findings provide evidence of another potential benefit of MrgD blockade over MasR blockade in the treatment of cirrhotic portal hypertension.

The role of RAS in the development of noncirrhotic portal hypertension is largely unknown; however, like cirrhotic portal hypertension, noncirrhotic portal hypertension is also characterized by excessive splanchnic vasodilatation.[31,32] We therefore investigated whether the RAS contributes to the pathogenesis of noncirrhotic portal hypertension by using a noncirrhotic

portal hypertensive rat model of PPVL. There was a marked reduction in SPVR in PPVL rats and an increased MBF, leading to the development of portal hypertension. A higher percentage of mesenteric portosystemic shunting in this model (Table S3) compared to cirrhotic animals has been shown to be associated with a high degree of passive dilatation of preexisting vascular channels and increased angiogenesis.[31,33,34] Nevertheless, the present study provides strong evidence that the receptors of the alternate RAS have no role in the pathogenesis of noncirrhotic portal hypertension or at least in the PPVL model as the expression of both MrgD and MasR were not altered in the mesenteric resistance vessels. This was further supported by receptor blockade studies demonstrating that none of the receptor blockers failed to improve SPVR, MBF, and thus portal pressure. Therefore, while the alternate RAS, acting through MrgD and MasR, may be an important regulator of splanchnic vasodilatation in cirrhosis, it may have an insignificant role in the PPVL rat model of noncirrhotic portal hypertension.

In conclusion, this is the first study to document the potential role of the vasodilatory Ang-(1–7) receptor MrgD in splanchnic vasodilatation and the development of portal hypertension in cirrhosis. We found that the effects of MrgD blockade on SPVR, MBF, and portal pressure in cirrhosis are significantly greater than those of MasR blockade, at least in some of the cirrhotic models. Importantly, in contrast to MasR, the MrgD gene and protein levels, which were up-regulated in mesenteric/splanchnic vessels of animal models of cirrhosis and patients with cirrhosis, were either low or undetectable in animal and human cirrhotic livers, supporting the concept that highly specific MrgD blockade may reduce splanchnic blood flow without increasing intrahepatic resistance. Thus, MrgD offers an attractive target for the design and development of novel therapeutics that can specifically block splanchnic vasodilatation in cirrhotic portal hypertension. However, in noncirrhotic portal hypertensive PPVL rats, MasR and MrgD blockades have no effect, suggesting the development of portal hypertension in the absence of cirrhosis is not regulated by the alternate RAS.

AUTHOR CONTRIBUTIONS

Conceptualization: Lakmie S. Gunarathne, Peter W. Angus, Chandana B. Herath. *Data curation*: Lakmie S. Gunarathne, Chandana B. Herath. *Formal analysis*: Lakmie S. Gunarathne, Indu G. Rajapaksha, Chandana B. Herath. *Funding acquisition*: Peter W. Angus, Chandana B. Herath. *Investigation*: Lakmie S. Gunarathne, Indu G. Rajapaksha, Tawar Qaradakhi, Anthony Zulli, Chandana B. Herath. *Methodology*: Lakmie S. Gunarathne, Chandana B. Herath. *Project administration*: Lakmie S. Gunarathne, Indu G. Rajapaksha, Chandana B. Herath. *Resources*: Lakmie S. Gunarathne, Indu G. Rajapaksha, Stephen Casey, Anthony Zulli, Peter W. Angus, Chandana B. Herath. *Supervision*: Peter W. Angus, Chandana B. Herath. *Validation*: Lakmie S. Gunarathne, Peter W. Angus, Chandana B. Herath. *Visualization*: Lakmie S. Gunarathne, Indu G. Rajapaksha. *Writing original draft*: Lakmie S. Gunarathne. *Review and editing*: Lakmie S. Gunarathne, Indu G. Rajapaksha, Harinda Rajapaksha, Jonel Trebika, Peter W. Angus, Chandana B. Herath.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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