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Title

Activation of the Mas Receptor by Angiotensin-(1–7) in the Renin–Angiotensin System Mediates Mesenteric Vasodilation in Cirrhosis

Short Title

Angiotensin-(1-7) regulates portal hypertension

Authors

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Abbreviations

ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme-2; Ang I, angiotensin I; Ang II, angiotensin II; Ang-(1-7) , angiotensin-(1-7); AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; BDL, bile duct ligation; bw, body weight; CCl_4, carbon tetrachloride; cDNA, complementary DNA; C_T, comparative threshold cycle; DNA, desoxyribonucleic acid; i.p., intraperitoneal; MAP, mean arterial pressure; MasR, Mas receptor; qPCR, quantitative real-time polymerase chain reaction; PP, portal pressure; RAS, renin-angiotensin system; RNA, ribonucleic acid.

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JG, CH, LB and PA assisted with concept and design of the ex-vivo experiments and performed and analysed the data from those experiments. SK, RS, MG, NM and JT performed and analysed the data for the in-vivo and human experiments. TW performed the human liver ACE2 and Mas receptor expression studies. JG drafted the manuscript and JG, SK, JT, TS, CH, LB and PA revised the manuscript.

Disclosures

No conflicts of interest exist for any of the authors.

Abstract

BACKGROUND & AIMS: Splanchnic vascular hypocontractility with subsequent increased portal venous inflow leads to portal hypertension. Although the renin–angiotensin system contributes to fibrogenesis and increased hepatic resistance in patients with cirrhosis, little is known about its effects in the splanchnic vasculature—particularly about those of the alternate system in which angiotensin (Ang)II is cleaved by the Ang-converting enzyme-2 (ACE2) to Ang-(1–7), which activates the G protein-coupled receptor Mas (MasR). We investigated whether this system contributes to splanchnic vasodilatation and portal hypertension in cirrhosis.

METHODS: We measured levels of renin–angiotensin system mRNA and proteins in splanchnic vessels from patients and rats with cirrhosis. Production of Ang-(1–7) and splanchnic vascular reactivity to Ang-(1–7) were measured in perfused mesenteric vascular beds from rats following bile-duct ligation. Ang-(1–7) and MasR were blocked in rats with cirrhosis, to examine splanchnic vascular haemodynamics and portal pressure response.

RESULTS: Levels of ACE2 and MasR were increased in splanchnic vessels from cirrhotic patients and rats, compared with healthy controls. We also observed an ACE2-dependent increase in Ang-(1–7) production. Ang-(1–7) mediated splanchnic vascular hypocontractility in ex vivo splanchnic vessels from rats with cirrhosis (but not control rats) via MasR stimulation. Identical effects were observed in the splanchnic circulation in vivo. MasR blockade reduced portal pressure, indicating that activation of this receptor in splanchnic vasculature promotes portal inflow to

contribute to development of portal hypertension Moreover, the splanchnic effects of MasR required nitric oxide. Interestingly, Ang-(1–7) also decreased hepatic resistance.

CONCLUSIONS: In the splanchnic vessels of patients and rats with cirrhosis, increased levels of ACE2 appear to increase production of Ang-(1–7); this leads to activation of MasR and splanchnic vasodilatation in rats. This mechanism could cause vascular hypocontractility in patients with cirrhosis, and might be a therapeutic target for portal hypertension.

Keywords: rat model, portal hypertension, BDL, haemodynamics

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Introduction

Portal hypertension is the major cause of morbidity and mortality in patients with cirrhosis. The development of portal hypertension and the porto-systemic collateral circulation results from an increase in hepatic vascular resistance and subsequent dilatation of the splanchnic vascular bed, together with an increase in portal inflow. These changes lead to activation of powerful vasoconstrictor mechanisms such as the sympathetic nervous system and the renin-angiotensin system (RAS) in an attempt to maintain systemic vascular filling and blood pressure [1]. However, this homeostatic response fails to restore normal haemodynamics due to the impairment of mesenteric vascular responsiveness to vasoconstrictors.

To date, the mechanisms responsible for this splanchnic vascular hypocontractility in cirrhosis have not been fully elucidated. Although angiotensin II (Ang II) aggravates hepatic fibrosis and increases intrahepatic resistance [2, 3], it fails to elicit adequate contraction of splanchnic vessels in human and experimental cirrhosis. It is now recognised that, in addition to the classical RAS pathway in which ACE generates the powerful vasoconstrictor Ang II, there is an alternate arm of the RAS via which Ang II is cleaved by the angiotensin-converting enzyme (ACE) homologue, angiotensin-converting enzyme-2 (ACE2) to angiotensin-(1-7) (Ang-(1-7)), which stimulates the Mas-receptor (MasR) to cause vasodilatation [4-6]. Recent studies have shown that hepatic ACE2 and MasR expression are upregulated in cirrhosis and high levels of Ang-(1-7) can be found in the systemic circulation of patients with cirrhosis [7-10]. Here, we show for the first time that there is upregulation of components of the alternate pathway of RAS (ACE2/Ang-(1-7)/MasR) in splanchnic vessels in experimental and

human cirrhosis, and that Ang-(1-7) mediates, via MasR, splanchnic vasodilatation and contributes to portal hypertension in two models of experimental cirrhosis. These findings reveal a new pathogenic mechanism explaining splanchnic vascular dysfunction in cirrhosis.

Experimental procedures

Human samples

Experimental procedures to obtain human vessels were approved by the Human Ethics Committee of the University of Bonn 2002/01. During liver transplantation, samples of hepatic arteries from patients with alcohol-induced cirrhosis were obtained (n=7) and compared to hepatic artery samples from non-cirrhotic organ donors, which served as controls (n=7). Liver samples were obtained from cirrhotic liver explants (n=8) and compared to healthy liver samples from patients undergoing other surgery (n=10).

Animals

Experimental procedures to obtain animals vessels were approved by Austin Health Animal Ethics Committee (AEC2010/04093 and AEC2009/04093) and animal research ethics committee for the University of Bonn (LANUV NRW, 8.87-50.10.31.08.287), and performed according to the principles of the Helsinki declaration. Details of animal models of cirrhosis can be found in supplementary methods. Liver function tests in the samples of BDL rats are described in Supplementary Table 2. The perfused mesenteric vascular bed preparation used in BDL rats was a modification of that originally described by McGregor [11]. Please see Supporting Methods for detailed description of surgical technique.

Ex-vivo perfused mesenteric vascular bed experiments

***Ex-vivo* Angiotensin peptide metabolism protocols:** The detailed protocol is described in the supplemental material. Briefly, following an equilibration phase of 15 min, Ang I (99 pmole bolus in 200 µL) was injected into the superior mesenteric

artery and effluent was collected at defined time points. The preparation was thoroughly flushed out with Krebs-Henseleit solution for a further 15 min and then Ang II (60 pmole bolus in 200 µL) was injected and effluent collected in a similar manner. Two experimental protocols were performed: without RAS inhibition, and in the presence of the ACE2 inhibitor, MLN-4760 (1×10^{-6} mol/L), each protocol contained 5-8 controls and 6-8 cirrhotic animals. Radioimmunoassay for Ang-(1-7) was performed as described in the supplemental material and previously [12].

***Ex-vivo* Mesenteric vascular resistance protocols:** The detailed method is described in the supplemental material. Briefly, vasoconstrictor responses to methoxamine were examined in control and cirrhotic animals with and without Ang-(1-7) (1×10^{-7} mol/L). The response to Ang-(1-7) in cirrhotic animals was further examined with A779, a MasR antagonist (1×10^{-7} mol/L), candesartan, an Ang II type 1 receptor (AT1R) blocker (1×10^{-6} mol/L), PD123319, an AT2R blocker (1×10^{-6} mol/L) and NG-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase (NOS) inhibitor (1×10^{-4} mol/L).

In-vivo haemodynamic experiments

In-vivo studies were performed in rats under ketamine anaesthesia (60mg/kg i.m.) as described in the supplemental material and previously [13]. Briefly, two different protocols were performed:

***In vivo* Ang-(1-7) protocol:** After stabilization, invasive measurement of PP and MAP and assessment of regional blood flows were preformed by application of microsphere technique. Thereafter, increasing doses (1, 10, 30 µg/kg bw) of Ang-(1-7) were administered intravenously, followed by application of the microsphere technique using red and white microspheres.

In vivo A779 protocol: Similarly to the previous protocol, PP, MAP and regional blood flows were assessed before and after i.v. injection of 10 μg/kg bw of A779.

qPCR analysis of RAS gene expression in rat and human vessels

Total RNA was extracted from mesenteric arterial beds from cirrhotic and control rats and human hepatic arteries from donors and recipients using TRI reagent (Sigma Aldrich, Sydney, Australia). Quantitative real time polymerase chain reactions (qPCR) were carried out using multiplexing as previously described [10]. The details of oligonucleotide probes and primers used in rat tissue are given in Supplementary Table 1. For human arteries, ready to use mix was used (hACE2: Hs01085333_m1; hACE: Hs00174179_m1; hMasR: Hs00267157_s1; hAT1R: Hs01096942_m1; hAngiotensinogen: Hs01586213_m1).

Western blotting and immunohistochemistry

Rat mesenteric arterial vascular beds and human hepatic arteries were subjected to Western blot analysis as described previously [13, 14]. Details of antibodies used are given in supplementary methods. β-actin and GADPH were used as loading controls. Blots were developed with enhanced chemiluminescence (ECL, Amersham, UK). Intensities of the digitally detected bands were evaluated densitometrically using Chemi-Smart (PeqLab, Biotechnologies, Erlangen, Germany).

Human liver samples were embedded in Tissue Tack (tissue freezing Medium, Leica Microsystems, Nussloch, Germany) and cryosections of 7μm were made by using the Cryostat HM 560 (Microm, Walldorf, Germany). The sections were transferred to object slides (Gerhard Menzel, Braunschweig, Germany) and dried over night at room temperature. The slides were stored at -20°C until further use. Sections were

incubated with primary and secondary antibodies as detailed in supplementary methods and fluorescence images were obtained with an Axio Observer A1 microscope fitted with a LD-Neofluar 40x and an Axio camera ICC1 (Zeiss, Jena, Germany) using the Axiovision Software (Release 4.8).

Statistics

Results are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated. Data were analysed using Student's unpaired and paired *t* test, and repeated measures ANOVA where appropriate. Data that violated the assumption of equal variance were log transformed prior to analysis. Statistical analyses and graphing were performed using GraphPad Prism 4.0 for Macintosh. $P < .05$ was considered statistically significant.

Results

Expression of MasR and ACE2 is increased in splanchnic vascular wall in cirrhotic rats and humans

The Ang-(1-7) receptor, MasR, protein was significantly increased in mesenteric arterial vessels from cirrhotic BDL rats (Figure 1A). The findings were similar in human hepatic arteries, where transplant recipient vessels (cirrhotic vessels) contained more MasR protein compared to non-cirrhotic donor vessels (control vessels) (Figure 1A).

In contrast, expression of AT1R in splanchnic vessels from cirrhotic animals and cirrhotic patients was not significantly different from control ($P = .48$ and $P = .20$ compared to respective controls). However, AT2R expression was increased by greater than three-fold in splanchnic vessels of cirrhotic animals (1.00 ± 0.2258 control vs 3.437 ± 1.102 cirrhotic, $P = .0244$).

In cirrhotic BDL animals there was significant upregulation of splanchnic vascular ACE2 expression, which was increased approximately five-fold compared to its expression in control vessels (Figure 1B). ACE2 was also increased in human cirrhotic vessels compared to controls (Figure 1B). In contrast to ACE2, expression of ACE was unchanged in rat and human cirrhotic vessels ($P = .51$ and $P = .60$ compared to respective controls).

Hepatic expression of MasR and ACE2 is increased in cirrhosis in humans

MasR protein was increased 2-fold in human cirrhotic liver (Figure 1C). Immunofluorescence demonstrated increased staining of MasR in human cirrhosis, which was restricted to the nucleus in control liver, but in cirrhosis was predominantly located in the cytoplasm (Figure 1C). There was no co-localisation of MasR with a-SMA in cirrhosis, but MasR staining showed a close relationship to a-SMA positive cells, implying that it is not expressed on myofibroblasts, and consistent with expression on endothelial cells. ACE2 expression was minimal in controls, but was substantially increased in human cirrhotic livers, and similarly to MasR, there was increased translocation to the cytoplasm in cirrhosis (Figure 1C).

Splanchnic vascular Ang-(1-7) formation is increased in cirrhosis due to ACE2 upregulation

There was brisk metabolism of both Ang I and Ang II in the perfused splanchnic circulation. Following bolus injection of Ang II, five times the quantity of Ang-(1-7) was formed in the splanchnic vessels of cirrhotic animals compared to controls (Figure 2A, B). We hypothesised that increased ACE2 within the splanchnic vascular wall was responsible for augmented formation of Ang-(1-7) in cirrhosis. This was supported by the finding that specific ACE2 inhibition with MLN-4760 markedly reduced the formation of Ang-(1-7) from Ang II in the vessels of cirrhotic animals, but had no significant effect in controls (Figure 2A, B) Furthermore, ACE2 inhibition completely abolished the difference in Ang-(1-7) production between control and cirrhotic vascular beds.

There was also significantly more Ang-(1-7) produced from Ang I in cirrhotic animals (Figure 2C, D), which was again clearly abrogated by ACE2 inhibition in the cirrhotic vascular bed. In contrast, ACE2 inhibition had no effect in controls.

Ang-(1-7) reduces vascular tone in the mesenteric vascular bed of cirrhotic rats

To investigate the functional role of Ang-(1-7) in the splanchnic vascular bed, we performed *ex-vivo* mesenteric perfusion studies in cirrhotic and control rats. As expected, methoxamine elicited an attenuated increase in perfusion pressure in cirrhotic vessels compared to controls (Figure 3A). Pre-incubation with Ang-(1-7) had no effect on vascular contractility in controls, but further blunted the methoxamine response in cirrhotic vessels (Figure 3A). A dose-response effect was observed to Ang-(1-7) in cirrhotic vessels, with a modest attenuation of mesenteric contractility observed at 1×10^{-9} mol/L and a significant greater attenuation at the higher dose of 1×10^{-7} mol/L (Pressure response to highest dose of methoxamine 55.42 ± 13.52 mm Hg at 1×10^{-9} mol/L Ang-(1-7) vs 42.86 ± 11.26 mm Hg at 1×10^{-7} mol/L Ang-(1-7) compared to 75.14 ± 7.192 mm Hg in the absence of Ang-(1-7)).

The vasodilatory response to Ang-(1-7) in cirrhosis is mediated by MasR

The MasR antagonist, A779, completely reversed the effect of Ang-(1-7) in cirrhotic vessels at all doses of methoxamine greater than 3×10^{-6} mol/L (Figure 3B). The AT2R blocker, PD123319, also significantly reduced the effects of Ang-(1-7) at 1×10^{-5}mol/L, 3×10^{-5} mol/L and 1×10^{-4} mol/L of methoxamine, without reaching statistical significance ($P = .077$) for the highest dose of methoxamine (Figure 3C). In contrast, AT1R blockade with candesartan did not have significant effects at any dose

of methoxamine (Figure 3D). None of the receptor blockers changed the baseline vascular tone of the preparation.

MasR blockade improves splanchnic vascular resistance, whereas Ang-(1-7) exacerbates splanchnic vasodilatation in cirrhosis

As expected, baseline splanchnic vascular resistance (SpVR) was lower and hepatic vascular resistance (HVR) and PP higher in the cirrhotic rats compared to sham (Figure 4). In keeping with our mesenteric perfusion data, Ang-(1-7) caused a significant reduction in SpVR in both models of experimental cirrhosis (Figure 4A). Moreover, Ang-(1-7) attenuated the high HVR in BDL rats, reducing it to sham control levels (Figure 4B). A similar finding, although less pronounced, was observed in CCl_4 rats (Figure 4B). The net effect of these changes was that, despite exacerbating splanchnic vasodilatation in cirrhosis, Ang-(1-7) significantly reduced PP in cirrhotic animals, with identical effects being observed in rats with cirrhosis due to BDL or CCl_4 (Figure 4C). In control animals no haemodynamic changes were observed (Figure 4A-C), suggesting that Ang-(1-7) has an effect that occurs specifically in liver cirrhosis. Details of haemodynamic changes following Ang-(1-7) are given in Supplementary Table 3.

To further investigate the relationship between PP and Ang-(1-7), we performed dose-response experiments in cirrhotic and control animals. Ang-(1-7) caused a significant dose-dependent reduction in PP in cirrhotic but not control animals (Figure 5). Interestingly, no significant changes were observed in the systemic circulation,

underlining that the site of action of Ang-(1-7) is the splanchnic and hepatic vessels (Figure 5B).

MasR blockade had beneficial effects on the splanchnic circulation in both groups of cirrhotic animals, counteracting pathological splanchnic vasodilatation in cirrhosis and elevating the SpVR to control levels (Figure 4D). In keeping with our results in Ang-(1-7) *in-vivo* experiments, MasR blockade produced a trend (P =.11) towards increased HVR in BDL rats (Figure 4E). However, there was no effect on hepatic resistance in CCl_4 rats (Figure 4E). Interestingly, MasR blockade significantly increased HVR in control rats (Figure 4E).

In CCl_4 animals, beneficial effects of MasR blockade in the splanchnic circulation in the absence of adverse effects in the hepatic circulation produced a significant improvement in PP, reducing it from mean 17.3 mmHg to mean 14.4 mmHg, but had no effect on portal pressure in controls or BDL animals (Figure 4F). Details of haemodynamic changes following A779 are given in Supplementary Table 4. There were no statistically significant differences in liver function tests between the different BDL groups.

The effects of Ang-(1-7) in the splanchnic vascular bed in cirrhosis are mediated by nitric oxide

The effects of physiological Ang-(1-7) and MasR stimulation have previously been reported to be due to NO release [15]. To determine whether this is also true in cirrhosis, we measured mesenteric arterial eNOS and iNOS protein following Ang-(1-7). eNOS phosphorylation was increased, particularly in BDL animals, following exposure to

Ang-(1-7) (Figure 6A). Total eNOS was unchanged, whereas iNOS expression was stimulated by Ang-(1-7) in BDL animals (Figure 6B). As a marker of the NO-effector protein kinase G (PKG) activity, we determined phosphorylation of the PKG substrate, vasodilator stimulated phospho-protein (VASP). There was increased VASP phosphorylation in mesenteric arteries of cirrhotic rats when stimulated by Ang-(1-7) (Figure 6C). Functional evidence that the effects of Ang-(1-7) are mediated by NO in cirrhosis was provided by perfusion studies, where, in conditions of NOS inhibition, Ang-(1-7) had no effect on mesenteric contractility (Figure 6D). L-NAME greatly increased the contractility of mesenteric vascular beds in cirrhotic animals, consistent with previous reports [16].

Discussion

This study uncovers a new pathophysiological mechanism of splanchnic vasodilatation, which contributes to portal hypertension in cirrhosis (Figure 7). Our results demonstrate that, in experimental and human cirrhosis, the expression and activity of splanchnic vascular ACE2 are increased. Consequently, local production of the vasodilator and MasR-agonist, Ang-(1-7), is elevated. Furthermore, locally increased production of Ang-(1-7), together with augmented splanchnic vascular expression of MasR, lead to an NO-dependent increase in portal vein inflow, a hallmark of portal hypertension. Thus, our findings suggest that intramural activation of ACE2/Ang-(1-7)/MasR axis in splanchnic vessels contributes to increased splanchnic blood flow and the development of portal hypertension.

A central tenet of current understanding is that there is activation of the classical ACE and Ang II-dependent arm of the RAS in patients with advanced liver disease and portal hypertension in response to vasodilatation and systemic hypotension [17]. However, recent research shows that, in addition to hepatic upregulation of this system, which mediates fibrosis and stellate cell contraction, the alternate ACE2-dependent arm of the intrahepatic RAS is also upregulated in experimental cirrhosis, and probably represents a regulatory mechanism that opposes the effects of Ang II in an autocrine or paracrine fashion [7, 9]. In the present study we demonstrate that splanchnic vessels in cirrhosis are highly active angiotensin peptide-metabolising tissues (Figure 2). This is consistent with the observation that, in cirrhotic patients at liver transplantation, the Ang-(1-7)/Ang II ratio is elevated in the splanchnic compared to the peripheral circulation, and splanchnic but not systemic angiotensin

peptide levels negatively correlate with systemic vascular resistance [8]. These previously published data, which strongly support our hypothesis, are complementary to our data and underline our suggested mechanism.

The results of the current study provide evidence that this increase in splanchnic Ang-(1-7) results from increased vascular expression of endothelial ACE2 [5, 18]. We found increased ACE2 protein in the mesenteric vasculature of cirrhotic animals, and in human hepatic arteries from liver transplant recipients. This corresponded to a functional increase in splanchnic ACE2 activity, as demonstrated by enhanced, ACE2-dependent, breakdown of Ang II to Ang-(1-7). These data indicate that increased splanchnic vascular expression of ACE2 in cirrhosis reduces the regional concentration of Ang II whilst increasing Ang-(1-7). Both of these effects might be expected to reduce vascular tone. Splanchnic hyporeactivity to Ang II in cirrhosis has been consistently documented both in animals and humans [19, 20]. In addition to other reported mechanisms such as increased AT1R desensitization [19 21], these data suggest that hypocontractility to Ang II might be at least partly explained by increased intramural local degradation of the endogenous vasoconstrictor Ang II to the vasodilator Ang-(1-7).

In this study we have demonstrated important effects of Ang-(1-7) in the splanchnic circulation, which are evident only in the setting of cirrhosis. While, consistent with previous findings [22], Ang-(1-7) elicited no measurable effect in normal vessels, it inhibited the contraction of cirrhotic vessels in a dose-dependent fashion, reflecting upregulation of MasR, ACE2 and MasR-dependent signalling pathways in these vessels. Our findings suggest that these effects of Ang-(1-7) in cirrhosis are mediated

by MasR, as demonstrated by the ability of the specific MasR blocker, A779, to inhibit the action of Ang-(1-7). The MasR is a G protein-coupled receptor, identified as an Ang-(1-7) receptor by Santos and colleagues in 2003 [23], and constitutively expressed on human endothelial cells [15]. Mas RNA has been identified in the testes, heart and kidney [24], in mesenteric microvessels [25], and in the cardiovascular areas of the brain [26]. The effects of Ang-(1-7)/MasR might be mediated by Gs protein and protein kinase (PK)A[27], which are known to stimulate eNOS-phosphorylation and NO-production [28, 29]. Indeed, Ang-(1-7), via MasR, has been shown to activate Akt-dependent pathways including eNOS phosphorylation and NO release [15]. This is consistent with our finding of increased eNOS phosphorylation after Ang-(1-7), which may be due to Gs/PKA or Akt stimulation. In addition, we found that iNOS expression was increased following Ang-(1-7) in BDL animals only. Whereas eNOS is known to be an important regulator of splanchnic vasodilatation in cirrhosis, the role of iNOS is not as clear. There is evidence that stimulation of splanchnic vascular iNOS expression may become more important in advanced cirrhosis, and this may explain why iNOS expression was increased in the BDL but not the CCl_4 model of cirrhosis [30]. In the present study, increased eNOS phosphorylation and iNOS expression in cirrhotic vessels after stimulation of MasR with Ang-(1-7) was accompanied by increased activity of PKG, a NO-effector, demonstrated by the phosphorylation of its substrate, VASP. This is consistent with increased NO production in these vessels. Furthermore, Ang-(1-7) vasodilation was completely eliminated in the presence of NOS inhibition, supporting the hypothesis that it is mediated by a NOS-dependent mechanism.

We also found that blockade of AT2R, the expression of which was also increased in cirrhosis, attenuated the effects of Ang-(1-7). This is consistent with previous findings showing that AT2R inhibition affects Ang-(1-7) activity, both *in-vitro* [31] and *in-vivo* [32], due to unknown mechanisms. Neither AT1R nor AT2R blockers are ligands for MasR [23], and Ang-(1-7) has very low affinity for both Ang II receptors [33]. Our results support the previously described concept of an interaction between MasR and AT2R, either due to receptor hetero-dimerisation or alterations in post-receptor signalling [25, 32]. The effects of AT2R inhibition might be more apparent in BDL vessels due to significantly increased expression of this receptor in this model. There was no significant abrogation of Ang-(1-7)-mediated vasodilatation in the presence of candesartan (Figure 3D), suggesting that there is no major cross talk between the Mas and AT1 receptors. Unfortunately it was not possible to study the effects of MasR blockade on AT1R as, during preliminary experiments, vasoconstriction responses to Ang II in cirrhotic vessels were small and non-reproducable. Tachyphylaxis to Ang II is well documented [34, 35], and is thought to be due to AT1R internalisation [36].

Our *in-vivo* studies confirm the pathophysiological role of Ang-(1-7) and MasR suggested by the *ex-vivo* findings. Firstly, they demonstrate that Ang-(1-7) reduces SpVR in cirrhotic animals, whereas MasR blockade has the opposite effect. These data strongly suggest that Ang (1-7), acting via the MasR, contributes towards splanchnic vasodilatation in cirrhosis, presumably due to increased expression of MasR. Our finding that MasR blockade increased mesenteric vascular tone by more than 100% in both animal models (Figure 4D) and in fact returned it to near control levels in BDL animals, suggests that this novel pathway plays a major role in mesenteric vasodilatation in cirrhosis and that its blockade might lower PP by

ameliorating splanchnic vasodilatation. Indeed, we found that it reduced PP by nearly 20% in CCl_4 animals. This degree of improvement in PP is more than that seen clinically with AT1R blockade and similar to that produced by beta blockers[37], suggesting that MasR blockers could provide a novel therapeutic strategy.

One aspect of this therapeutic approach would be concomitant haemodynamic effects on the hepatic circulation. In cirrhotic animals, Ang-(1-7) reduced HVR, and therefore, despite the previously noted adverse effects on the splanchnic circulation, PP decreased in a dose-dependent fashion. We have previously demonstrated that hepatic MasR expression is greatly increased in BDL cirrhosis [10], and the present study confirms this in humans, and also demonstrates increased cytoplasmic translocation in cirrhosis. Consistent with this finding, HVR was increased in BDL animals following MasR blockade. By contrast, MasR blockade had no effect on liver haemodynamics in animals with cirrhosis induced by CCl4. Nonetheless, increased portal delivery of Ang-(1-7) to the liver as a result of augmented splanchnic ACE2 activity, might be a compensatory, albeit inadequate, mechanism to counteract hepatic vasoconstriction in cirrhosis.

Thus our findings demonstrate potentially conflicting effects of strategies targeting the MasR and suggest that these may vary in different forms of cirrhosis. Whilst MasR blockade reduces mesenteric hyper-perfusion in both models, it increases HVR and reduces hepatic artery flow in BDL cirrhosis, and this may have harmful effects. In contrast, in both models, Ang-(1-7) worsens mesenteric vasodilatation but lowers PP in association with reduced HVR and increased portal perfusion. Indeed, manipulation of this system may be more effective when used in combination with

other established therapies. For example, MasR blockers may improve the efficacy and tolerability of beta blockade in the treatment of portal hypertension by increasing splanchnic resistance and preventing systemic arterial hypotension. Similarly, by reducing vasodilatation, MasR blockade would be expected to increase the tolerability and efficacy of AT1R blockers.

In conclusion, our results uncover a mechanism by which the alternate ACE2-dependent axis of the RAS plays a role in the pathophysiology of splanchnic hyperperfusion, and thereby of portal hypertension in cirrhosis. This system is a potential target for the treatment of portal hypertension. Manipulation of the RAS in the form of AT1R blockade is of proven benefit in the reduction of PP by blocking the vasoconstrictive effects of Ang II in the liver [3, 12], but its clinical use is severely limited by systemic effects such as hypotension, particularly in advanced cirrhosis [37, 38]. Selective modulation of MasR in splanchnic and hepatic tissue should be investigated as a means to reduce PP. Another avenue for pharmacological intervention is combination therapy with AT1R and MasR blockade, which could have synergistic beneficial effects on liver and splanchnic haemodynamics.

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Figure legends

Figure 1 Expression of Mas receptor (MasR) (Panel A) and ACE2 (Panel B) in mesenteric arteries of cirrhotic animals (BDL) (n=18) compared to controls (n=13) and in hepatic arteries of cirrhotic human liver transplant recipients (n=9) compared to non-cirrhotic donors (n=7). Panel C depicts MasR and ACE2 expression in human cirrhotic liver (n=10) compared to healthy control liver (n=8) **P* < .05, ***P* < .01, ****P*< .005 vs control. Where non-contiguous blots, run on the same gel, have been spliced together, a white divider has been inserted. MasR blots were stripped before reprobing for control protein. Upper panels in immunofluorescence are costained with 4',6-diamidino-2-phenylindole (DAPI) in blue and the target protein in red; lower panels demonstrate additional costaining with alpha-smooth muscle actin (a-SMA) in green. Data are shown as means + SEM.

Figure 2 Formation of angiotensin-(1-7) (Ang-(1-7)) in control (closed triangles) and cirrhotic (closed squares) splanchnic vascular beds, and following incubation with MLN-4760 (1 x 10^{-6} mol/L), a specific ACE2 inhibitor (ACE2i) (open triangles and squares).

Panels A and C show the concentration of Ang-(1-7) in portal effluent at time points relative to (A) 60 pmole bolus Ang II or (C) 99 pmole bolus Ang I. **P* < .05, ***P* < .01, ****P* < .0001 cirrhotic vs control; #*P*< .05 cirrhotic vs cirrhotic with ACE2i. Panels B and D show the total amount of Ang-(1-7) produced as calculated from the area under the curve (AUC). **P* < .05, ***P* < .01 between groups indicated by bracket. n = 6-8 in each group. Data are shown as mean + SEM.

Figure 3 The effect of angiotensin-(1-7) (Ang-(1-7)) on mesenteric vascular contractility in cirrhotic BDL and control rats. A) Pressure response to methoxamine following incubation with vehicle or Ang-(1-7) in control and cirrhotic vessels. **P* < .05, cirrhotic with vehicle vs cirrhotic with Ang-(1-7); #*P* < .05, ##*P* < .01, control with vehicle vs cirrhotic with vehicle. n = 9-10 in control groups, n = 6 in cirrhotic groups. **B-D)** the effect in cirrhotic vessels of **B)** MasR antagonist, A779 (1 x 10^{-7} mol/L), **C)** AT2R antagonist, PD123319 (1 x 10^{-6} mol/L) and **D)** AT1R antagonist, candesartan (1 x 10^{-6} mol/L) on the effect of Ang-(1-7) **P* < .05, ***P* < .01, RAS blockade group vs Ang-(1-7) group. n = 6-7 in each group. Data are shown as means + SEM.

Figure 4 *In-vivo* haemodynamic changes in rats with cirrhosis induced by bile duct ligation (BDL), or CCl_4 intoxication compared to sham, in response to Ang-(1-7) (10µg/kg) (Panels A-C) or MasR blockade (A779 10µg/kg) (Panels D-F). **P* < .05, ***P* < .01, ****P* < .005 between groups enclosed by bracket; xxx *P* < .005 BDL after Ang-(1-7) vs BDL before; % *P* < .05 CCl_4 after Ang-(1-7) vs CCl_4 before. n = 6-7 sham, 8-9 BDL, 7-10 CCl_4. Data are shown as mean + SEM.

Figure 5 Portal pressure (A) and mean arterial pressure (B) in BDL, CCl_4 and control rats in response to increasing dose of Ang-(1-7) (1-30 µg/kg). *** P < .0001 BDL; # P < .05 CCl_4; ns=not significant by repeated measures ANOVA. n=9, control; 11, BDL; 8, CCl_4. Data are shown as means + SEM.

Figure 6 The mechanisms of Ang-(1-7)-mediated splanchnic vascular hypocontractility in cirrhosis. A-C) Expression of **A)** phosphorylated eNOS, **B**)

iNOS and **C**) phosphorylated VASP in mesenteric arteries of cirrhotic BDL and CCl_4 animals following Ang-(1-7) compared to cirrhotic controls *P < .05, **P < .01 vs control. N=3-15 per group. **D)** Pressure response to methoxamine following incubation with vehicle or Ang-(1-7) in BDL cirrhotic vessels, and following incubation with the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (1 x 10^{-4} mol/L). $$P$< .0001 vehicle vs L-NAME; #P< .0001 vehicle vs L-NAME + Ang-(1-7). N=7-9 in each group Data are shown as means + SEM.

Figure 7 The vascular intramural renin-angiotensin system in cirrhosis. In the left panel is depicted a non-cirrhotic splanchnic vessel, where the circulating and the locally active RAS are in equilibrium, regulating the vascular wall tone (balanced contraction and relaxation). In cirrhosis there is a shift towards increased generation of angiotensin-(1-7) due to augmented ACE-2 expression and activity. This, together with an upregulation of MasR, results in more generation of NO, which shifts the equilibrium towards more splanchnic vasodilatation, and is at least partly responsible for splanchnic hyperperfusion in portal hypertension.

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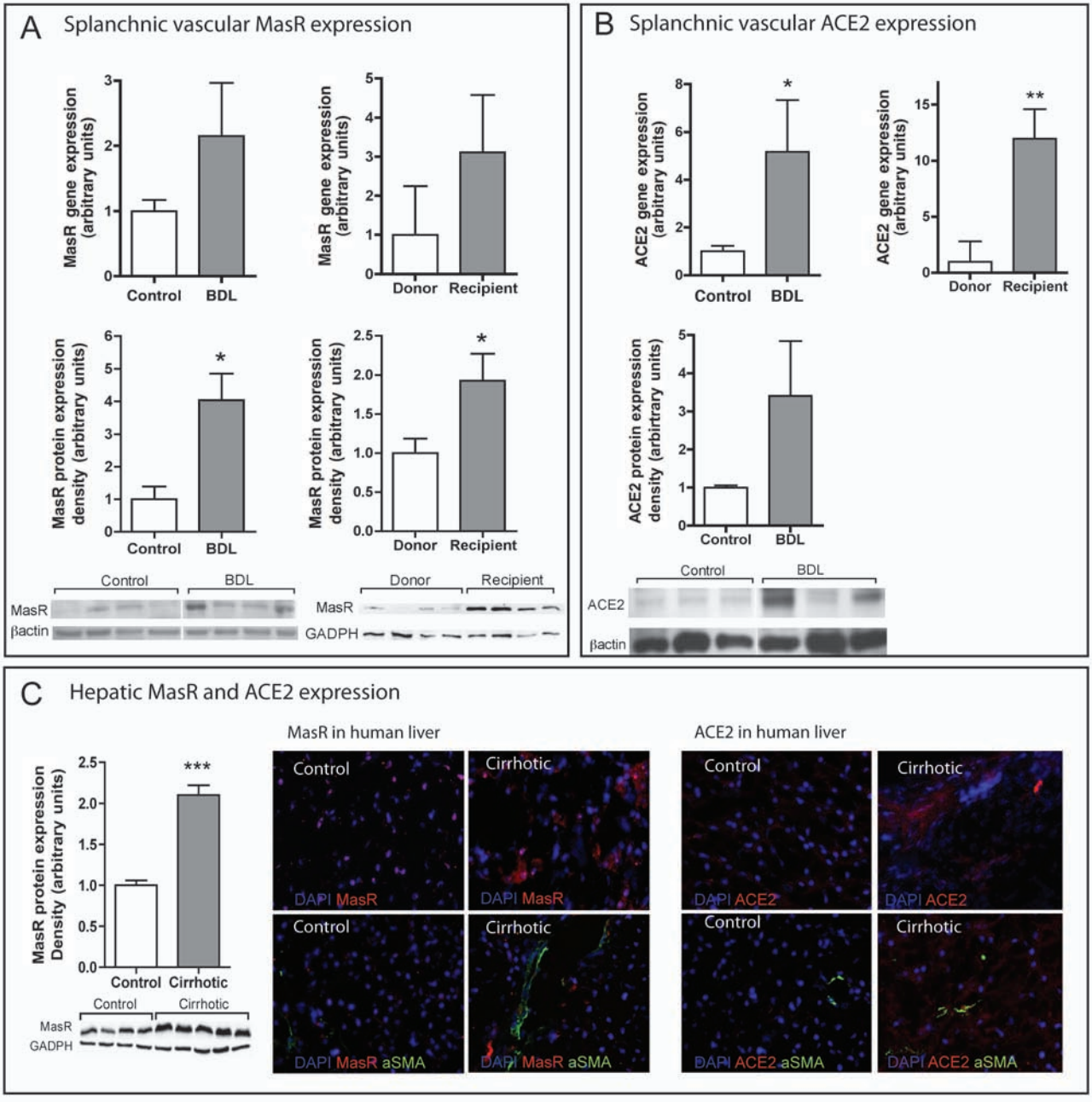
Author names in bold designate shared co-first authorship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Splanchnic vascular MasR expression
MasR gene expression (arbitrary units)
Control
BDL
MasR gene expression (arbitrary units)
Donor
Recipient
MasR protein expression density (arbitrary units)
*
Control
BDL
MasR protein expression density (arbitrary units)
*
Donor
Recipient
Control
BDL
MasR
βactin
Donor
Recipient
MasR
GADPH
B
Splanchnic vascular ACE2 expression
ACE2 gene expression (arbitrary units)
*
Control
BDL
ACE2 gene expression (arbitrary units)
**
Donor
Recipient
ACE2 protein expression density (arbitrartary units)
Control
BDL
Control
BDL
ACE2
βactin
C
Hepatic MasR and ACE2 expression
MasR protein expression Density (arbitrary units)
***
Control
Cirrhotic
Control
Cirrhotic
MasR
GADPH
MasR in human liver
Control
Cirrhotic
DAPI MasR
DAPI MasR
Control
Cirrhotic
DAPI MasR aSMA
DAPI MasR aSMA
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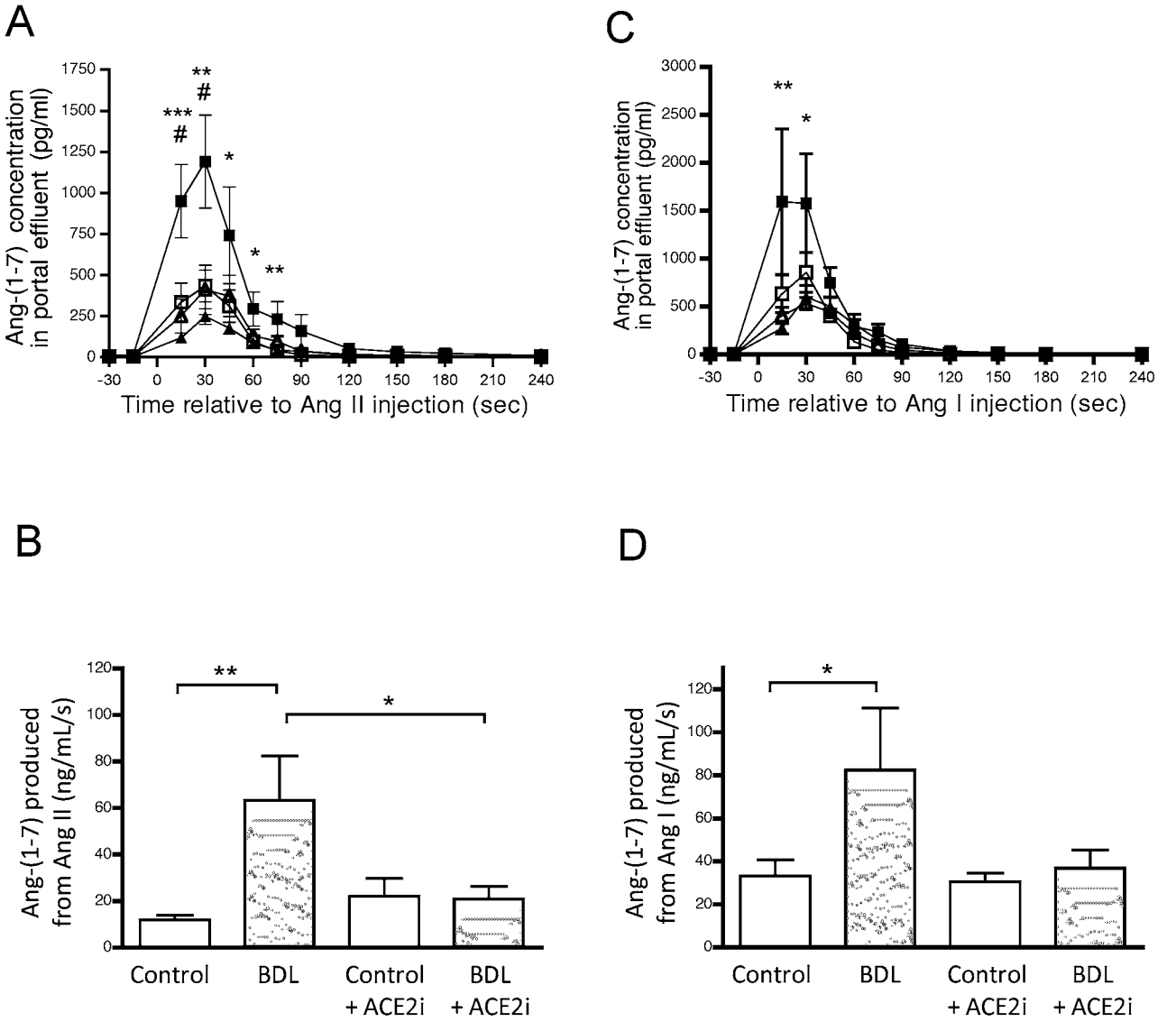
Control
Cirrhotic
DAPI ACE2 aSMA
DAPI ACE2 aSMA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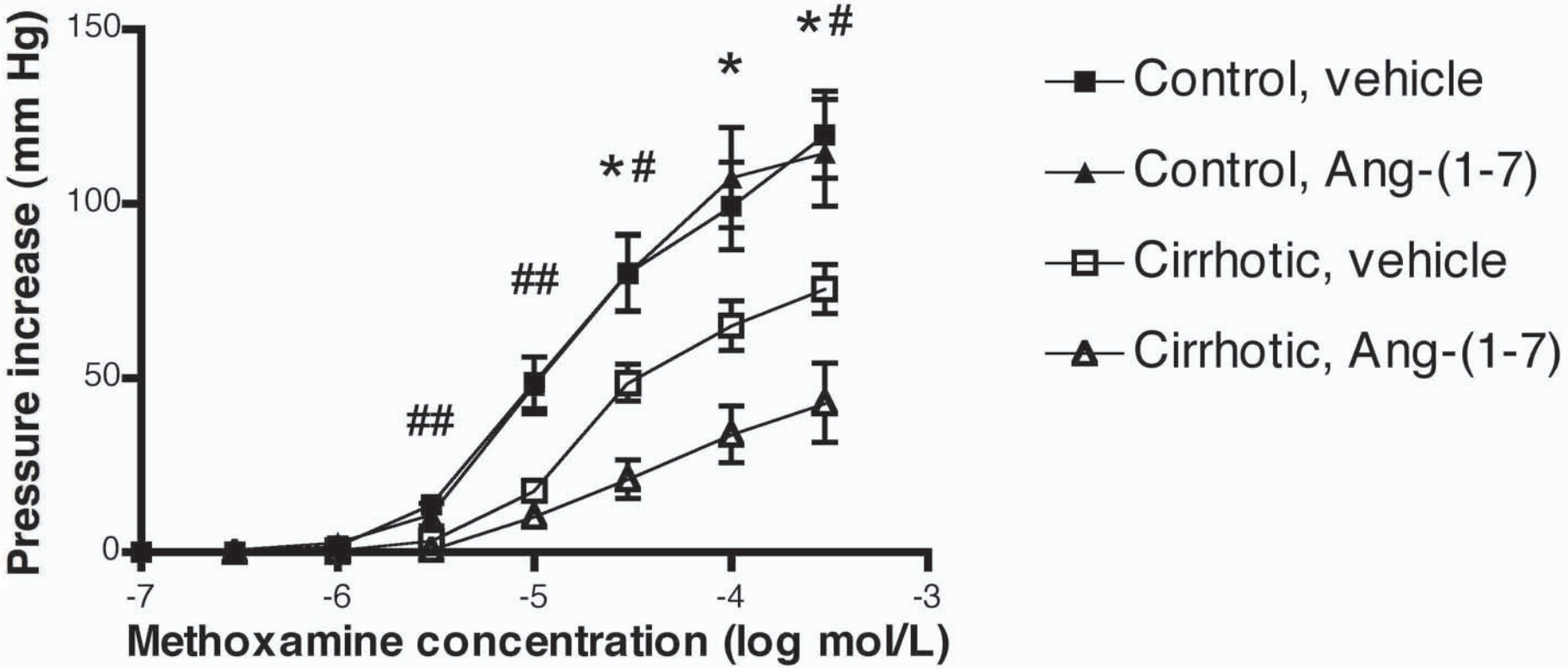
Ang-(1-7) concentration in portal effluent (pg/ml)
Time relative to Ang II injection (sec)
C
Ang-(1-7) concentration in portal effluent (pg/ml)
Time relative to Ang I injection (sec)
B
Ang-(1-7) produced from Ang II (ng/mL/s)
Control
BDL
Control + ACE2i
BDL + ACE2i
D
Ang-(1-7) produced from Ang I (ng/mL/s)
Control
BDL
Control + ACE2i
BDL + ACE2i

A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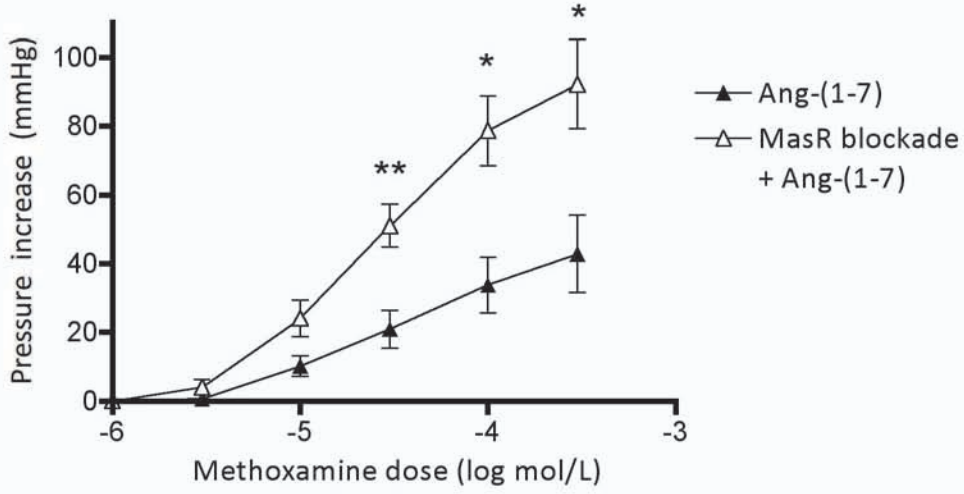
150
100
50
0
-7
-6
-5
-4
-3
Methoxamine concentration (log mol/L)
##
##
*#
*
*#
Control, vehicle
Control, Ang-(1-7)
Cirrhotic, vehicle
Cirrhotic, Ang-(1-7)

B


Pressure increase (mmHg)
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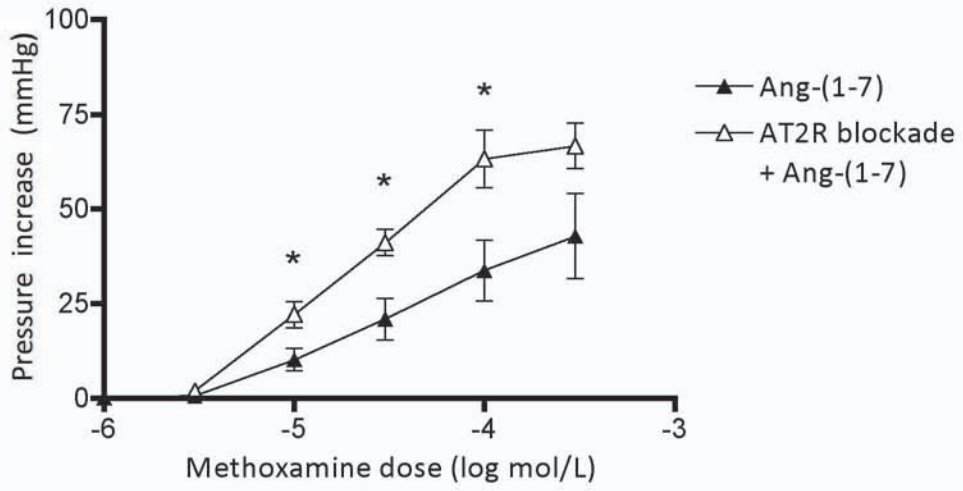
80
60
40
20
0
-6
-5
-4
-3
Methoxamine dose (log mol/L)
**
*
*
Ang-(1-7)
MasR blockade
+ Ang-(1-7)

C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75
50
25
0
-6
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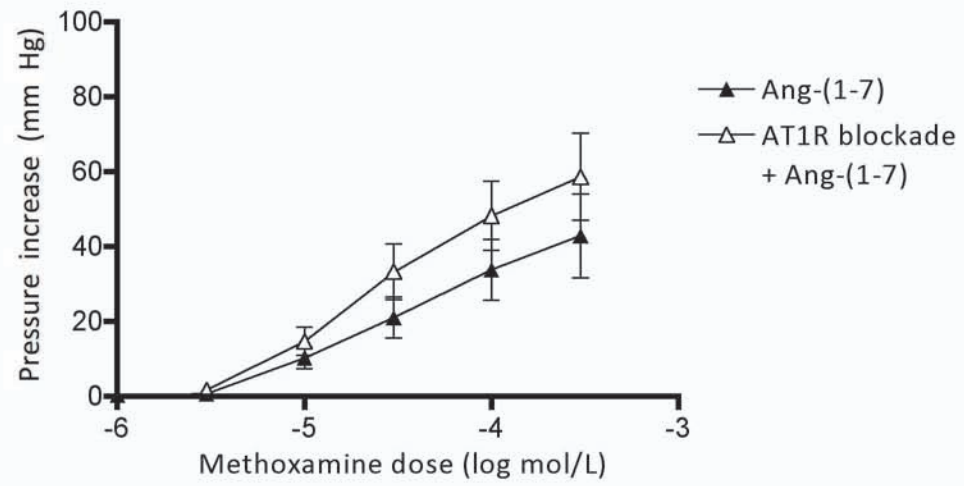
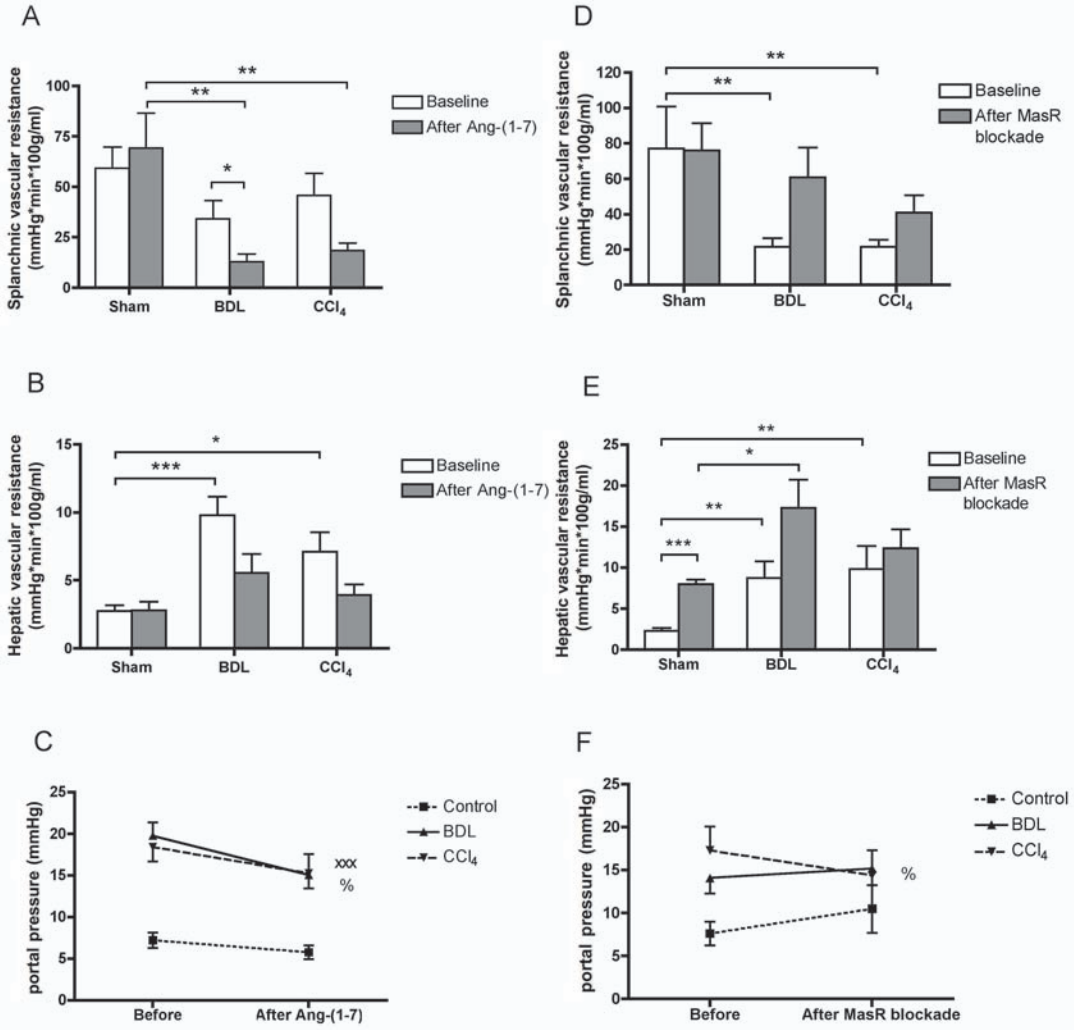
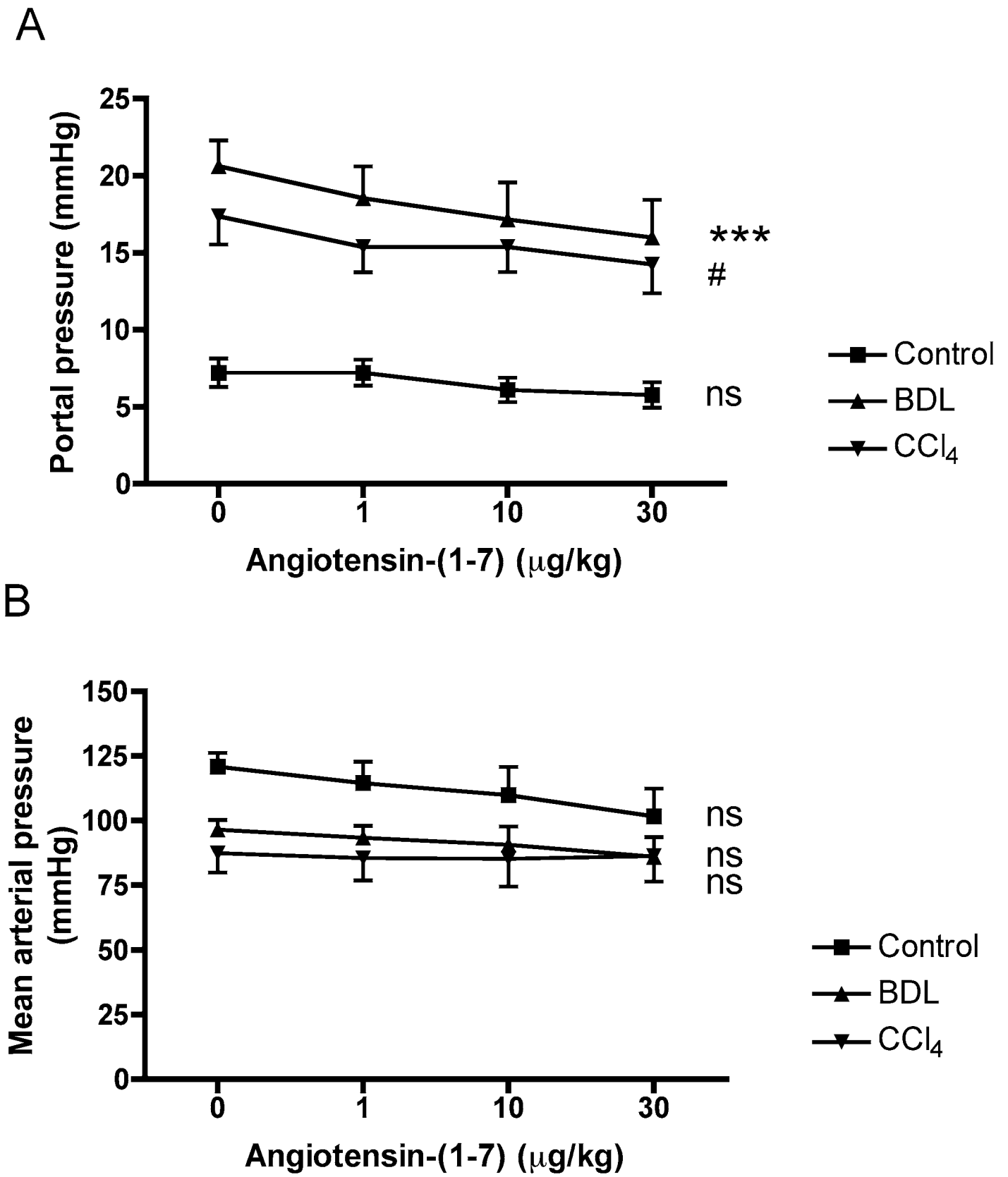
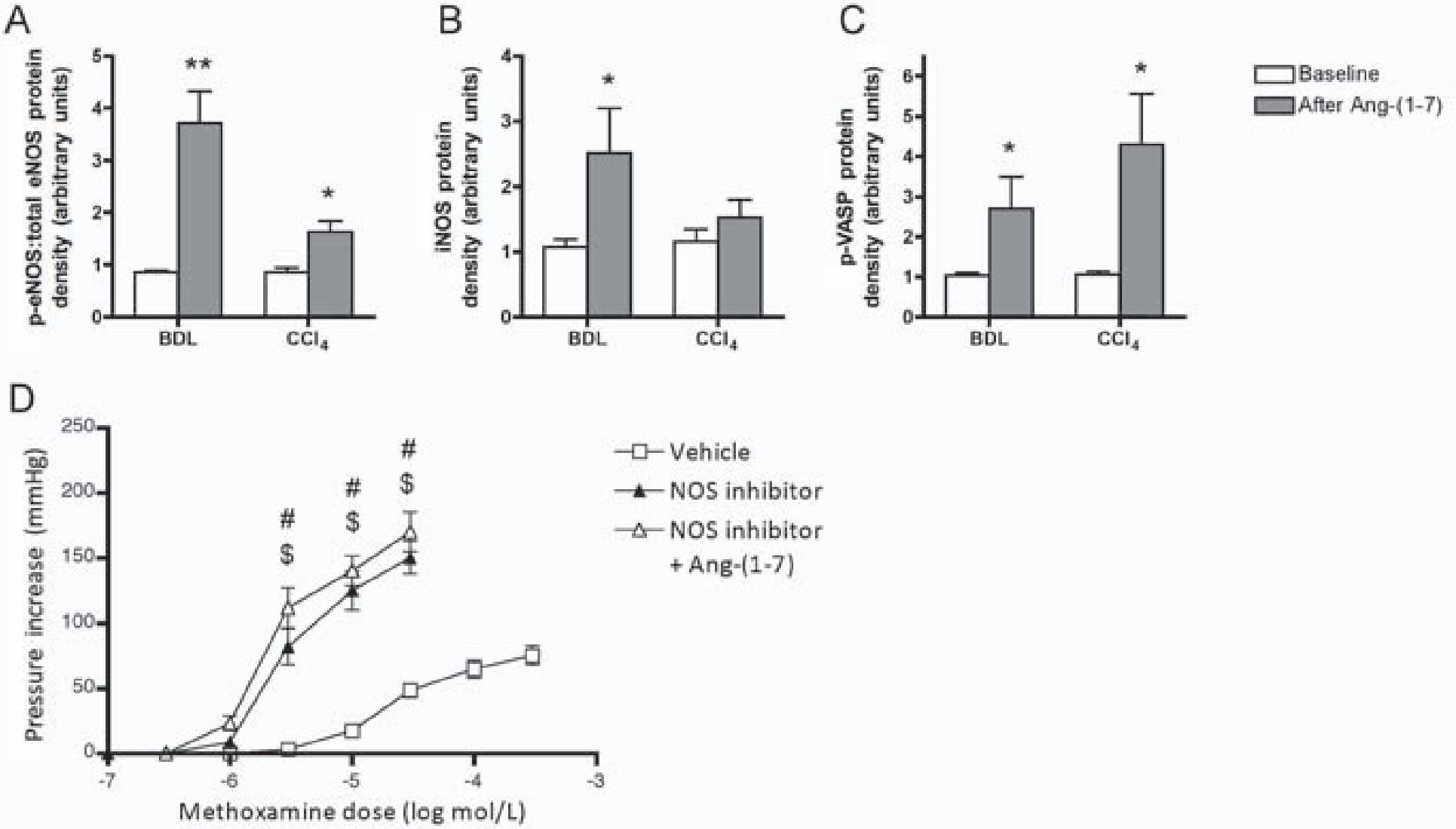
-3
Methoxamine dose (log mol/L)
*
*
*
Ang-(1-7)
AT2R blockade
+ Ang-(1-7)

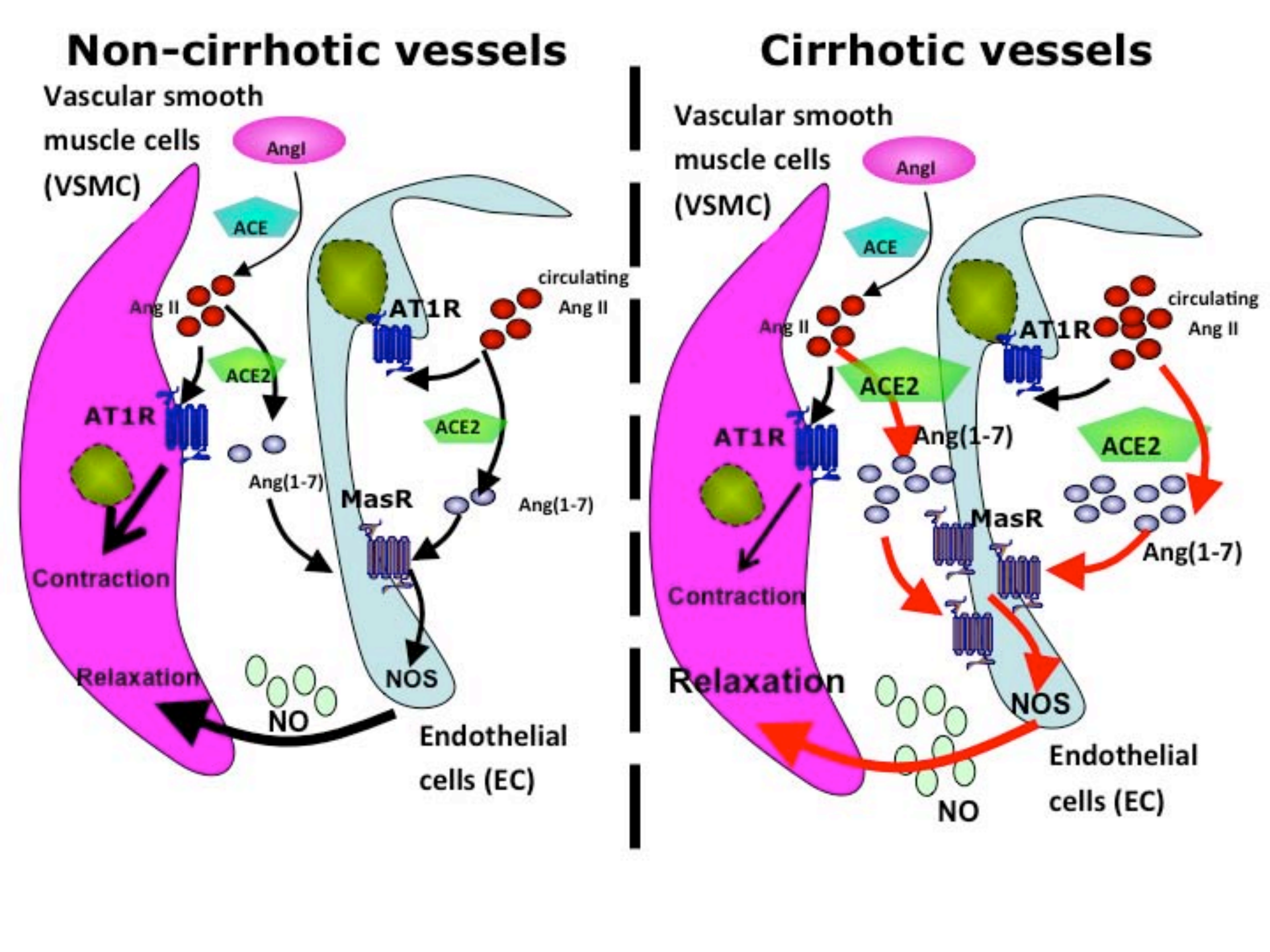
D


Pressure increase (mm Hg)
100
80
60
40
20
0
-6
-5
-4
-3
Methoxamine dose (log mol/L)
Ang-(1-7)
AT1R blockade
+ Ang-(1-7)


A
Splanchnic vascular resistance (mmHg*min*100g/ml)
100
75
50
25
0
**
**
*
Baseline
After Ang-(1-7)
Sham
BDL
CCl4
D
Splanchnic vascular resistance (mmHg*min*100g/ml)
120
100
80
60
40
20
0
**
**
Baseline
After MasR blockade
Sham
BDL
CCl4
B
Hepatic vascular resistance (mmHg*min*100g/ml)
15
10
5
0
*
***
Baseline
After Ang-(1-7)
Sham
BDL
CCl4
E
Hepatic vascular resistance (mmHg*min*100g/ml)
25
20
15
10
5
0
**
*
**
***
Baseline
After MasR blockade
Sham
BDL
CCl4
C
portal pressure (mmHg)
25
20
15
10
5
0
Control
BDL
CCl4
xxx
%
Before
After Ang-(1-7)
F
portal pressure (mmHg)
25
20
15
10
5
0
Control
BDL
CCl4
%
Before
After MasR blockade


A
Portal pressure (mmHg)
25
20
15
10
5
0
0
1
10
30
Angiotensin-(1-7) (μg/kg)
***
#
ns
Control
BDL
CCl4
B
Mean arterial pressure (mmHg)
150
125
100
75
50
25
0
0
1
10
30
Angiotensin-(1-7) (μg/kg)
ns
ns
ns
Control
BDL
CCl4


A
p-eNOS:total eNOS protein density (arbitrary units)
**
*
BDL
CCl4
B
iNOS protein density (arbitrary units)
*
BDL
CCl4
C
p-VASP protein density (arbitrary units)
*
*
BDL
CCl4
Baseline
After Ang-(1-7)
D
Pressure increase (mmHg)
Methoxamine dose (log mol/L)
Vehicle
NOS inhibitor
NOS inhibitor + Ang-(1-7)
#
$


Non-cirrhotic vessels
Vascular smooth muscle cells (VSMC)
AngI
ACE
Ang II
ACE2
AT1R
Ang(1-7)
Contraction
Relaxation
circulating Ang II
AT1R
ACE2
MasR
Ang(1-7)
NOS
NO
Endothelial cells (EC)
Cirrhotic vessels
Vascular smooth muscle cells (VSMC)
AngI
ACE
Ang II
ACE2
AT1R
Ang(1-7)
Contraction
Relaxation
circulating Ang II
AT1R
ACE2
MasR
Ang(1-7)
NOS
NO
Endothelial cells (EC)