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Angiotensin-(1-7) reduces the perfusion pressure response to angiotensin II and methoxamine via an endothelial nitric oxide mediated pathway in cirrhotic rat liver

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Running title: Angiotensin-(1-7) reduces perfusion pressure in cirrhotic liver

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Recent studies have shown that in cirrhosis portal angiotensin-(1-7) (Ang-(1-7)) levels are increased and hepatic expression of angiotensin converting enzyme 2 (ACE2) and the Mas receptor are up-regulated, but the effects of Ang-(1-7) on hepatic haemodynamics in cirrhosis have not been studied. This study investigated the effects of angiotensin-(1-7) (Ang-(1-7)) on vasoconstrictor-induced perfusion pressure increases in cirrhotic rat livers. Ang II or the alpha 1 agonist methoxamine (MTX) were injected in the presence or absence of Ang-(1-7) and the perfusion pressure response recorded. Denudation of vascular endothelial cells with sodium deoxycholate was used to investigate the contribution of endothelium to the effects of Ang-(1-7). Ang-(1-7) alone had no effect on perfusion pressure. However, it reduced the maximal vasoconstriction response and area under the pressure response curve to Ang II and MTX by more than 50% ($P<0.05$). This effect of Ang-(1-7) was not blocked by Mas receptor inhibition with A779 or by Ang II type 1 and type 2 receptor and bradykinin B_2 receptor blockade and was not reproduced by the Mas receptor agonist AVE0991. D-Pro^7-Ang-(1-7), a novel Ang-(1-7) receptor antagonist, completely abolished the vasodilatory effects of Ang-(1-7) as did inhibition of endothelial nitric oxide synthase (eNOS) with L-NAME, guanylate cyclase blockade with ODQ and endothelium denudation. The functional inhibition by D-Pro^7-Ang-(1-7) was accompanied by significant ($P < 0.05$) inhibition of eNOS phosphorylation. This study shows that Ang-(1-7) significantly inhibits intrahepatic vasoconstriction in response to key mediators of increased vascular and sinusoidal tone in cirrhosis via a receptor population present on the vascular endothelium that is sensitive to D-Pro^7-Ang-(1-7) and causes activation of eNOS and guanylate cyclase-dependent NO signalling pathways.

Keywords: Hepatic fibrosis, hepatic resistance, renin angiotensin system, vasodilatation

IN CIRRHOSIS, PORTAL HYPERTENSION results from increases in both intrahepatic vascular resistance and mesenteric blood flow (32). Much of the increase in intrahepatic resistance is due to fixed obstruction of the portal vascular bed resulting from tissue fibrosis and disturbance of the normal hepatic architecture. However there is also a dynamic component mediated by contraction of perivascular stellate cells, other myofibroblasts and portal vascular smooth muscle cells (23, 37). It is this dynamic component of intrahepatic resistance that is potentially amenable to pharmacological therapies.

There is considerable evidence to suggest that angiotensin II (Ang II) contributes significantly to the dynamic intrahepatic component of portal hypertension (23, 37). Circulating levels of Ang II are elevated in cirrhosis (21, 29), and Ang II induces the contraction and proliferation of hepatic stellate cells (2-4). Furthermore, we have shown that Ang II infusion greatly increases intrahepatic resistance to portal flow and that this response is increased in cirrhosis (20, 29). These findings help explain the results of clinical studies which have shown that both Ang II type 1 receptor (AT1R) blockers (ARBs) and angiotensin converting enzyme (ACE) inhibitors can reduce portal pressure in both experimental models of portal hypertension and human cirrhosis (19, 48, 49). However the use of these drugs has been limited by their adverse effects on blood pressure and renal perfusion in patients with advanced liver disease (48).

The classical view of the renin angiotensin system (RAS) is of a linear cascade in which ACE is a key enzyme, converting angiotensin I (Ang I) to the potent vasoconstrictor and profibrotic peptide Ang II, which acts via the AT1R. It is now known that there is alternate arm of the RAS in which ACE2, a homologue of ACE, degrades Ang II and generates angiotensin-(1-7) (Ang-(1-7)), which has a number of effects that appear to oppose those of Ang II. These effects are mediated in part by the Mas receptor, a novel endogenous G protein-coupled receptor (GPCR) (14, 22, 24, 34, 40, 47). This ACE2/Ang-(1-7)/Mas axis is

thought to intrinsically regulate the RAS system by reducing Ang II levels and producing Ang (1-7) thus counter-balancing the potentially harmful effects of Ang II. There is substantial evidence to suggest that Ang-(1-7) is anti-fibrotic in several cell types and tissues such as liver, heart, breast and the lungs (9, 17, 24, 29, 44). It has also been documented that Ang-(1-7) is a vasodilator in several vascular beds (5, 8, 28, 33, 40, 46).

As rats develop hepatic fibrosis, there is upregulation of all components of this alternate system in the liver, and a major increase in Ang-(1-7) levels (20, 21, 29) in conjunction with increased expression of classic RAS components. However the role of the alternate RAS in portal hypertension and the therapeutic potential of drugs targeting the alternate RAS in the management of portal hypertension has not been studied. Given the body of literature demonstrating its beneficial vasodilatory effects in cardiovascular physiology(43), in the current study, we investigated whether Ang-(1-7) could vasorelax the intrahepatic vasculature in cirrhosis, an environment where there is increased vascular tone mediated by Ang II and other vasoconstrictors. We therefore studied the effects of Ang-(1-7) on hepatic vascular resistance and the pressure response to Ang II and the α-adrenergic agonist methoxamine (MTX) using *in situ* perfused cirrhotic rat liver preparation and compared the observed pressure responses to endothelial nitric oxide synthase (eNOS) phosphorylation status.

MATERIALS AND METHODS

Chemicals and drugs. Indomethacin, PD123319, N^G-nitro-*L-arginine* methyl-ester (L-NAME), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (1) (ODQ), MTX, sodium deoxycholate, sodium nitroprusside and dextrose were purchased from Sigma-Aldrich, Sydney, Australia. JE049 and AVE0991 were gifts from Sanofi-Aventis Deutschland GmbH, Germany. Candesartan was a gift from Dr Lindsay Brown, The University of Queensland, Australia. D-Ala^7-Ang-(1-7) (A779), Ang-(1-7), Ang II were purchased from Auspep Pty

Ltd, Parkville, Victoria, Australia. D-Pro^7-Ang-(1-7) was purchased from Mimotopes, Victoria, Australia. Bovine serum albumin (BSA) was purchased from Bovogen Biologicals, Victoria, Australia.

Animal model of cirrhosis and portal hypertension. Experimental procedures were approved by the Animal Ethics Committee of Austin Health and performed according to the National Health and Medical Research Council (NHMRC) of Australia Guidelines for animal experimentation, and the principles of the Helsinki declaration. Eight-week-old male Sprague Dawley rats (300-350 g) were housed in a controlled environment (12 hour light/dark, temperature 22°C to 24°C), and fed standard rat chow (Norco, Lismore NSW, Australia) and water ad libitum. After one week acclimatization, the rats were anaesthetised with an i.p. injection of ketamine/xylazine mixture (75mg/kg and 10mg/kg body weight respectively, Therapon Pty Ltd, Victoria, Australia) and given a single dose of carprofen (5 mg/kg, Lyppard Victoria Pty Ltd, Victoria, Australia) subcutaneously prior to surgery in order to limit post-operative discomfort. Bile duct ligation (BDL) was performed as previously described (21). Briefly, a midline abdominal incision was made, and the common bile duct was doubly ligated with 5/0 silk and transacted between the two ligations. The abdominal wall was closed in two layers using 4/0 silk. After 4 weeks of bile duct obstruction, rats were prepared for liver perfusion experiments as described below. Immediately after the completion of experiments, a liver sample was fixed in 4% PFA for histological examination and a sample was snap-frozen in liquid nitrogen and stored at -80°C until extracted for protein.

In-situ perfused rat liver preparation. In-situ rat liver perfusion was performed as previously described (20). Briefly, the rat was anaesthetized with intraperitoneal administration of pentobarbital (60mg/kg body weight, Boehringer Ingelheim, Artarmon, NSW, Australia). The abdominal and thoracic cavities were opened and the portal vein and

supra-diaphragmatic inferior vena cava (IVC) were cannula following occlusion of the IVC above the right renal vein. During portal vein cannulation, the liver was flushed with heparinised (400 IU) saline. Following surgery, the rat was transferred to a thermostatically controlled cabinet and kept at 37.5^{o}C +/- 0.5^{o}C. Livers were perfused through the portal vein using using a 14 gauge cannula with oxygenated (95% O_2, 5% CO_2) Krebs Henseleit solution with 1% BSA and 0.1% dextrose using both non-recirculating and recirculating systems. The non-recirculation was performed in the first 20 min and 10 min of washing period (Figure 1). perfusion pressure had stabilized after the first 20 min of perfusion. Portal flow was kept constant at 28 mL per min. Viability of the preparation was determined by macroscopic and histological appearance of the liver, together with oxygen consumption and stability of perfusion pressure (20).

Experimental protocol. Control experiments were performed in livers from healthy rats (n=7) to examine the effects of Ang –(1-7) on resting vascular tone and the response to Ang-(1-7) in the normal liver. The remaining experiments were performed on *in-situ* perfused BDL rat liver four weeks after surgery. The experimental protocol is shown in Figure 1. During the 70 min experimental period, each liver preparation was sequentially given five Ang II boluses (each 60 pmole) administered into the portal vein cannula in 0.2 mL over 10 sec period as in our previous study (20).

Perfusion pressures were measured every 2 min during the 20 min stabilization period and every 15 sec for 1 min immediately after each bolus injection and every 30 sec thereafter using a vertically positioned graduated fluid-filled column open to atmospheric pressure. The ACE inhibitor lisinopril (0.7 μM) was used in the perfusion medium throughout the experimental period to prevent Ang-(1-7) breakdown (20). There were 12 experimental groups with each group consisting of 4-8 BDL livers.

With the system in a recycling mode, the first Ang II bolus was given at time zero (after the 20 min of stabilization period) and the pressure response recorded. Ang-(1-7) (0.7 μM) was added to the perfusate before the second Ang II bolus was injected. Four minutes after the second Ang II bolus the liver preparation was thoroughly flushed out (non-recirculation) with Krebs Henseleit solution for 10 min and changed to recirculation again and the third Ang II bolus was administered. Then in separate experiments various blockers/antagonists were added into the perfusate and incubated until the end of the experiment (see Figure 1). Five minutes after the addition of different blockers/antagonists a fourth Ang II bolus was given. This was followed by the addition of Ang-(1-7) (0.7 μM) into the perfusate. Twelve minutes after the addition of Ang-(1-7) the fifth Ang II bolus was given. Since the effect of Ang-(1-7) was evident after completion of several experiments (see Figs. 2, 3), three rather than five Ang II boluses were used to elucidate the pathways responsible for the effect of Ang-(1-7) in some groups (see Figs. 5, 6).

In these experiments, the following blockers/antagonists were used to block cellular receptors or intracellular pathways potentially involved in Ang-(1-7) signalling; cyclooxygenase inhibitor indomethacin (125 nM), the bradykinin B_2 receptor (BK-B_2) blocker JE049 (2.7 μM), the AT1R blocker candesartan (0.7 μM), the Ang II type 2 receptor (AT2R) blocker PD123319 (0.7 μM), the NOS inhibitor L-NAME (70 μM), the guanylate cyclase inhibitor ODQ (7 μM). Mas receptor blocker A779 (7 μM) and a novel Ang-(1-7) receptor antagonist D-Pro^7-Ang-(1-7) (7 μM) were used to block Ang-(1-7) receptors including the Mas receptor. The non-peptide Mas receptor agonist AVE0991 (0.7 μM) was used to examine the role of the Mas receptor. There was one additional group in which both AT1R and Mas receptor were blocked. The doses of lisinopril, candesartan, PD123319, JE049, A779, AVE0991, D-Pro^7-Ang-(1-7), L-NAME and ODQ were based on our preliminary investigations and published studies (1, 7, 8, 15, 26, 39, 45, 46).

In order to determine whether Ang-(1-7) affected the response to another major mediator of hepatic vascular tone and to dissect out the involvement of AT1R in the Ang-(1-7) signalling pathway, we administered the α-adrenergic agonist MTX in two groups using the same design as for Ang II; one group of livers was perfused in the presence of the AT1R blocker candesartan and the other with candesartan plus the Mas receptor blocker A779.

We also investigated whether Ang-(1-7)-induced effects observed in the prior experiments were mediated via the vascular endothelium by using endothelium denuded cirrhotic rat liver preparations. The livers were perfused with 0.3% sodium deoxycholate (10) for 45 sec, followed by washing for 7 min. An Ang II bolus was injected before and after sodium deoxycholate treatment and the pressure response was recorded. The livers were then incubated with Ang-(1-7) (0.7 μM) for 12 min and a second Ang II bolus was injected and pressure response recorded. At the end of the experiment, the vasorelaxing activity of the liver preparation was tested by adding the NO donor sodium nitroprusside (55 μM) into the perfusate and incubated for 5 min and then a further Ang II bolus was injected and the pressure response recorded. Moreover endothelial denudation with deoxycholate was tested using an endothelium-dependent vasodilator, acetylcholine (18). This experiment was conducted in a recirculation system except for 7 min washing period of non-recirculation system.

Western Blotting for phosphorylated and total eNOS. Frozen liver tissue (50-100 mg) was minced, resuspended in buffer containing 22 mM Tris-HCl pH 7.4, 0.22 M EGTA, 0.11 M EDTA, 1.375 M NaF, 0.11 M Na orthovanadate, 2.2 M β-glycerolphosphate, 0.2 M N-ethylmalaimide, Noindet P-40, to which 1 μg/mL pepstatin, 5 μg/mL leupeptin and 10 μg/mL aprotinin were added, and homogenised at high speed for 1 min with the Basic Ultra-Turrax T10 (IKA, North Carlifonia, USA) and centrifuged at 13200 rpm at 4˚C for 30 minutes. The resultant supernatant was harvested and stored in aliquots at minus 80˚C. Total protein was

quantified using PIERCE BCA Protein Assay Kit (Thermo FIsher Scientific, Massachusetts, USA). Samples (50 ug protein) were loaded and run on a 10% sodium dodecyl sulphate polyacrylamide gel system and transblotted onto immunoblot PVDF membrane (BioRad, California, USA) using a transfer tank at 100V for 1 hour. At the end of the transfer, filters were washed twice in TBS and blocked with 5% non-fat skim milk powder in TBS containing 0.1% Tween20 for 1 hour at room temperature. The anti-phospho eNOS Ser^{1177} antibody (Cell Signaling Technology, Massachusetts, USA) diluted to a concentration of 1/1000 with 5% BSA in TBS-T was incubated overnight at 4˚C. The membrane was then washed thoroughly three times in TBS-T. Positive bands were developed using PIERCE ECL western Blotting Substrate (PIERCE) in which horseradish peroxidase (HRP) labeled secondary goat anti-rabbit antibody (DAKO, Glostrup, Denmark) was diluted at 1/2000 with 5% BSA in TBS-T, followed by one hour incubation at room temperature. Exposed Amersham hyperfilm (GE Healthcare, Buckinghamshire, UK) of bands representing phospho-eNOS protein were quantified using a Molecular Imager Gel Doc XR+ System (BioRad, Carlifonia, USA). Total eNOS was used as a loading control and probed following the same procedure with 1/1000 concentration of eNOS primary antibody (Cell Signaling Technology).

Statistical analysis. In the perfusion studies, statistical significance between the baseline-corrected total area under the curve (AUC) was determined using ANOVA designed to account for repeated measures. The analyses used the percentage increase in perfusion pressure response relative to the pressure response from the control Ang II bolus without treatment. When considerable variation was observed the data were log transformed to stabilize the variation before they were used in the analyses. ANOVA was used for comparison of means of protein expression data. Data are presented as mean ± SEM. A *P*

value of less than 0.05 was considered statistically significant. All statistical analyses were carried out using the SAS computer package (SAS, Statistics, Version 9.2, Cary, NC, USA).

RESULTS

Liver injury and fibrosis. As in previous studies, the livers of BDL rats 4 weeks after surgery displayed extensive liver fibrosis with bridging and nodule formation. These changes were accompanied by significantly elevated plasma gamma-glutamyl transpeptidase (GGT), alanine aminotransaminase (ALT), alkaline phosphatase (ALP) and bilirubin levels, and significantly higher Ishak score and percentage area stained for picrosirius red compared with healthy livers (20, 21)

Effects of Ang II on hepatic perfusion pressure. Ang II at a dose of 60 pmole bolus reliably produced perfusion pressure increases in both normal (data not shown) and BDL livers (Figs. 2-3 & 5-8). Peak perfusion pressure increases were observed 45 sec after each Ang II bolus in all groups and are shown in centimetre (H_2O) in top panels of each Figure while respective areas under angiotensin II-induced response curves are shown in the bottom panels. As shown in Figure 1, the first and third bolus injections of Ang II were given in the absence of Ang-(1-7) and without blockers and/or antagonist. There was no evidence of tachyphylaxis to Ang II between these 2 injections in any of the groups.

Ang-(1-7) reduces Ang II mediated hepatic vasoconstriction. In healthy livers (n=7) Ang-(1-7) reduced portal perfusion pressure and the total area under Ang II curve by almost 50% ($P < 0.05$) (mean 10433±1499 SEM and mean 5351±825 SEM [% increase/sec], in the absence or presence of Ang-(1-7), respectively. Importantly, despite the increased resting vascular resistance in the cirrhotic liver, infusion of Ang-(1-7) still resulted in a major reduction in Ang II mediated vasoconstriction, producing a more than 50% reduction ($P < 0.005$) in the AUC of the Ang II-induced increase in perfusion pressure (Fig. 2A, 2B).

In cirrhosis Ang-(1-7) reduces Ang II mediated vasoconstriction via Mas receptor antagonist A779 insensitive mechanism. In cirrhosis, the Mas receptor antagonist A779 alone had no effect on vasoconstriction in response to Ang II and did not prevent the major reduction in Ang II mediated vasoconstriction induced by Ang-(1-7) (Fig. 2A, 2B). To further investigate whether the effect of Ang-(1-7) was mediated through the Mas receptor, another group of livers were pre-treated with the Mas receptor agonist AVE0991 in place of Ang-(1-7). However, AVE0991 had no effect on Ang II-induced vasoconstriction and the combination of AVE0991 and A779 also had no effect (Fig. 2C, 2D). In a marked contrast, another Ang-(1-7) antagonist D-Pro^7-Ang-(1-7) (39) completely abolished the effects of Ang-(1-7) (Fig. 2E, 2F).

Role of the AT1R, AT2R and BK-B_2 receptors. The AT2R antagonist PD123319 (Fig. 3A, 3B) and BK-B_2 receptor antagonist JE049 (Fig. 3C, 3D) alone had no effect on Ang II-mediated vasoconstriction and did not prevent the reduction in the pressure response after incubation with Ang-(1-7).

In order to investigate whether Ang-(1-7) reduces the vasoconstriction and perfusion pressure increase induced by other mediators of vascular tone in cirrhosis, and if its vasodilatory effects occur via interaction with the AT1R, we used the α-adrenergic agonist MTX to constrict the liver and candesartan as an AT1R blocker. Bolus MTX rapidly increased perfusion pressure in a similar fashion to Ang II and this response was significantly ($P < 0.05$) inhibited by Ang-(1-7) with more than 50% reduction in the AUC (Fig. 4A, 4B). Candesartan alone neither affected the MTX-induced pressure change nor blocked inhibition of the MTX response by Ang-(1-7) (Fig. 4A, 4B). As the AT1R and Mas receptor have been shown to interact during ligand binding (8, 27) we investigated the effect of blocking both receptors on MTX-induced perfusion pressure changes. The combination of AT1 and Mas receptor blockers had no effect on MTX mediated vasoconstriction. However in the presence

of these 2 inhibitors Ang-(1-7) did not significantly reduce the response to Ang II (Fig. 4C, 4D).

The vasodilatory effects of Ang1-7 are NOS and guanylate cyclase dependent. We then investigated the intracellular mechanisms that might be responsible for the effects of Ang-(1-7). As in the prior experiments, Ang-(1-7) reduced the Ang II-induced perfusion pressure response by more than 50% ($P < 0.0005$). However, the addition of L-NAME completely abolished the effect of Ang-(1-7) (Fig. 5A, 5B). In a similar fashion, the guanylate cyclase inhibitor ODQ completely abolished the effect of Ang-(1-7) (Fig. 5C, 5D). In contrast, the cyclooxygenase inhibitor indomethacin did not modify the effect of Ang-(1-7) (Fig. 6A, 6B).

It has been shown that in other vascular beds Ang-(1-7) causes eNOS phosphorylation at Ser^{1177} resulting in an increase in enzyme activity and NO production by endothelial cells. In the presence of the various receptor blockers outlined above, we therefore examined the effects of Ang-(1-7) on hepatic Ser^{1177} phosphorylated eNOS levels using an anti-phospho eNOS Ser^{1177} antibody. As shown in Figure 7A and 7B, the level of eNOS Ser^{1177} phosphorylation detected correlated with the vasoactive effects of Ang-(1-7) in all groups. Mas receptor antagonism with A779 or AT1R blockade with candersartan neither affected the perfusion pressure response nor eNOS phosphorylation. In contrast, when combined with the AT1R blocker candesartan, Mas antagonism with A779 resulted in a significant ($P < 0.05$) reduction in phosphorylated eNOS levels which was accompanied by inhibition of the Ang-(1-7) response (see Fig. 4C, 4D).

Ang-(1-7)-induced vasodilatation is mediated via the endothelium. There was no significant reduction in the Ang II response after denudation of the hepatic endothelium with sodium deoxycholate. Importantly, in sodium deoxycholate-infused livers Ang-(1-7) no longer inhibited Ang II mediated vasoconstriction (Fig. 8A, 8B). We then confirmed that the cirrhotic livers maintained their vasodilatory activity after sodium deoxycholate infusion by

291 showing a significant ($P < 0.05$) reduction in Ang II pressure response to infusion of the NO
292 donor sodium nitroprusside (Fig. 8A, 8B). Moreover a lack of response to endothelium-
293 dependent vasodilator, acetylcholine, was confirmed in methoxamine-constricted
294 endothelium denuded livers (data not shown).

295 ## DISCUSSION

296 It is well documented that in cirrhosis there is enhanced activity of both the RAS and
297 sympathetic nervous system, leading to production of the potent vasoconstrictor Ang II and
298 activation of adrenergic receptors, respectively, and these are major contributors to increased
299 sinusoidal resistance (20, 21, 31, 34). In the *ex-vivo* perfused liver, in the absence of both
300 intrinsic sympathetic tone and circulating vasoconstrictors, much if not all of the increase in
301 sinusoidal resistance to portal flow is likely to reflect the fixed component of portal resistance
302 rather than vascular tone that can be modulated by vasodilators such as Ang-(1-7). However
303 the current study demonstrates for the first time that in the cirrhotic rat liver Ang-(1-7)
304 produces an approximately 50% reduction in the response to both Ang II and MTX. This
305 effect was greater than the effects of Ang-(1-7) observed in other vascular beds, where in
306 general it reduces vasoconstriction response by 20% (8, 17, 28, 33, 40, 46).

307 An important focus of our study was on the cellular receptors that mediate the effects of
308 Ang-(1-7) since in addition to the putative Ang-(1-7) receptor Mas, other types of receptor
309 populations are involved in its action, depending on the vascular bed under investigation (5,
310 8, 33, 40, 46). Recent evidence has shown that there is marked upregulation of Mas receptor
311 expression in the cirrhotic liver (21). However, our study suggested that in the perfused liver
312 Mas alone was not the primary receptor responsible for the vasodilatory effect of Ang-(1-7)
313 but that it acts via a receptor subtype or population that is sensitive to D-Pro^7-Ang-(1-7).

Mas is a specific G protein-coupled receptor that is thought to be responsible for many Ang-(1-7) actions (28, 40). Indeed, studies in several vascular beds have established that activation of this receptor produces vasodilatory, antihypertrophic, antifibrotic, and antithrombotic effects that oppose those of Ang II (14, 28, 36, 42). However, it has also been shown that in some tissues Ang-(1-7)-induced vasodilatation is not blocked by the specific Mas receptor antagonist A779 (46) or that combined treatment with Mas and other receptor antagonists is required to block its effects (8). In line with these observations, the present study demonstrated that in the cirrhotic rat liver, Mas is not the sole receptor that mediates the vasodilatory action of Ang-(1-7) because the specific Mas receptor antagonist A779 could not block its effects. Furthermore, the effects of Ang-(1-7) were not reproduced by the non-peptide Mas agonist AVE0991, which has been shown to stimulate the Mas receptor at concentrations commensurate with those used in the present study (35).

A number of other mechanisms have been shown to contribute to Ang-(1-7) mediated vasodilation in different organs and under differing pathophysiological conditions. For example, vasodilatory prostacyclins appear to be involved in the response to Ang-(1-7) in spontaneously hypertensive rats, in rat mesenteric resistance vessels and in the mouse aorta (5, 11, 25, 28, 33). The bradykinin B_2 receptor and AT2R also appear to contribute to its effects in coronary, mesenteric, renal and cutaneous vascular beds (6, 16, 30, 38, 41). Using specific receptor and pathway blockers we found no evidence that any of these pathways contribute significantly to the effects of Ang-(1-7) in the cirrhotic rat liver. In addition, we produced evidence suggesting that Ang-(1-7) does not act via the AT1R or by competing with Ang II for this receptor by showing that Ang-(1-7) also markedly inhibited methoxamine mediated vasoconstriction and these effects were not inhibited by AT1R antagonism. Combined treatment with Mas and AT1R antagonists, on the other hand, reduced the vasodilatory response to Ang-(1-7) in association with a reduction in hepatic

phosphorylated eNOS levels (see Figures 4C, 4D and 7A, 7B). Important functional interactions between the Mas receptor and AT1R have been identified in previous studies (8, 27). In line with this, the present findings suggest an interaction between the two receptors in the cirrhotic liver, implying that inhibition of AT1R may expose the Mas receptor to its antagonist A779. The complete inhibition of the vasodilatory effect of Ang-(1-7) by D-Pro^7-Ang-(1-7) agrees with the findings of Santos and colleagues who showed that the specific Mas receptor antagonist A779 did not block the vasodilatation caused by Ang-(1-7) in the aorta from Sprague Dawley rats but complete inhibition was achieved by pre-incubation of rat aorta with D-Pro^7-Ang-(1-7) (46). Taken together, our findings suggest that either a distinct population of novel receptor subtypes or combination of recognized receptors, all sensitive to D-Pro^7-Ang-(1-7) may be primarily responsible for the vasodilatory action of Ang-(1-7) in the cirrhotic rat liver. However, it may also be possible that blockade of effects of Ang-(1-7) using novel selective Ang-(1-7) receptor antagonist D-Pro^7-Ang-(1-7) may have mediated through the Mas receptor but this hypothesis remains to be confirmed.

Our findings are also consistent with previous studies that have indicated Ang-(1-7) produces vasodilatation via activation of eNOS. The activity of eNOS is regulated through coordinated phosphorylation of specific sites where phosphorylation at Ser^{1177} results in an increase in enzyme activity and NO production (12). Thus, in the present study, D-Pro^7-Ang-(1-7) and combined treatment with A779 and candesartan significantly blocked Ang-(1-7)-induced phosphorylation at Ser^{1177} (see Figure 7A, 7B). The use of frozen liver tissue may reduce phosphorylated-eNOS protein levels; however, we found that phosphorylated eNOS expression was highly reproducible within each experimental group and the results were in line with functional data obtained with various antagonists/blockers. Moreover our results indicate that it is eNOS phosphorylation in the vascular endothelium that is regulated by Ang-(1-7) by demonstrating a complete inhibition of the vasodilatory effects of Ang-(1-7) in

endothelium denuded liver preparations. Importantly, the NO donor sodium nitroprusside significantly reduced the pressure response to Ang II in the endothelium denuded liver, confirming that the livers maintained their vasodilatory activity. In order to examine intracellular mechanisms associated with Ang-(1-7)-induced vasodilatation, we used the non-specific NOS inhibitor L-NAME, to block NO production by competitive inhibition of its natural substrate L-arginine and the specific guanylate cyclase inhibitor ODQ to block NO signaling through the activation of smooth muscle cell membrane-associated guanylate cyclase. We found that both interventions completely blocked the vasodilatory effects of Ang-(1-7).

The present study provides experimental data suggesting that manipulation of the alternate RAS in the liver could be of benefit in portal hypertension. However, *in vivo* application of Ang-(1-7) has been shown to enhance NO availability and mesenteric vasodilatation (13, 38). Thus, whilst systemic administration of Ang-(1-7) may have beneficial effects on the hepatic circulation, this might be outweighed by its propensity to aggravate systemic and splanchnic vasodilatation. Further studies *in vivo* will be required to address this possibility.

In conclusion, the present study demonstrates that in the cirrhotic liver Ang-(1-7) has a significant vasodilatory effect that markedly reduces vasoconstriction mediated by both Ang II and MTX. This action of Ang-(1-7) was not significantly inhibited by a range of specific blockers including the Mas receptor antagonist A799, but was sensitive to D-Pro^{7}-Ang-(1-7), suggesting that a novel receptor population or a combination of receptors is involved in its effects. Our findings indicate that activation of this receptor population on the vascular endothelium causes eNOS phosphorylation and NO production which in turn activates the NO-dependent guanylate cyclase pathway leading to vasodilatation. These findings suggest that strategies targeting the intrahepatic alternate arm of the RAS and its signalling pathways may have a therapeutic role in the management of portal hypertension

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DISCLOSURES

The authors have nothing to disclose.

Figure Legends

Fig. 1. Schematic representation of the experimental plan adopted in the present study. After 15 minutes of perfusion, the angiotensin converting enzyme (ACE) inhibitor lisinopril was added to perfusate to prevent angiotensin II (Ang II) breakdown. Five consecutive boluses of Ang II (60 pmole) were used to increase perfusion pressure, and between the boluses, various agonists, receptor and enzyme blockers were used. The perfusion pressure response to Ang II was measured in the absence (bolus 1) or presence (bolus 2) of angiotensin-(1-7) (Ang-(1-7)). The preparation was thoroughly washed after the second Ang II bolus injection to flush out remaining Ang-(1-7) before commencement of the second set of Ang II boluses in the absence (bolus 3) or presence of only the blockers (bolus 4) or both blockers and Ang-(1-7) (bolus 5). The arrow indicates the time of Ang II bolus injections. In some experiments, Ang II was replaced with methoxamine bolus (300 nmole) injections.

Fig. 2. Perfusion pressure changes in response to angiotensin II (Ang II) in cirrhotic rat liver. Panels A, C and E show absolute pressure changes (cm H_2O) in response to Ang II and the panels B, D and F show the baseline-corrected total area under Ang II response curve after each of the five boluses. Ang II reproducibly increased portal resistance and pressure. This response was significantly attenuated by Ang-(1-7). The effect of Ang-(1-7) was not blocked by A779 (panels A & B). Panels C & D show the effect of Mas receptor agonist AVE0991 on Ang II-induced perfusion pressure response. AVE0991 failed to mimic the action of Ang-(1-7). In contrast, D-Pro^7-Ang-(1-7) completely blocked the effect of Ang-(1-7) (panels E & F). Each panel represents the mean±SEM profile from four or five cirrhotic rat liver preparations.

Fig. 3. Perfusion pressure changes in response to angiotensin II (Ang II) in cirrhotic rat liver. Top panels show absolute pressure changes (cm H_2O) in response to Ang II and the bottom panels show the baseline-corrected total area under Ang II response curve after each of the five boluses. The angiotensin II type 2 receptor (AT2R) blocker PD123319 had no effect on Ang II-induced vasoconstriction. The inhibitory effect of Ang-(1-7) on the vasoconstriction response to Ang II was not blocked by PD123319 (panels A & B). Panels C & D show the effect of the bradykinin B_2 receptor blocker JE049 on Ang II-induced vasoconstriction. JE049 had no effect on the response to Ang II and failed to block the effect of Ang-(1-7). Each panel represents the mean±SEM profile from four or five cirrhotic rat liver preparations.

Fig. 4. Perfusion pressure changes in response to methoxamine in cirrhotic rat liver. Top panels show absolute pressure changes (cm H_2O) in response to methoxamine and the bottom panels show the baseline-corrected total area under methoxamine response curve after each of the five or three boluses. Methoxamine (300 nmole) increased perfusion pressure and this response was significantly reduced by Ang-(1-7). The angiotensin II type 1 receptor (AT1R) blocker candesartan had no effect on methoxamine-induced vasoconstriction. The inhibitory effect of Ang-(1-7) on the vasoconstriction response to methoxamine was blocked by candesartan (panels A & B). Panels C & D show the effect of a combined treatment with AT1 and Mas receptor blockers. There was no significant effect of Ang-(1-7) on the response to methoxamine in the presence of both candesartan and A779. Each panel represents the mean±SEM profile from four or five cirrhotic rat liver preparations.

Fig. 5. Perfusion pressure changes in response to angiotensin II (Ang II) in cirrhotic rat liver. Top panels show absolute pressure changes (cm H_2O) in response to Ang II and bottom panels show the baseline-corrected total area under Ang II response curves after each of the

five or three boluses. The nitric oxide synthase (NOS) inhibitor L-NAME (panels A & B) and guanylate cyclase inhibitor ODQ (panels C & D) completely abolished the effect of Ang-(1-7) on Ang II-induced vasoconstriction. Each panel represents the mean±SEM profile from five or six cirrhotic rat liver preparations.

Fig. 6. Perfusion pressure changes in response to angiotensin II (Ang II) in cirrhotic rat liver. Top panel shows absolute pressure changes (cm H_2O) in response to Ang II and bottom panel shows the baseline-corrected total area under Ang II response curve after each of the five boluses. Five consecutive Ang II boluses were given in the presence or absence of Ang-(1-7) and indomethacin. Indomethacin had no effect on the response to Ang II and did not block Ang-(1-7)-induced vasorelaxation. Each panel represents the mean±SEM profile from four cirrhotic rat liver preparations.

Fig. 7. Western blot analysis of phosphorylated endothelial nitric oxide synthase (phospho-eNOS) at SER^{1177} position in cirrhotic rat livers treated with the various receptor blockers and Ang-(1-7). Panel A is a representative western blot image and panel B shows quantitative phospho-eNOS protein expression compared to total eNOS (eNOS) as loading control. Phospho-eNOS expression was abundant in the perfused livers treated with angiotensin-(1-7) (Ang-(1-7)). Novel Ang-(1-7) receptor antagonist D-Pro^7-Ang-(1-7) and combined treatment with Mas receptor antagonist A779 and AT1R blocker candesartan significantly reduced phospho-eNOS level compared with AT1, AT2, BK-B_2 and Mas receptor blockers. In panel B, each group represents the mean±SEM profile from four to seven perfused cirrhotic rat liver preparations. AT1R: angiotensin II type 1 receptor, AT2R: angiotensin II type 2 receptor, BK-B_2: bradykinin B_2 receptor.

Fig. 8. Perfusion pressure changes in response to angiotensin II (Ang II) in cirrhotic rat liver. Top panel shows absolute pressure changes (cm H_2O) in response to Ang II and bottom panel shows the baseline-corrected total area under Ang II response curve. Endothelium denudation with sodium deoxycholate had no effect on Ang II-induced vasoconstriction but completely abolished the inhibitory effect of Ang-(1-7) on Ang II-induced vasoconstriction. However the nitric oxide (NO) donor sodium nitroprusside significantly reduced Ang II-induced vasoconstriction. Each panel represents the mean±SEM profile from six to eight cirrhotic rat liver preparations.

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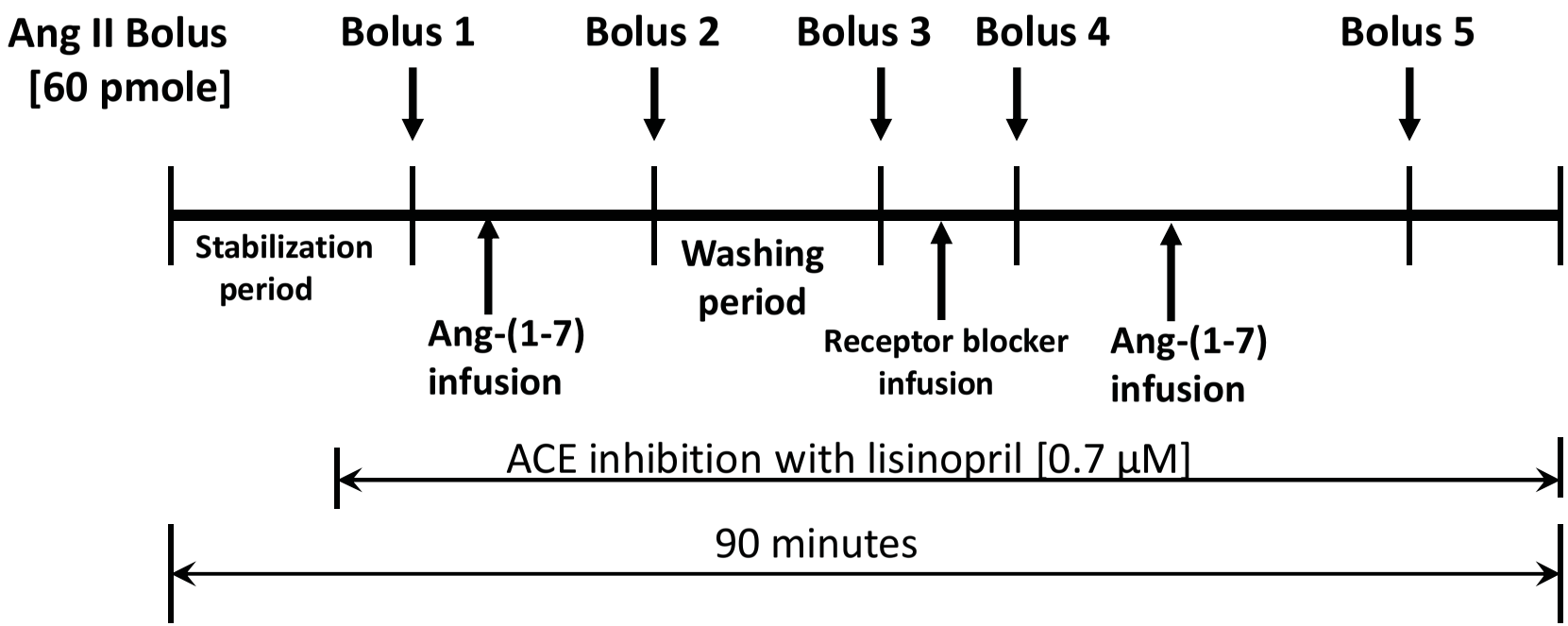
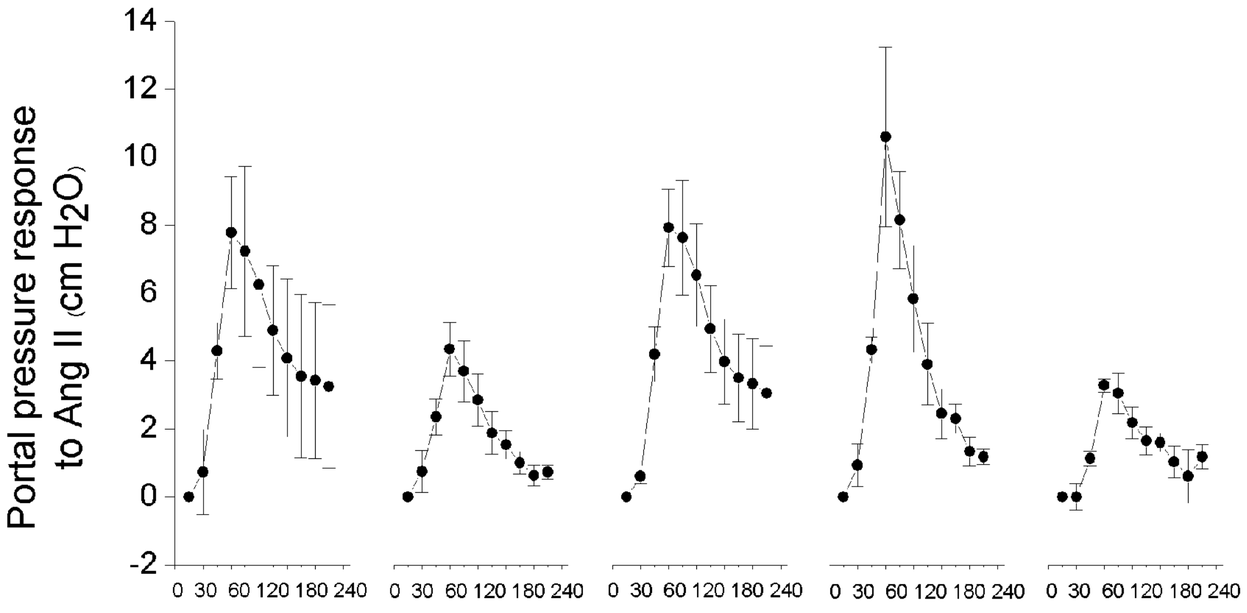

Ang II Bolus
[60 pmole]
Bolus 1
Bolus 2
Bolus 3
Bolus 4
Bolus 5
Stabilization
period
Ang-(1-7)
infusion
Washing
period
Receptor blocker
infusion
Ang-(1-7)
infusion
ACE inhibition with lisinopril [0.7 µM]
90 minutes

A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12
10
8
6
4
2
0
-2
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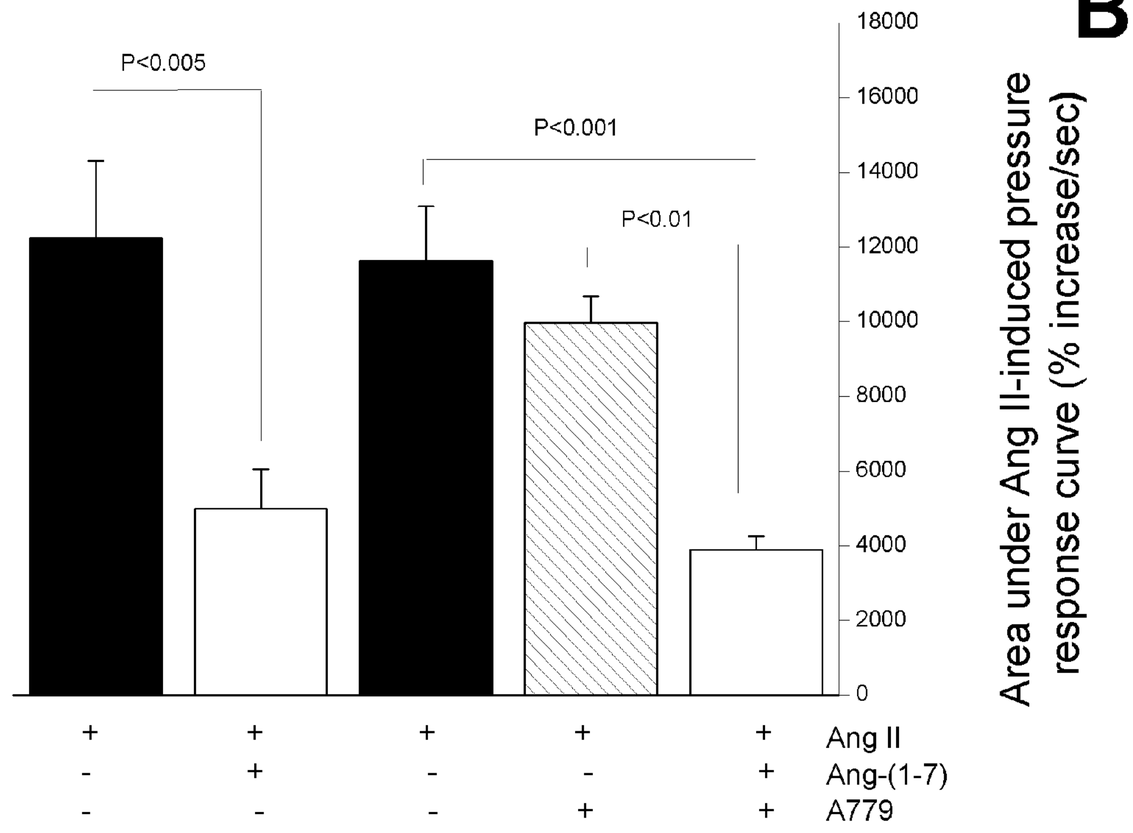
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240

B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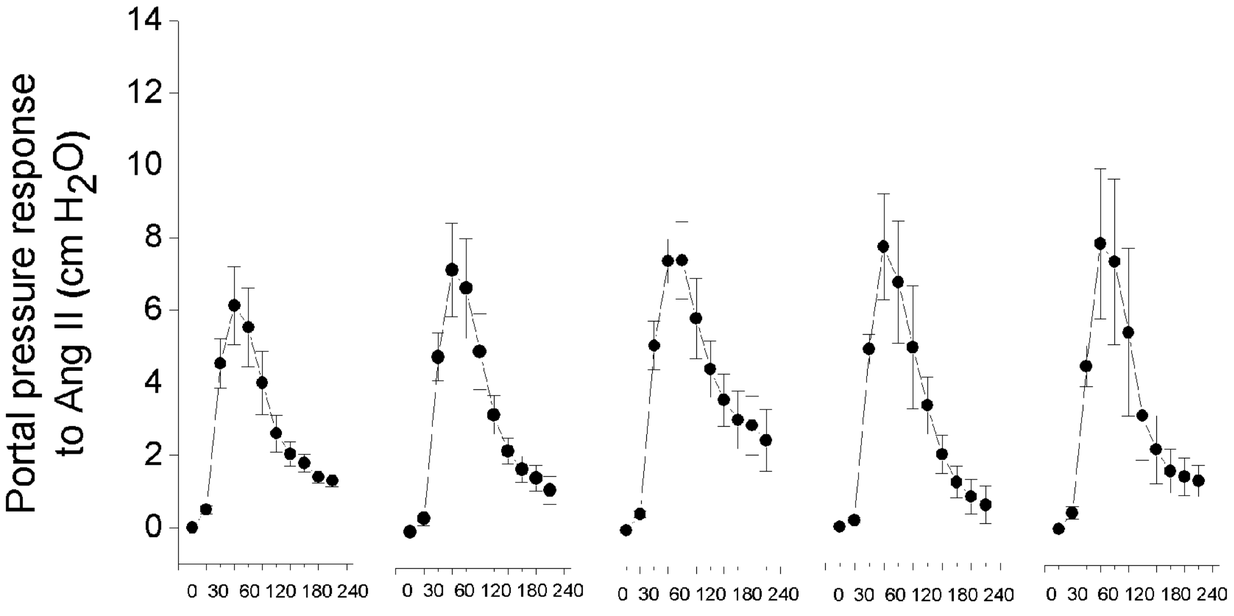
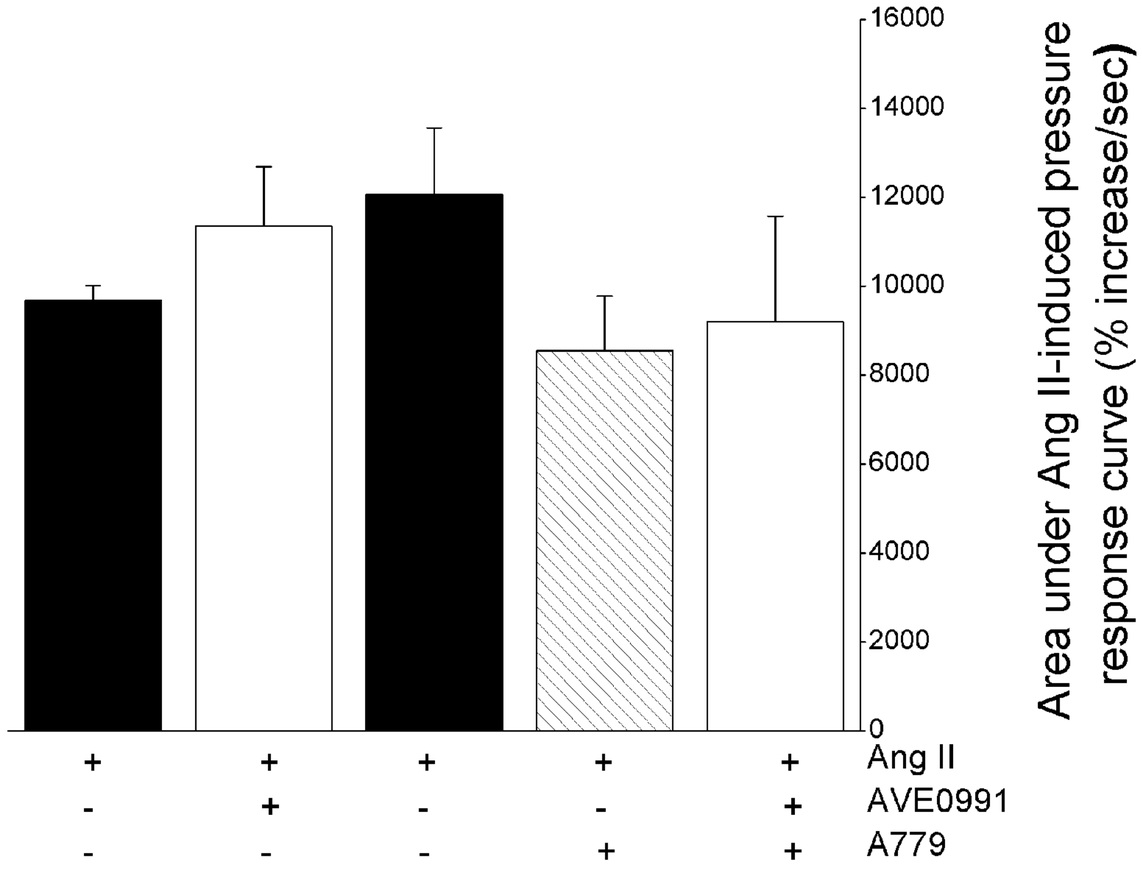
P<0.001
P<0.01
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
+ + + + + Ang II
- + - - + Ang-(1-7)
- - - + + A779
Area under Ang II-induced pressure response curve (% increase/sec)

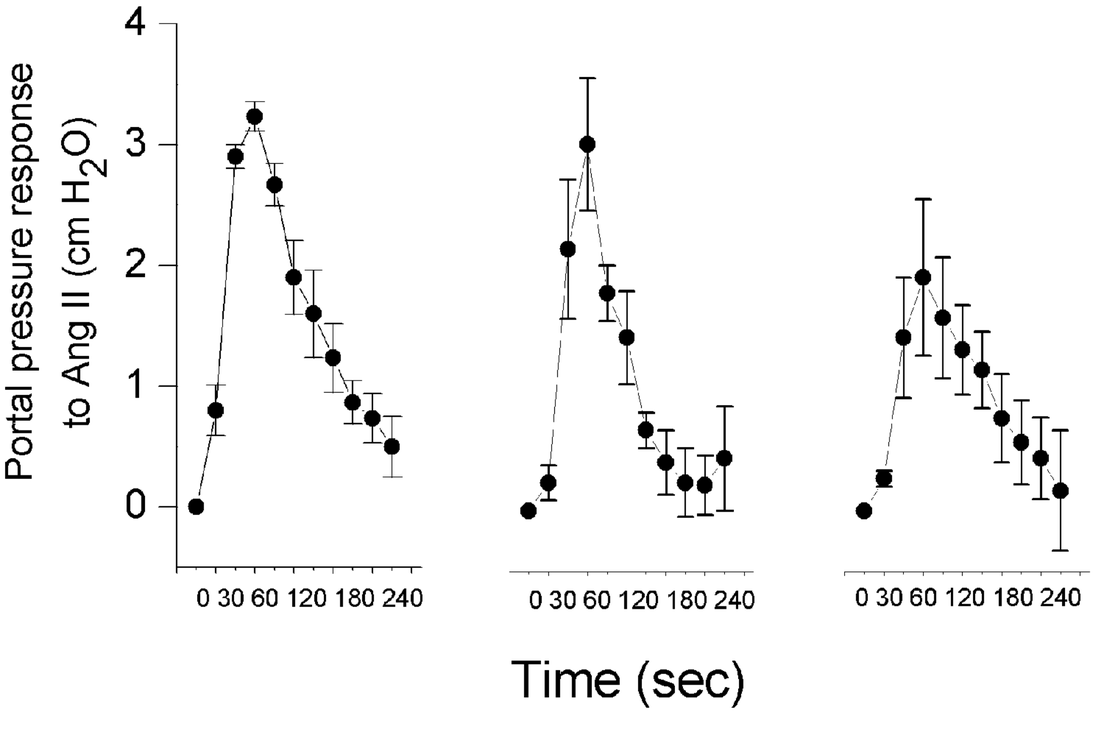
C


Portal pressure response to Ang II (cm H2O)
14
12
10
8
6
4
2
0
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240

D


16000
14000
12000
10000
8000
6000
4000
2000
0
+ + + + + Ang II
- + - - + AVE0991
- - - + + A779
Area under Ang II-induced pressure response curve (% increase/sec)

E


Portal pressure response to Ang II (cm H2O)
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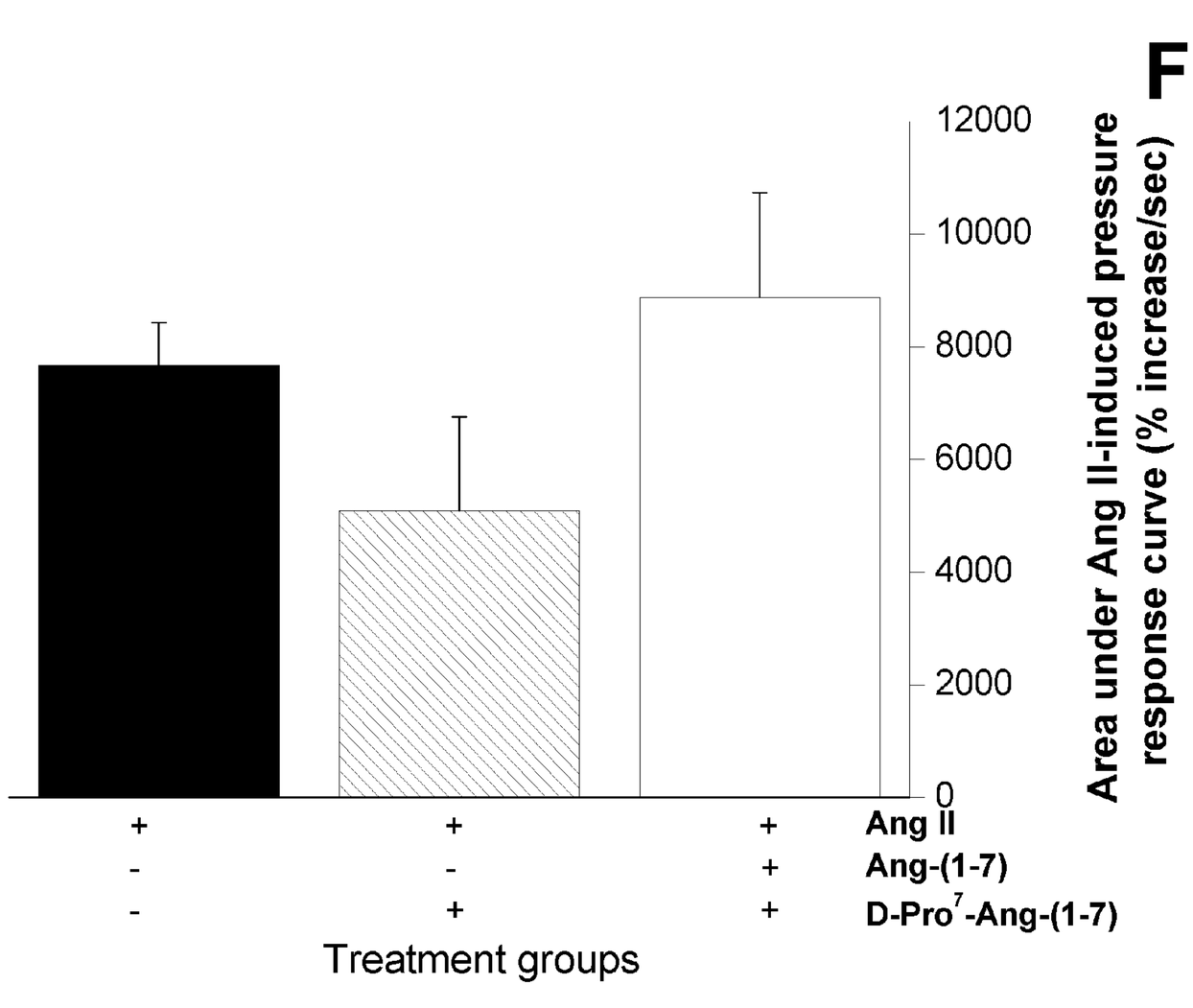
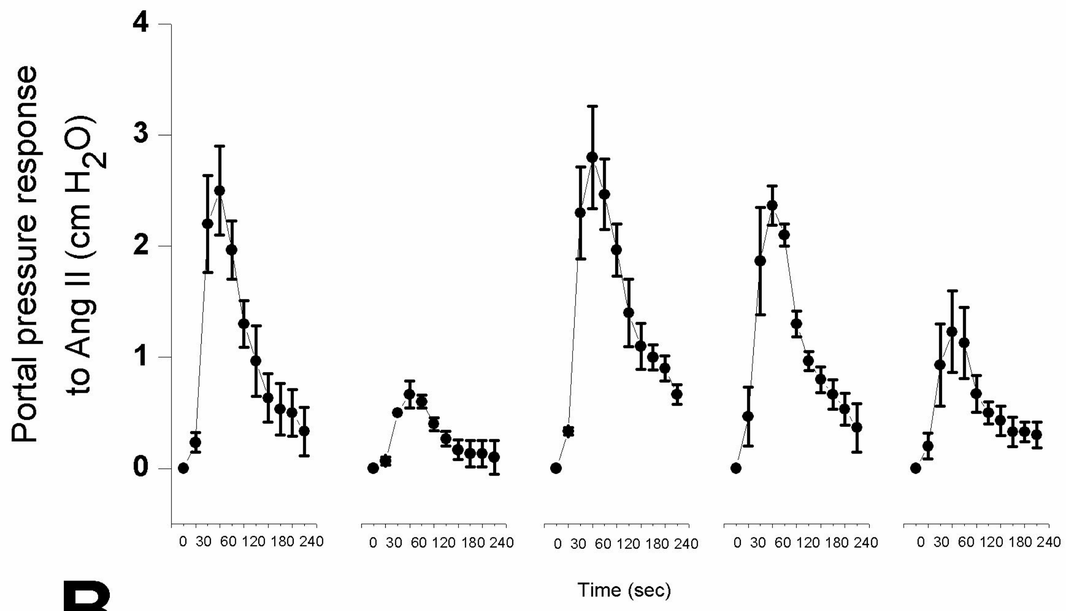
3
2
1
0
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240
Time (sec)

F


12000
10000
8000
6000
4000
2000
0
+ + + Ang II
- - + Ang-(1-7)
- + + D-Pro7-Ang-(1-7)
Treatment groups
Area under Ang II-induced pressure response curve (% increase/sec)

A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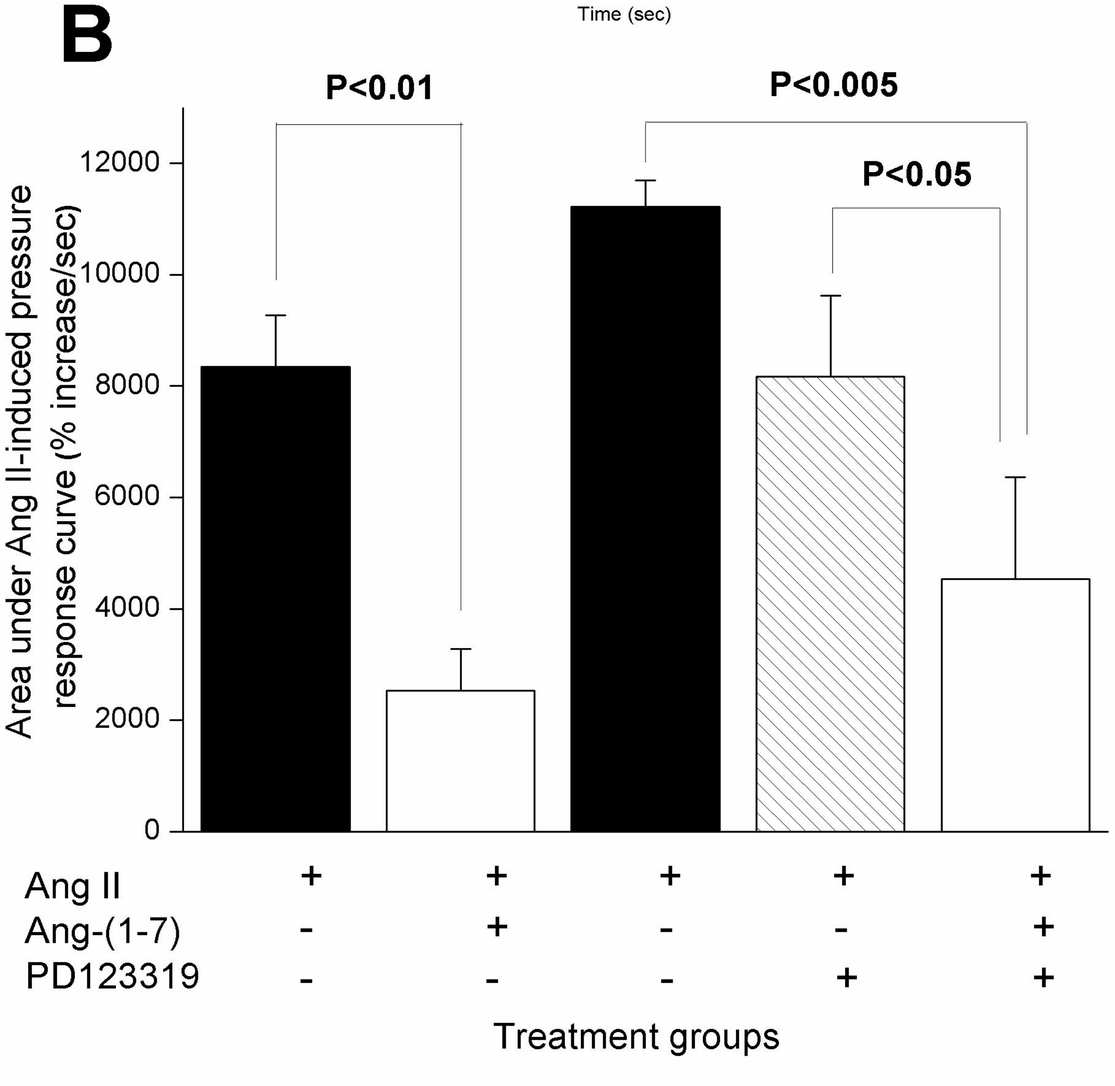
0 30 60 120 180 240
Time (sec)

B


P<0.01
P<0.05
P<0.005
Area under Ang II-induced pressure response curve (% increase/sec)
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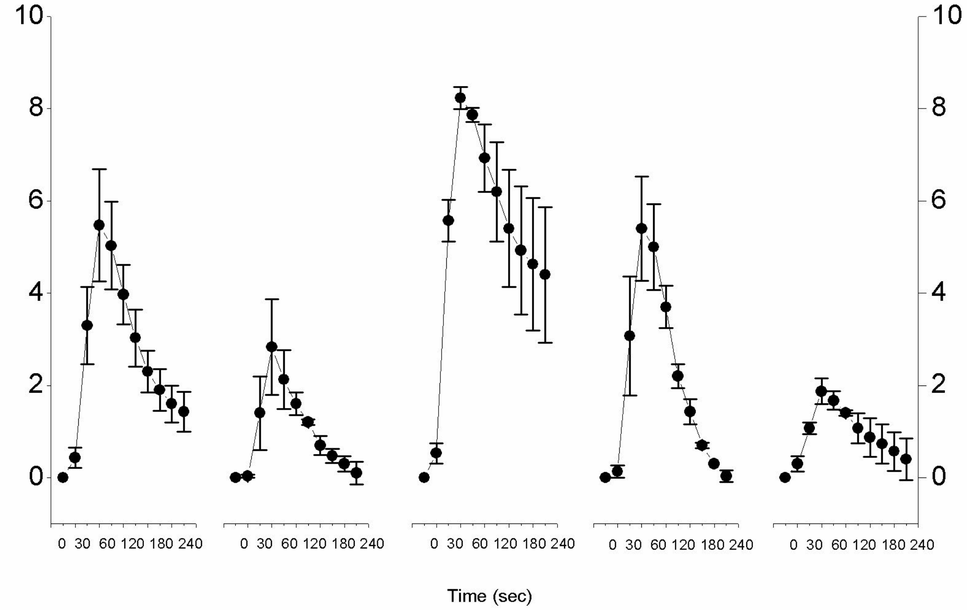
Ang II + + + + +
Ang-(1-7) - + - - +
PD123319 - - - + +
Treatment groups

C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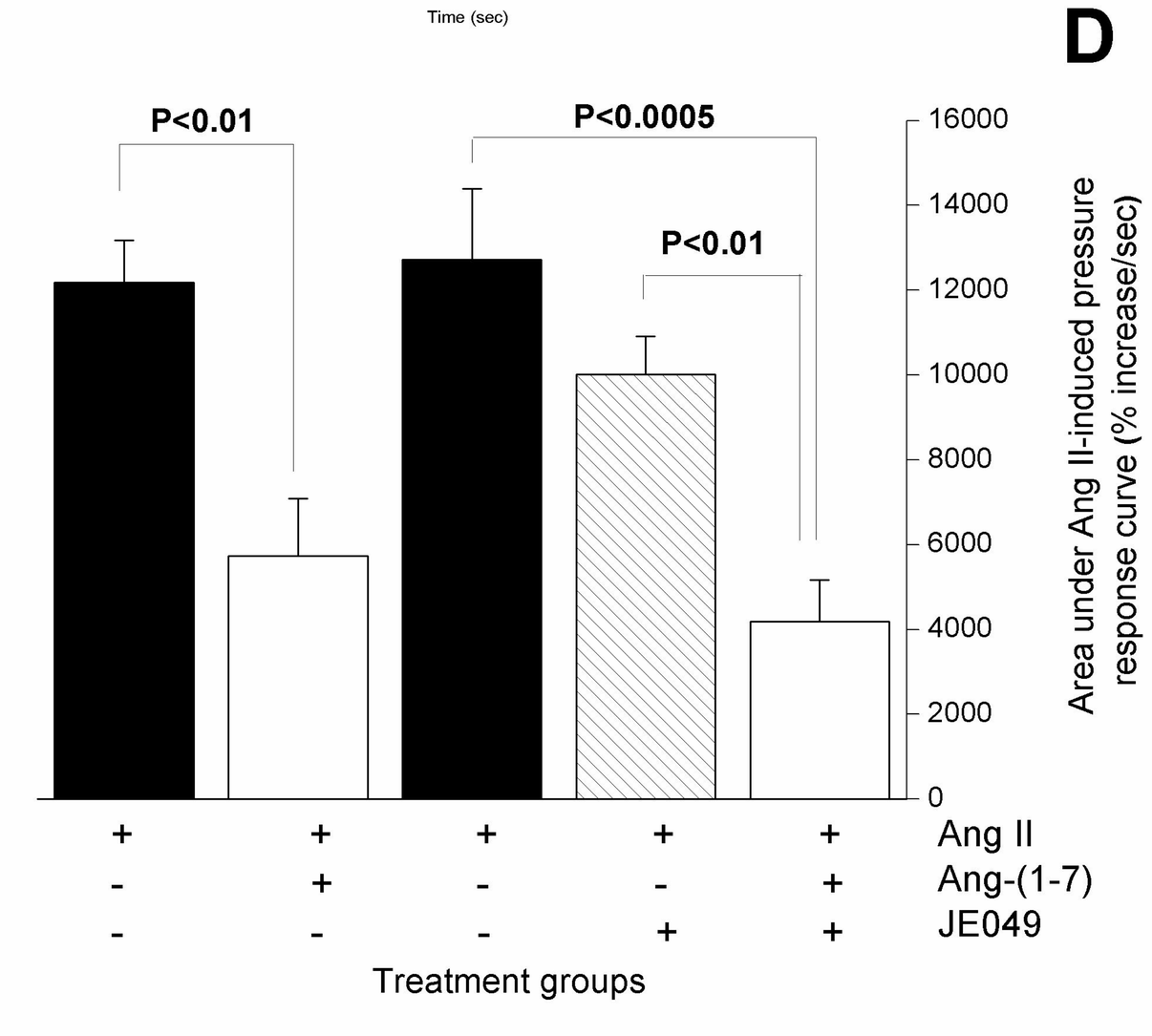
0 30 60 120 180 240
Time (sec)

D


P<0.01
P<0.0005
P<0.01
Area under Ang II-induced pressure response curve (% increase/sec)
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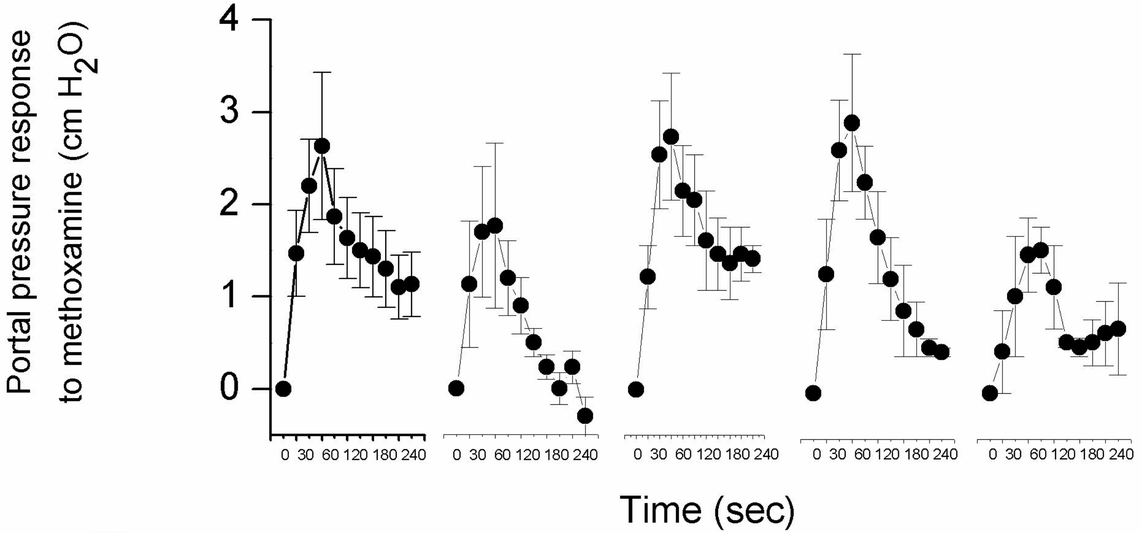
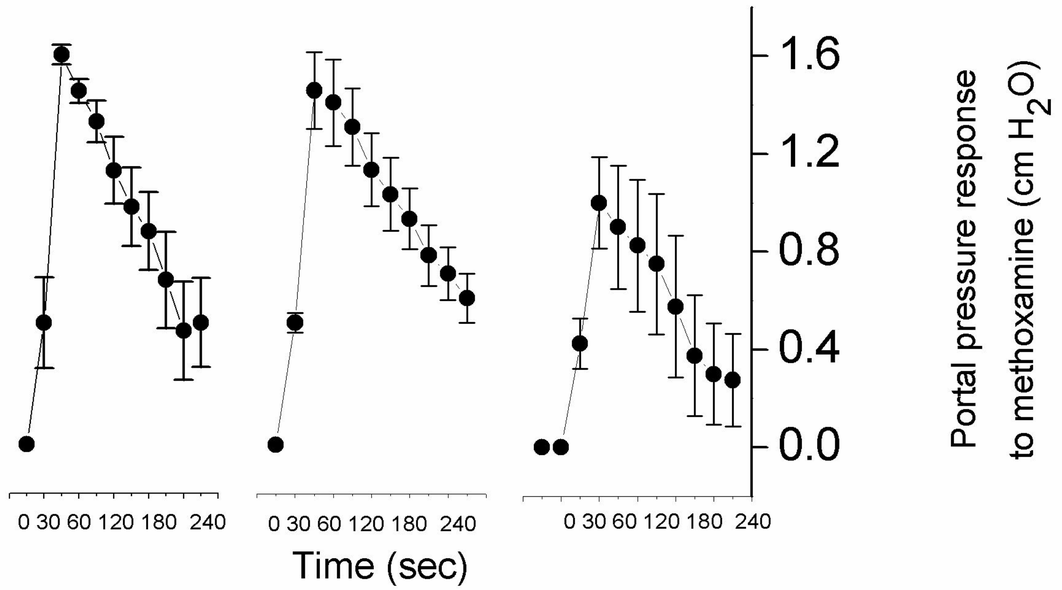
Ang II + + + + +
Ang-(1-7) - + - - +
JE049 - - - + +
Treatment groups

A


Portal pressure response to methoxamine (cm H_2O)
4
3
2
1
0
0 30 60 120 180 240
Time (sec)

C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1.2
0.8
0.4
0.0
Portal pressure response to methoxamine (cm H_2O)
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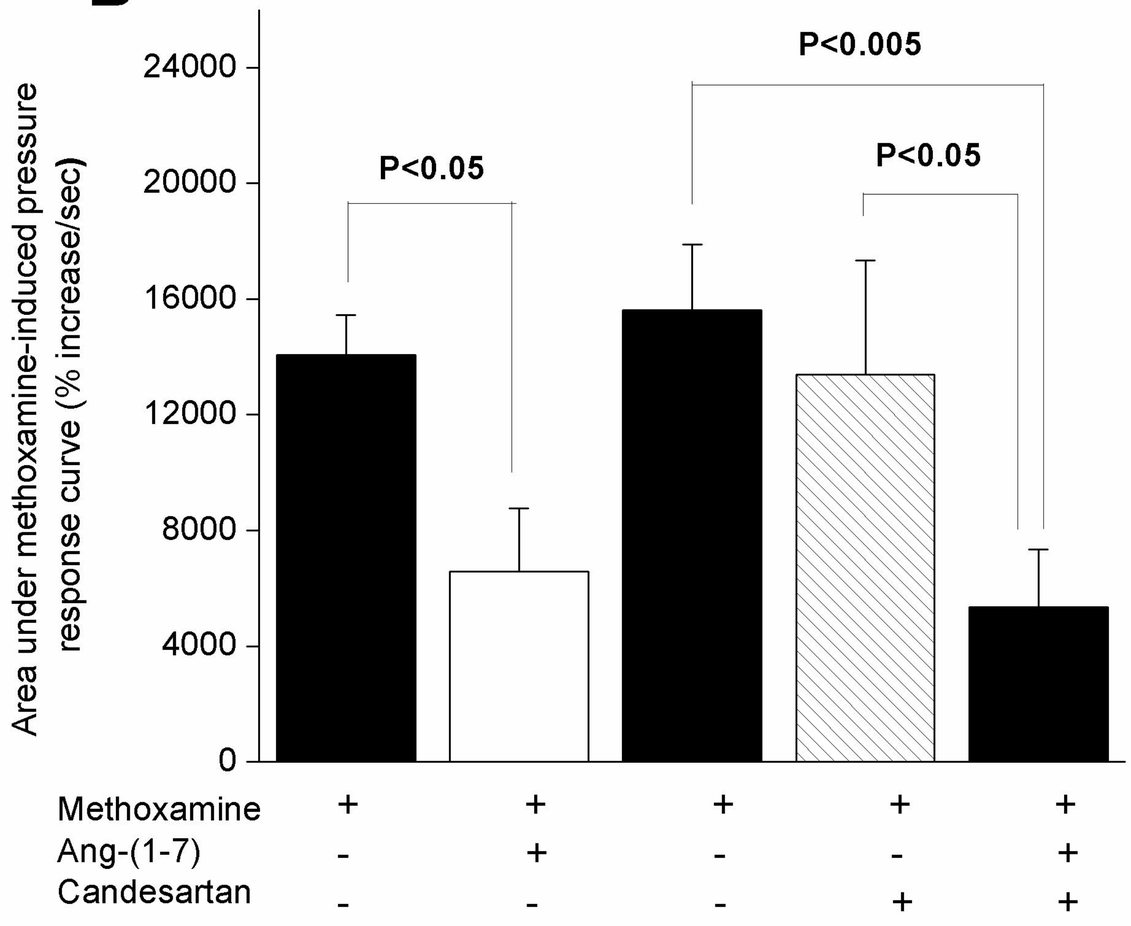
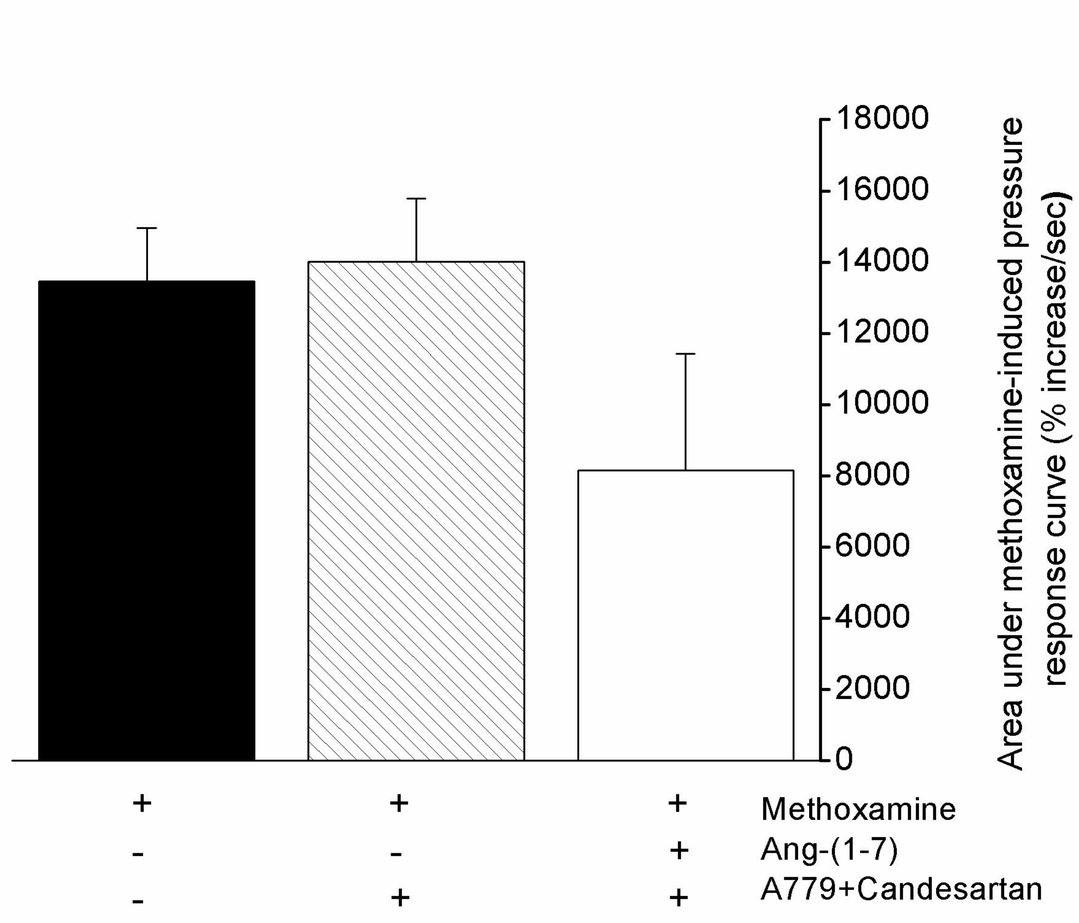
Time (sec)

B


P<0.005
P<0.05
P<0.05
24000
20000
16000
12000
8000
4000
0
Area under methoxamine-induced pressure response curve (% increase/sec)
Methoxamine + + + + +
Ang-(1-7) - + - - +
Candesartan - - - + +
Treatment groups

D


18000
16000
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8000
6000
4000
2000
0
Area under methoxamine-induced pressure response curve (% increase/sec)
+ + + Methoxamine
- - + Ang-(1-7)
- + + A779+Candesartan
Treatment groups

A

Portal pressure response to Ang II (cm H_2O)

10
8
6
4
2
0

0 30 60 120 180 240

Time (sec)

C

Portal pressure response to Ang II (cm H_2O)

8
6
4
2
0

0 30 60 120 180 240

Time (sec)

B

Area under Ang II-induced pressure response curve (% increase/sec)

20000
16000
12000
8000
4000
0

P<0.0005

Ang II	+	+	+	+	+
Ang-(1-7)	-	+	-	-	+
L-NAME	-	-	-	+	+

Treatment groups

D

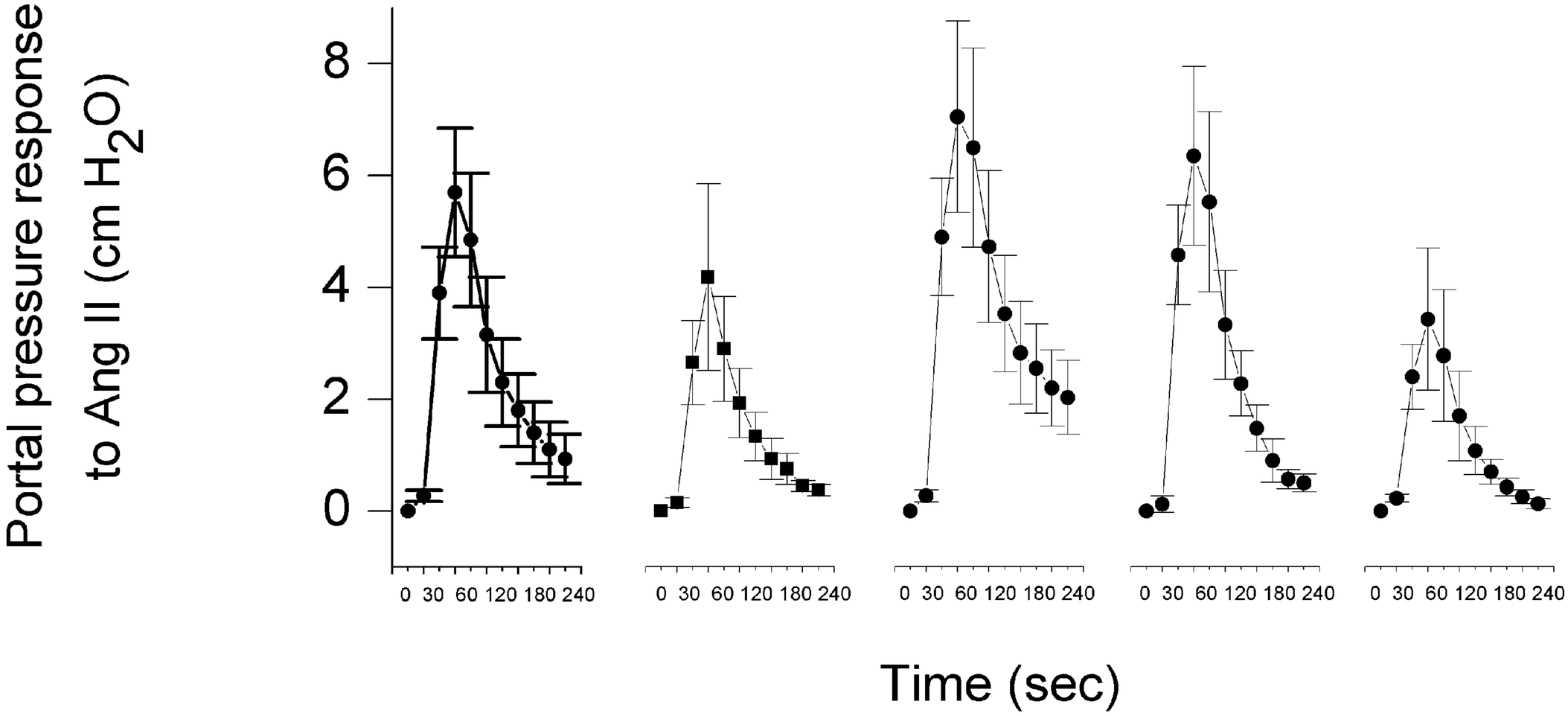
Area under Ang II-induced pressure response curve (% increase/sec)

20000
16000
12000
8000
4000
0

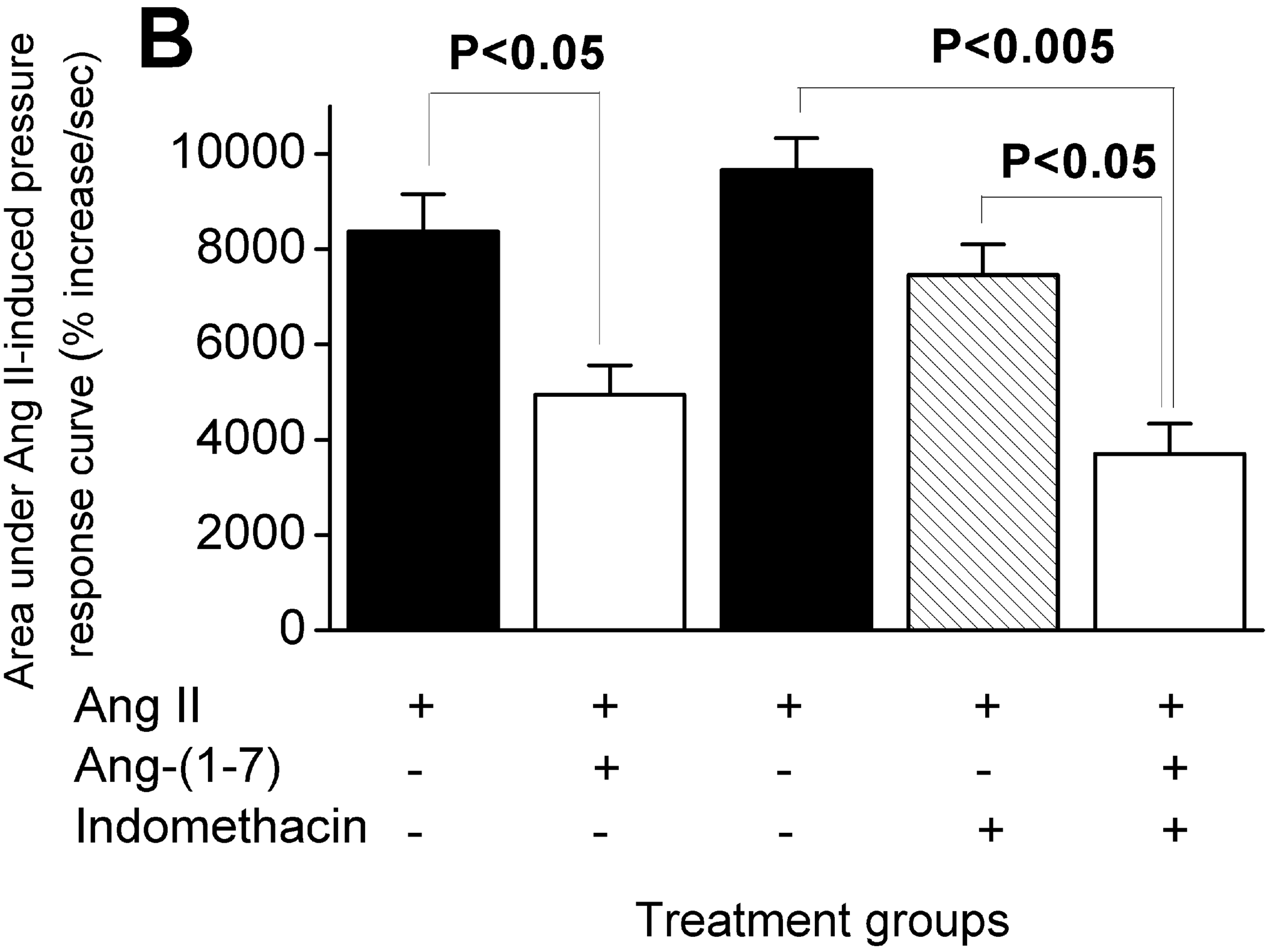
+	+	+	**Ang II**
-	-	+	**Ang-(1-7)**
-	+	+	**ODQ**

Treatment groups

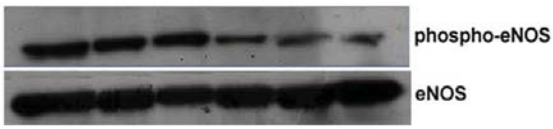
A


Portal pressure response to Ang II (cm H_2O)
8
6
4
2
0
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240
Time (sec)

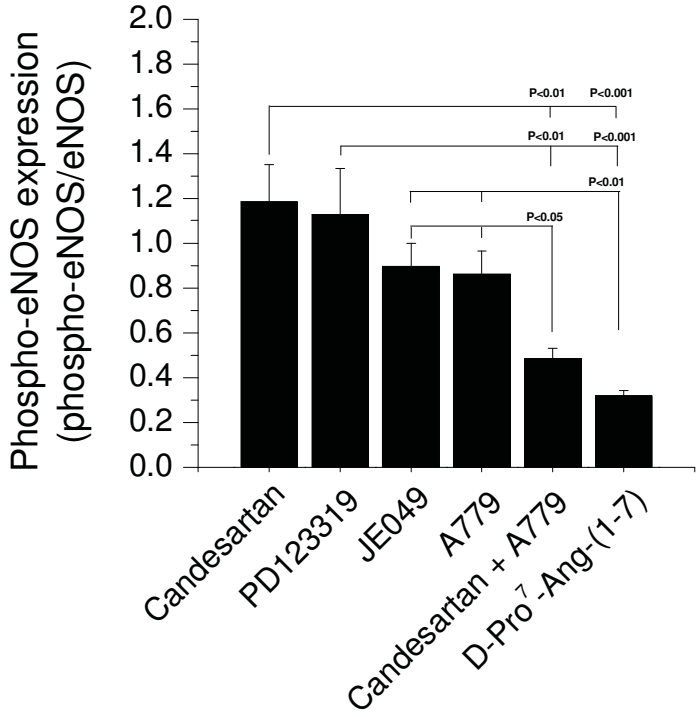
B


Area under Ang II-induced pressure response curve (% increase/sec)
P<0.05
P<0.005
P<0.05
10000
8000
6000
4000
2000
0
Ang II + + + + +
Ang-(1-7) - + - - +
Indomethacin - - - + +
Treatment groups

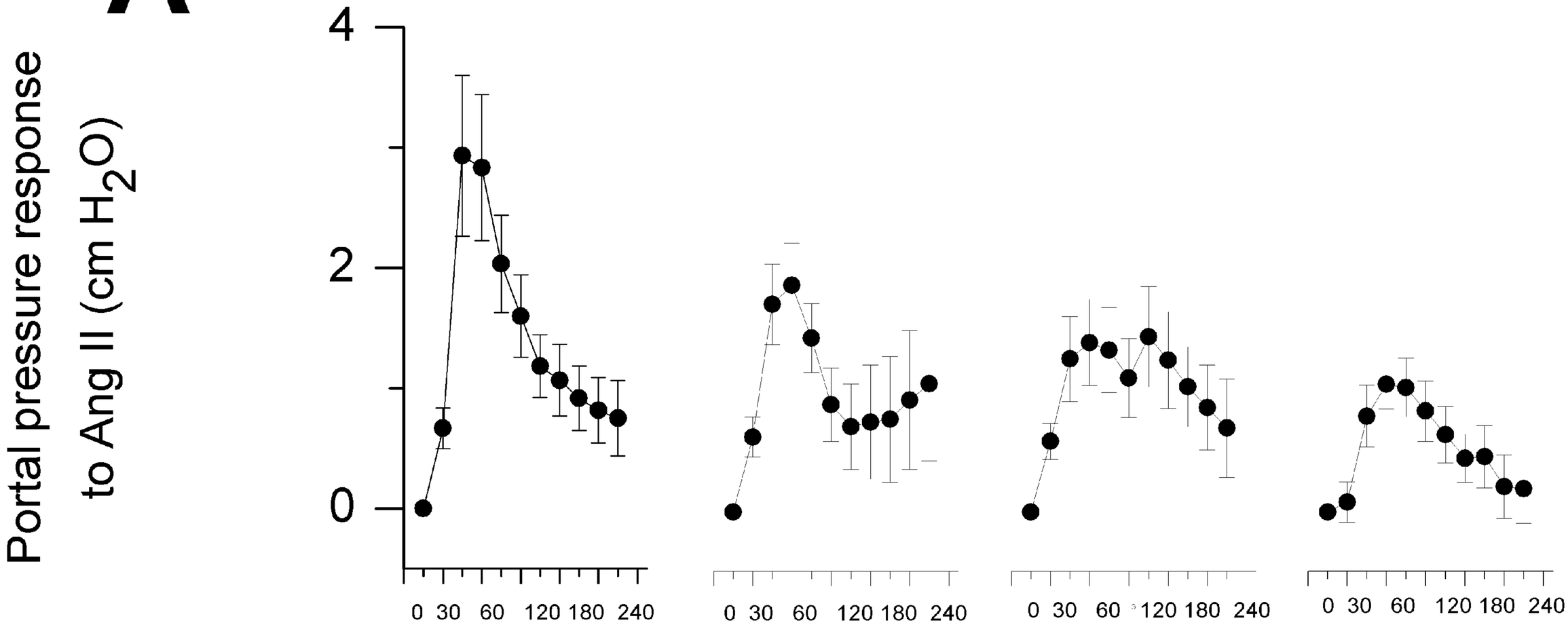
A


phospho-eNOS
eNOS

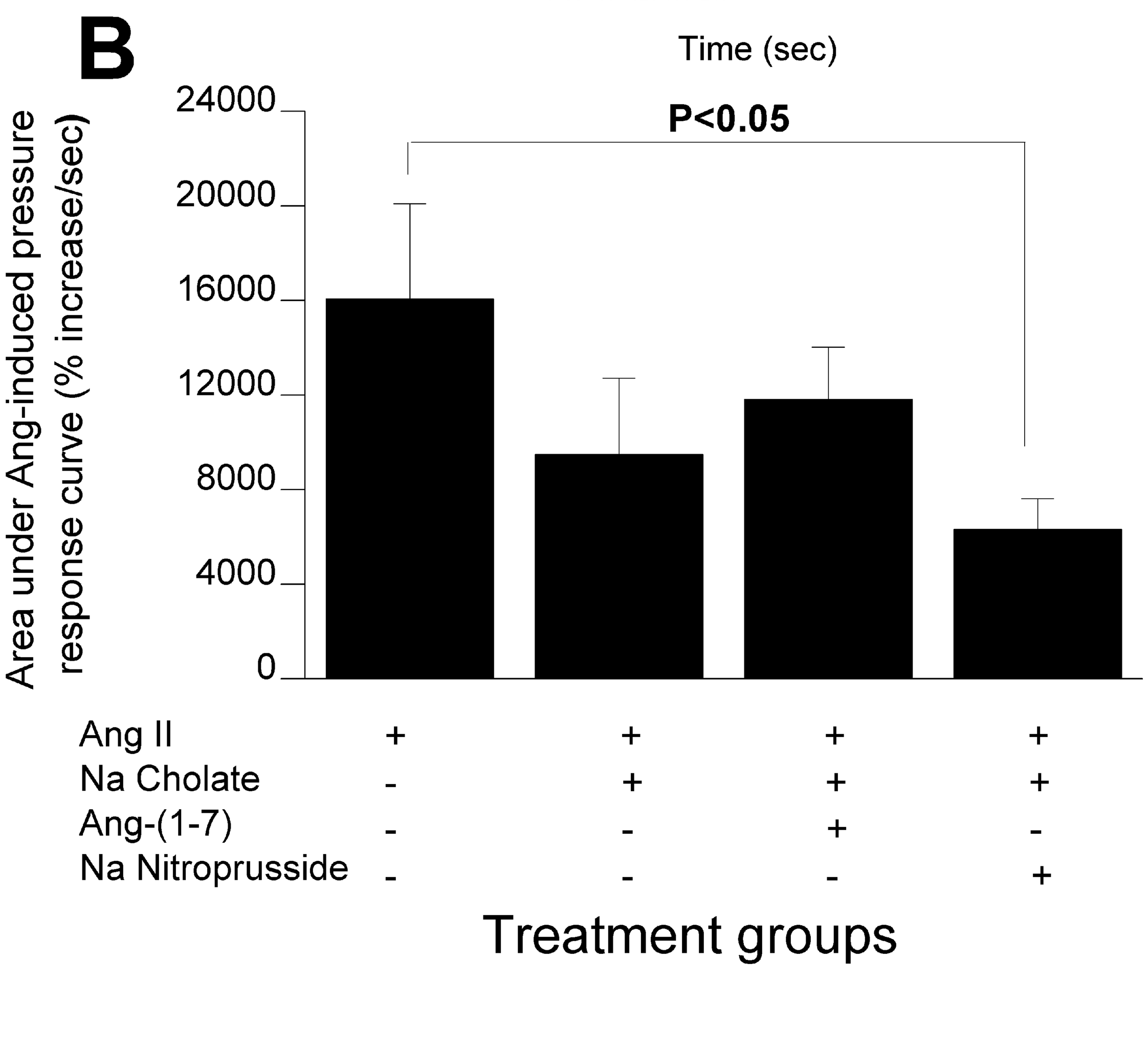
B


Phospho-eNOS expression
(phospho-eNOS/eNOS)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P<0.01
P<0.001
P<0.01
P<0.001
P<0.01
P<0.05
Candesartan
PD123319
JE049
A779
Candesartan + A779
D-Pro7-Ang-(1-7)

A


Portal pressure response to Ang II (cm H_2O)
4
2
0
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240
0 30 60 120 180 240
Time (sec)

B


Area under Ang-induced pressure response curve (% increase/sec)
24000
20000
16000
12000
8000
4000
0
P<0.05
Ang II + + + +
Na Cholate - + + +
Ang-(1-7) - - + -
Na Nitroprusside - - - +
Treatment groups