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THE ROLE OF THE HEME OXYGENASES IN ABNORMALITIES OF THE MESENTERIC CIRCULATION IN CIRRHOTIC RATS

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ABSTRACT

Carbon monoxide (CO), a product of heme metabolism by heme-oxygenase (HO), has biological actions similar to those of nitric oxide (NO). The role of CO in decreasing vascular responses to constrictor agents produced by experimental cirrhosis, induced by carbon tetrachloride (CCl_4), was evaluated before and after inhibition of HO with tin-mesoporphyrin (SnMP) in the perfused superior mesenteric vasculature (SMV) of cirrhotic and normal rats and in normal rats transfected with the human HO-1 (HHO-1) gene. Perfusion pressure and vasoconstrictor responses of the SMV to potassium chloride (KCl), phenylephrine (PE) and endothelin-1 (ET-1) were decreased in cirrhotic rats. SnMP increased SMV perfusion pressure and restored the constrictor responses of the SMV to KCl, PE and ET-1 in cirrhotic rats. The relative roles of NO and CO in producing hyporeactivity of the SMV to PE in cirrhotic rats were examined. Vasoconstrictor responses to PE were successively augmented by stepwise inhibition of CO and NO production, suggesting a complementary role for these gases in the regulation of reactivity of the SMV. Expression of constitutive (HO-2), but not of inducible (HO-1) HO, was increased in the SMV of cirrhotic rats as was HO activity. Administration of adenovirus containing HHO-1 gene produced detection of HHO-1 RNA and increased HO activity in the SMV within 7 days. Rats transfected with HO-1 demonstrated reduction in both perfusion pressure and vasoconstrictor responses to PE in the SMV. We propose that HO is an essential component in mechanisms that modulate reactivity of the mesenteric circulation in experimental hepatic cirrhosis in rats.

The heme-oxygenase (HO) system is the rate limiting step in heme degradation, releasing carbon monoxide (CO), biliverdin and free iron. Three HO isoforms (HO-1, HO-2, HO-3) have been identified that can carry out this reaction (Abraham, 1987; McCoubrey, 1992; Shibahara, 1993; McCoubrey, 1997). HO-1 is a 32 kDa heat shock protein (Shibahara, 1987) which is inducible by noxious stimuli (Lutton, 1992; Neil, 1995; Lu, 1998). HO-2, a 36 kDa protein, is constitutively expressed and found in abundance in brain, testis, liver (Trakshel, 1988; Maines, 1986) and endothelium (Zakhary, 1996). HO-3, although related to HO-2, is a product of a different gene with lesser ability than HO-2 to catalyze heme degradation (McCoubrey, 1997).

HO-2, which is localized in endothelial cells and adventitial nerves of blood vessels (Zakhary, 1996), functions in circulatory regulation. Furchgott and Jothianandan (1991) reported that CO, arising from the metabolism of heme by HO, dilated blood vessels, while inhibition of HO by tin-mesoporphyrin (SnMP) produced vasoconstriction (Kozma, 1999) and raised blood pressure (Johnson, 1995; Johnson, 1996). Heme, by activating HO, has been shown to dilate the ductus arteriosus (Coceani, 1997). Moreover, contiguous, if not overlapping, spheres of activity within the vasculature of nitric oxide (NO) and CO were suggested by the study of Zakhary (1996). The authors concluded that "the similarity of NOS and HO-2 localizations and functions in blood vessels and the autonomic nervous system implies complementary and possibly coordinated physiological roles for these two mediators." CO was shown to relax blood vessels via an endothelial-independent pathway linked to guanylate cyclase as its effects were accompanied by the accumulation of guanosine 3',5'-cyclic monophosphate (cGMP) levels in vascular smooth muscle (Christodoulides, 1995). Further, inhibition of guanylate cyclase attenuated the vasodilator effect of CO (Wang, 1997). In isolated gracilis muscle arterioles of rats which express HO-2,

HO inhibitors decreased the diameter of pressurized vessels (Kozma, 1999), likely by inhibiting potassium channels as a result of decreased CO production as has been reported for the thick ascending limb in response to inhibition of HO (Liu, 1999).

Mesenteric arteriolar vasodilatation and increased portal inflow play a major role in the development of portal hypertension in cirrhosis (Vorobioff, 1984; Shah, 1998). Various vasodilator compounds including glucagon, adenosine, prostacyclin, NO, α-calcitonin gene-related peptide, and adrenomedullin have been proposed as mediators of the mesenteric vascular abnormalities that occur both in cirrhosis and in prehepatic portal hypertension (Shah, 1998; Gatta,1999). In cirrhotic rats, inhibition of NO synthesis greatly reduced mesenteric blood flow and hyporeactivity of the mesenteric circulation to vasoconstrictor agents (Sieber, 1993). In rats with prehepatic portal hypertension, HO inhibition modified the hyporeactivity of the mesenteric circulation to vasoconstrictors (Fernandez, 2001).

The present study was designed to determine whether: 1) Augmentation of HO contributes to mesenteric vascular abnormalities in cirrhotic rats; 2) Gene transfer reproduces these alterations in normal rats; 3) NOS-NO and HO-CO systems interact in the mesenteric circulation to affect vascular tone and reactivity. To achieve these goals, the isolated perfused superior mesenteric vasculature (SMV) was studied in terms of: 1) Reactivity to constrictor agents in normal rats and in those with carbon tetrachloride (CCl_4)-induced cirrhosis; 2) Effects of inhibition of HO with SnMP in each group; 3) Effects on the SMV produced by transfection with the human HO-1 (HHO-1) gene. The participation of NO in the development of hyporeactivity of the mesenteric vasculature in cirrhotic rats was also examined. This issue was addressed by determining enhancement of the vascular responses

to constrictor agents as modified by successive inhibition of CO and NO production, and then reversing the order of inhibition, NO preceding CO. We were able to identify a significant component of each system, nitric oxide synthase (NOS)-NO and HO-CO, that contributed to the vascular hyporeactivity of cirrhotic rats, suggesting "complementary and possibly coordinated physiological roles" (Zakhary, 1996) for CO and NO. Our results indicate a key role for HO in the control of the mesenteric circulation as inhibition of HO activity with SnMP normalized mesenteric perfusion pressure and restored towards normal the response of the mesenteric vasculature to vasoconstrictor agents. We also found, quite unexpectedly, an increase in HO-2, the constitutive enzyme rather than HO-1, the inducible enzyme in the SMV in cirrhotic portal hypertension. Finally, HO activity augmented by HO-1 gene transfer, decreased mesenteric perfusion pressure and reduced the reactivity of the SMV to vasoconstrictor agents.

MATERIALS AND METHODS

Materials

Phenylephrine (PE) was purchased from Sigma Chemical Co., St. Louis, MI, and dissolved in distilled water. Endothelin-1 (ET-1) was purchased from Peninsula Laboratories, Belmont, CA, and dissolved and stored in 0.1% acetic acid at –20^{o}C. Stannous-mesoporphyrin (SnMP) was purchased from Porphyrin Products, Inc. Utah, USA. N^{G}-nitro-L-arginine methyl ester (L-NAME) was purchased from Sigma Chemical Co., St. Louis, MI, and dissolved in distilled water.

Animals

Adult male Sprague-Dawley rats (150-160 g b.w.) were purchased from Charles River Laboratories Inc, Wilmington, MA, and housed in an animal facility with controlled normal day-night cycle. The rats were maintained on a standard diet of Purina chow and were allowed free access to water and food until the night before experiments. Experiments were performed in accordance with the guidelines set forth by The American Physiological Society for ethical treatment of animals.

Induction of Cirrhosis

Cirrhosis was induced in animals (approx. 170 g b.w.) by combined treatment with CCl_4 and phenobarbital, the latter given in drinking water (Sacerdoti, 1991). After one week of phenobarbital administration, CCl_4 was given by gavage, starting with a dose of 30 μl, and then increasing doses were administered according to changes in body weight. CCl_4 was administered once a week and then twice a week for 8-10 weeks. Treatment was terminated

one week prior to conducting experiments. Rats on phenobarbital during the time of treatment were used as controls. Free access to standard chow was allowed throughout the study. In all the CCl_4-treated rats used for study, portal pressure was measured by a Statham transducer after direct cannulation of the portal vein before the experiments; rats with portal pressure < 8 mmHg were excluded from the study.

Perfusion of isolated mesenteric artery (MacGregor preparation)

The superior mesenteric artery was cannulated with a PE-60 catheter and perfused with 25 ml of warm Krebs-Hanseleit buffer. After the superior mesenteric artery was isolated with its mesentery, the gut was excised near its mesenteric border. The mesenteric arterial bed was then brought in a 37°C water-jacketed container and perfused at a constant rate of 0.8 ml/min/100g b.w. with oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution (NaCl 118.5 mmol/L; KCl 4.7 mmol/L; $CaCl_2$ 2.5 mmol/L; KH_2PO_4 1.2 mmol/L; $MgSO_4$ 1.2 mmol/L; $NaHCO_3$ 25.0 mmol/L; dextrose 11.1 mmol/L; pH 7.4 at 37°C. The effluent was constantly collected, and the pressure measured by a Statham transducer.

Vascular responses to agonists and antagonists

After an initial 30-minute basal period, concentration-response curves were constructed with potassium chloride (KCl), -5.22 to – 3.6 log mol, PE, -9.3 to –6.3 log mol, and ET-1, -11.7 to –10.5 log mol, in the presence and absence of HO inhibition with SnMP (10 μM). This concentration of SnMP does not inhibit NOS activity nor activity of soluble guanylate cyclase (Meffert, 1994; Zakhary, 1996). It does, however, inhibit completely the increase in HO activity produced by either heme or $SnCl_2$ in endothelial cells as well as smooth muscle cells (Li Volti, 2002). SnMP was added to the buffer 10 min before vasopressors were

examined. Concentration response curves were constructed by adding increasing bolus amounts (20-50 μl) of agonists. The maximum response was obtained within 60 sec; perfusion pressure returned to baseline values in 2-3 min after KCl and PE and after 10-15 min after ET-1. Increasing doses were added after return to the baseline pressure.Experiments performed to determine the differential effects of inhibition of HO and NOS in cirrhotic animals were done using single doses of PE, -7.3 log mol. After the initial equilibration period, the responses to PE were evaluated without inhibitors, and then after inhibition of HO with SnMP (10 μM) (n= 6) or L-NAME (100 μM) (n= 6). After restoration of baseline conditions, the second inhibitor was added to the perfusate and responses to the agonists were again evaluated. L-NAME was dissolved in saline. In rats transfected with the HHO-1 gene, the vasoconstrictor responses of the SMV to PE, -8 log mol, were evaluated after an equilibration period, before and after inhibition of HO with SnMP (10 μM).

Western blot analysis

For the detection of HO-1 and HO-2 protein, microsomes were prepared from tissues as previously described (Sacerdoti,1988); 20 μg of microsomal protein was analyzed by Commassie staining of the gels and by immunoblot analysis of proteins transferred to nitrocellulose membrane. Briefly, membrane blots were blocked in 5% non-fat milk overnight to block non-specific binding of antibodies. The blots were then incubated with mouse monoclonal anti-HO-1 or HO-2 antibodies (1:2000) (StressGen Biotechnology, Vancouver, Canada) for one hour and washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) three times. The blot was subsequently incubated with rabbit anti-mouse IgG antibody (1:4000) for one hour. The blot was washed three times with TBST and

immunoreactive proteins were visualized by means of enhanced chemiluminescence (ECL) reagent and exposure to an autoradiographic film.

Microassay for measurement of HO activity

HO activity was measured as previously described (Chernick, 1989). Briefly, tissue homogenates (100 μg protein) were incubated in a NADPH generating system, $MgCl_2$ 20 μM, heme 30 μM and 1μCi/ml of ^{14}C-heme. HO activity was measured as the conversion of heme to bilirubin in the presence of biliverdin reductase. ^{14}C-bilirubin was extracted with chloroform and radioactivity was measured. Results were expressed as cpm/mg protein.

Construction of the Adenovirus vector containing human HO-1 cDNA

HHO-1 cDNA with adenovirus vector was constructed using a mammalian transfection kit (Stratagene; La Jolla, CA), previously described by Abraham, (1995b). Briefly, the human cDNA plasmid was constructed as follows: a Xho1-Xba1 fragment of a HHO-1 expression vector, pRc/CMVHO-1, was cloned between Xho1 and Xba1 of the pBacPAC8 to generate pBacPAC8 HHO-1. The Bg1II-BamH1 fragment of the pBacPAC8HHO was then introduced into the Bg1II site of pAdBg1II. The human embryonic cell line, 293 cells (ATCC#1573-CRL), was co-transfected with 10 μg of EcoR1-digested adenoviral HHO-1 and 1 μg of Cla1-digested dL7001DNA by calcium phosphate co-precipitation, using a mammalian transfection kit. HHO-1 adenovirus construct was replicated and encapsulated into an infectious virus as described (Abraham, 1995a). The virus was concentrated by centrifugation by using Ultrafree-MC filters (Millipore, Bedford, MA). The viral titers were determined by plaque assay using 293 cells. The viral stock was stored at –80^{o}C until use.

In vivo gene transfer

Eight rats were injected intracardiacally with HHO-1 adenovirus suspension (10^{12} pfu/300g b.w.). Four rats were treated with the adenovirus without the HO-1 gene. The animals were acclimatized for one week prior to the start of the experiment, and experiments were performed one week after viral administration. Hemodynamic studies were performed as described above in 6 rats treated with the gene and 4 rats treated with the empty virus.

RNA extraction and RT-PCR

SMV were homogenized in liquid nitrogen and total RNA was extracted with guanine thiocynate, first purified with phenol-chloroform followed by an additional purification with chloroform, and then precipitated with isopropanol (Chomczynski and Sacchi, 1987). Total RNA was resuspended in autoclaved diethyl pyrocarbonate-water and quantified using Beckman DU7400 spectrophotometer. To verify the quality of RNA, 10 μg of total RNA was denatured and separated by 1% agarose gel (containing 2.2 M formaldehyde) electrophoresis. The RNA was visualized under UV light with the help of Ethidium bromide. Primers were designed to amplify specific fragments of the HHO-1 and/or rat HO-1. The primers used did not recognize HO-2 sequences. The RT-PCR for HHO-1 was performed by using the Advantage RT-PCR kit (Clonetech Laboratories, Inc., Palo Alto, CA), and the primers designed to amplify a PT-PCR product with a predicted size of 555 base pairs (bp). Briefly, 1 μg of total RNA was reverse transcribed using oligo (dT^{18}) primer to synthesize the second strand and to obtain the cDNA. The reverse transcription was performed at 42^{o}C for one hour, and the reaction tubes were placed on ice. The PCR of HHO-1 was performed by adding PCR mix prepared with sense primer (5'CAGGCAGAGAATGCT GAGTTC-3'), antisense primer (5'-quenched by heating it at

95^{o}C for five minutes and then the GATGTTGAGCAGGAACGCAGT-3'), dNTP, Taq polymerase, ^{32}P-dCTP and reaction buffer. To amplify the endogenous rat HO-1, the primers were designed from the rat cDNA sequence (sense primer: 5'-TGAAGGAGGCCACCAAGGAG-3'; antisense primer: 5'-CCCCTGAGAGGTCACCCAGG-3'), which amplify a 356-bp fragment. RT-PCR was performed as described above.

Statistical analysis

Results were expressed as means ± SEM. Concentration-response data for PE and KCl derived from each vessel were fitted separately to a logistic function by non-linear regression and the maximum asymptote of the curve (R_{max}) and concentration of agonist producing 50% of the maximal response (EC_{50}), which was expressed as negative log mol, were calculated using commercially available software (Prism 2.01, GraphPAD Software, San Diago, CA). Concentration-response data were analyzed by two-way analysis of variance. Differences between groups were evaluated by unpaired Student's t-test. The effects of inhibition of HO on the action of vasoconstrictors were evaluated by paired t-test. Statistical significance was set at $p< 0.05$.

RESULTS

We examined the effects of inhibition of HO on perfusion pressure in the isolated perfused SMV obtained from normal and cirrhotic rats. We addressed the effects of inhibition of HO with SnMP (10 μM) on responses of mesenteric arteries to three vasopressor agents: KCl, PE and ET-1. Figure 1 shows the baseline perfusion pressure in normal and cirrhotic rats with and without SnMP. Mesenteric perfusion pressure was lower in cirrhotic than in normal rats ($p<0.05$). Further, inhibition of HO did not significantly increase (6 ± 3 %) mesenteric perfusion pressure in control animals (n=14), whereas in cirrhotic rats a significant increase in pressure of 20 ± 3 % was observed (n=14).

Next we examined whether inhibition of HO by SnMP (10 μM) can restore normal vasopressor responses to KCl, PE, and ET-1 which were depressed in cirrhotic animals. The vasoconstrictor responses to KCl, PE, and ET-1 were shifted to the right, the maximal response was blunted, and the EC_{50} for KCl was increased (Figures 2-4). SnMP significantly increased the response to KCl in normal (n=8) and in cirrhotic rats, and restored to normal the responsiveness to KCl in the SMV (n=8) (Figure 2). SnMP did not affect the vasoconstrictor response to the sympathomimetic agent, PE, in normal rats (n=8) and restored to normal the responses of the SMV to the vasoconstrictor agent in cirrhotic rats (n=8) (Figure 3). SnMP increased the vasoconstrictor responses of SMV to ET-1 in cirrhotic, but not in normal rats (n=8); in cirrhotic rats, the responses to ET-1 in SMV were restored to normal (n=8) (Figure 4). These results indicate that SnMP-induced inhibition of HO in cirrhotic rats restores towards normal the responsiveness of the SMV to vasopressor agents.

We also examined the contributions of NO and CO to attenuating the response to PE in the SMV in view of reports that NO contributes to hyporeactivity of the SMV to pressor agents in cirrhotic rats (Sieber, 1993). SnMP was used to inhibit HO because of its demonstrated selectivity at the concentration employed (10 μM) and its solubility (Zakhary, 1996). It was important that SnMP did not inhibit NOS as our study was directed at the vascular effects of CO in the SMV of cirrhotic rats. The relative contribution of HO-CO and NOS-NO to producing hyporeactivity of the SMV of cirrhotic rats to PE was assessed by determining the effects of inhibiting each system separately, i.e., HO-CO with SnMP and NOS-NO with L-NAME, followed by inhibition of the other system, then reversing the order of inhibition of CO and NO production (Figure 5A and B). After achieving a significant reduction in vascular hyporeactivity by inhibition of either HO or NOS, additional reduction was obtained on inhibiting the other gas-producing system. For example, in Figure 5A, the constrictor response of the SMV of cirrhotic rats to PE was increased by inhibition of HO activity with SnMP and was further enhanced by inhibition of NOS activity with L-NAME. Then, the order of inhibition of HO and NOS activities was reversed in Figure 5B. After the combined inhibition of HO and NOS, the constrictor responses of the SMV of cirrhotic rats to PE were fully restored. Moreover, the effects of either SnMP or L-NAME in restoring to normal the vasoconstrictor responses to PE (Figure 5) in cirrhotic rats, were similar irrespective of the order of administration of SnMP or L-NAME. These findings are in accord with the findings of Thorup (1999) that CO "may synergize with NO in eliciting vasorelaxation and modulating basal vascular tone" by effects on vascular mechanisms as, for example, those served by guanylyl cyclase and cGMP.

We examined whether responsiveness of mesenteric arteries of cirrhotic rats to vasopressor agents was associated with increased expression of HO-1 and HO-2 in this vascular bed. Western blot analysis of mesenteric blood vessels obtained from normal and cirrhotic rats (Figure 6A), demonstrated significantly higher levels of HO-2, but not of HO-1, in vascular tissues obtained from cirrhotic rats (lanes 5-8) compared to those from normal rats (lanes 1-4). HO upregulation was quantified by densitometry-based analysis of HO-1 and HO-2 proteins after blotting the ratio of each sample. As seen in the densitometry analysis (Figure 6B), HO-2 was increased significantly in SMV obtained from cirrhotic rats when compared to those of control rats.

To assess the effect of the increase in HO-2 protein on HO activity, cell homogenates obtained from SMVs of control and cirrhotic rats were used to measure the enzyme activity as indicated by C^{14} heme catabolism to C^{14} bilirubin (Figure 7). Bilirubin formation was significantly increased in SMV samples obtained from cirrhotic rats compared to those obtained from control rats ($p< 0.05$). These findings help explain: 1) the hyporeactivity of the mesenteric vasculature to vasoconstrictor agents; and 2) why inhibition of HO helps restore the responsiveness to vasoconstrictor agents of the SMV obtained from cirrhotic animals.

To examine further our hypothesis that increased HO levels in mesenteric arteries in experimental cirrhosis are responsible for vasodilation and decreased reactivity of SMV to vasoconstrictor agents, we injected 8 normal rats with adenovirus containing the HHO-1 gene (10^{12} pfu/300 g body weight). These preparations of adenovirus-mediated HO-1 gene construct were shown to increase HO-1 gene expression within 7 days of infection (Abraham, 2000). The ability of adenovirus-mediated HHO-1 to infect mesenteric blood

vessels was assessed after 7 days and total RNA from SMV was assessed for the presence of the HHO-1 by RT/PCR and enzyme activity (Figure 8). Positive signals were obtained for the HHO-1 gene when amplified with HHO-1 primers within 7 days of infection. Injection of adenovirus mediated HHO-1 gene transfer resulted in the expression of HO-1 in mesenteric blood vessels. The HHO-1 mRNA signal was not detectable in mesenteric arteries infected with empty adenoviral constructs (Figure 8, lane 2). To determine the quality of total RNA of SMVs, RT-PCR analysis was performed using GAPDH-specific primers. Amplification of GAPDH/PCR products occurred in all RNA tested (Figure 8). HO protein in tissues obtained from rats infected with adenoviral constructs was significantly higher (1240 ± 200 vs. 187 ± 13 cpm/mg protein, $n=4$, $p< 0.01$) than in tissues obtained from rats infected with empty adenoviral constructs.

Finally, we examined the effect of HHO-1 transfection on the mesenteric circulation. As is evident in Figure 9, perfusion pressure and the increase in perfusion pressure of the SMV induced by PE were attenuated in animals transfected with the HHO-1 gene. Inhibition of HO with SnMP partially reversed these abnormalities. No difference was evidenced in rats treated with the empty virus. These results support our hypothesis that increased HO expression and activity in cirrhosis contribute to the aberrant responses of the SMV to vasoconstrictor agents.

DISCUSSION

Portal hypertension, a major complication of cirrhosis, is the basis for the development of esophageal varices, ascites, renal failure, and hepatic encephalopathy (Gatta, 1999). Mesenteric arterial resistance is normally high, whereas in cirrhosis, dilatation of the mesenteric vasculature is associated with an increase in portal inflow, a major determinant of portal hypertension (Shah, 1998; Gatta, 1999). Further, in experimental cirrhosis, the responsiveness of the mesenteric circulation to pressor agents is greatly impaired (Sieber, 1993; Gagano, 1997). A number of endogenous vasoactive compounds, previously enumerated, has been proposed to mediate this lack of vascular responsiveness (Shah, 1998; Gatta, 1999).

In the present study, we have shown in rats with CCl_4-induced hepatic cirrhosis that the resulting attenuation of mesenteric vascular constrictor responses to diverse agonists could be reversed by inhibition of HO, highlighting the potential importance of the HO-CO system to the regulation of circulatory tone and vascular reactivity. Quite unexpectedly, the HO isoform responsible for mesenteric vascular hyporeactivity was shown to be the constitutive enzyme HO-2, not HO-1, the inducible enzyme. This finding is not unique as HO-2 levels are increased by estrogens in endothelial cells (Tschugguel, 2001) and by corticosterone in the brain (Weber, 1994). Further, this finding stands in contrast to the induction of HO-1 activity in response to portal hypertension produced by ligation of the portal vein (Fernandez, 2001) and helps in distinguishing critical differences between these two experimental approaches, CCl_4-induced cirrhosis vs portal vein constriction, to the production of altered responses of the mesenteric vasculature. For example, in contrast to CCl_4-induced cirrhosis, HO inhibition in rats with ligated portal veins did not alter mesenteric vascular hyporeactivity to an α_1-adrenergic agonist (Fernandez, 2001). Blockade of NO production

was required to reverse the hyporeactivity to methoxamine in this model of portal hypertension.

We also examined potential interactions of the NOS-NO and HO-CO systems in the mesenteric vasculature in view of the demonstrated contribution of NO to splanchnic vascular hyporeactivity in cirrhotic rats (Sieber, 1993). Our findings suggest that these systems are complementary in the mesenteric circulation of the cirrhotic rat, which agrees with the "coordinated functions of NO and CO" proposed by Zakhary et al. (1996) and is in accord with the presence in endothelial cells of both NOS and HO (Zakhary, 1996), as well as the ability of each system to activate soluble guanylate cyclase and increase cGMP (Abraham, 2002). Alternatively, rather than overlapping spheres of activity of NO- and CO-mediated vascular effects reflecting colocalization of NOS and HO isoforms in the endothelium, segmental separation within a vasculature -- eNOS predominating in large arteries and HO-2 in small arteries and microvessels -- may also give rise to coordinated and complementary responses of the NOS-NO and HO-CO systems (Zakhary, 1996). A recent study demonstrated that, despite eNOS gene deletion, mice with portal hypertension developed a hyperdynamic circulation, indicating the importance of other vasodilator systems in the production of splanchnic circulatory changes in portal hypertensive animals (Iwakiri, 2002).

The possibility that SnMP inhibits NOS as well as HO under conditions of our study is unlikely as weak inhibition of NOS with SnMP can be obtained only at a 10 times higher concentration (100 μM) than that used in our study (10 μM) (Meffert, 1994; Zakhary, 1996). Further, SnMP, in the concentration used in our study (10 μM), was shown to be without

effect on eNOS activity in the vasculature despite colocalization of HO-2 and eNOS in the endothelium of the several vascular beds studied (Zakhary, 1996). This specificity of SnMP relative to HO when compared to NOS has also been reported for the CNS (Meffert, 1994). Further, the data presented in Figure 5A and B show that prior administration of SnMP does not notably affect the action of L-NAME in augmenting the constrictor response of the SMV to PE, indicating that the activity of NOS is not, to a readily detectable degree, affected by prior treatment with SnMP under the conditions of our study. Nonetheless, an effect of NO on HO gene expression and activity may occur over the long-term as NO has been shown to induce HO-1 in endothelial cells in vitro (Hartsfield, 1997). On the other hand, transfection of rat endothelial cells with HHO-1, which induced a four-fold increase in HO activity, did not affect either eNOS protein or NO production (Abraham, 2002).

Transfection of normal rats with HHO-1 provided additional support for the role of CO as an essential component in mechanisms that modulate reactivity of the mesenteric vasculature in experimental cirrhosis. The transfection was highly effective and HHO-1 mRNA expression was evident in the PCR analysis of the mesenteric vasculature (Figure 8). Further, HO activity in mesenteric arteries from transfected rats was significantly increased compared to the activity in rats treated with the empty virus. Transfected rats showed hemodynamic characteristics similar to or even more pronounced than those of cirrhotic animals; viz., perfusion pressure and reactivity of the mesenteric vasculature to constrictor agents were decreased (Figure 9). We have reported that adenovirus-mediated HHO-1 gene transfection was associated with a decrease in microsomal heme and release of CO (Abraham, 2000). Moreover, retrovirus-mediated HO gene transfer into endothelial cells, as we have shown, increases cGMP and protects against oxidant-induced cell injury (Yang, 1999). The increase

in HO activity produced by this strategy may prove useful as a means of modifying abnormalities of regional vascular beds.

Taken together, our findings support a role for HO-CO that may synergize with NOS-NO in regulating the mesenteric circulation; they provide a conceptual basis for examining abnormalities of vascular beds in human hepatic cirrhosis. The systemic circulation, in addition to the splanchnic vasculature, is affected by hepatic cirrhosis associated with a decline in systemic vascular resistance in human subjects (Schrier,1998) associated with increased CO production, the magnitude of which was correlated with the severity of the cirrhosis (De Las Heras, 2003). Inhibition of HO has also been reported to reverse vascular hyporeactivity to norepinephrine in cirrhotic patients (Sacerdoti, 2002). CO lowers blood pressure in rats by participating in the regulation of basal tone in resistance blood vessels (Johnson, 1995; Kozma, 1999). In normal rats, inhibition of HO increased blood pressure (Johnson, 1995), while in the SHR, but not in the normotensive Wistar Kyoto rat (WKY), stimulation of HO with $SnCl_2$ decreased blood pressure (Sacerdoti, 1989). The present study has clinical implications: inhibition of HO may serve as a therapeutic option in cirrhotic patients, possibly improving not only portal hypertension but also systemic hemodynamics and, as a consequence, preserving renal function and reducing portal hypertension.

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Footnote:

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FIGURE LEGENDS

Figure 1. Effect of inhibition of heme-oxygenase (HO) with tin mesoporphyrin (SnMP) on perfusion pressure of superior mesenteric vasculature (SMV) in normal (n= 14) and cirrhotic (n= 14) rats. Results are presented as mean±SEM. *= $p< 0.05$ vs. controls; = **$p< 0.05$ vs. baseline.

Figure 2. Pressor responses to bolus injections of potassium chloride (KCl) into the superior mesenteric vasculature (SMV) in normal (■ n= 8) and cirrhotic (○ n= 8) rats, before (A) and after (B) inhibition of heme-oxygenase (HO) with tin mesoporphyrin (SnMP) (10 μM). *= $p< 0.05$ vs. cirrhotic rats (dose-response) and the EC_{50} and R_{MAX} values of normal rats.

Figure 3. Pressor responses to bolus injections of phenylephrine (PE) into the superior mesenteric vasculature (SMV) in normal (■ n= 8) and cirrhotic (○ n= 8) rats, before (A) and after (B) inhibition of heme-oxygenase (HO) with tin mesoporphyrin (SnMP) (10 μM). *= $p< 0.05$ vs. cirrhotic rats (dose-response) and the EC_{50} and R_{MAX} values of normal rats.

Figure 4. Pressor responses to bolus injections of Endothelin-1 (ET-1) into the superior mesenteric vasculature (SMV) in normal (■ n= 8) and cirrhotic (○ n= 8) rats, before (A) and after (B) inhibition of heme-oxygenase (HO) with tin mesoporphyrin (SnMP) (10 μM). *= $p< 0.05$ vs. cirrhotic rats.

Figure 5 A-B. Effect of inhibition of heme-oxygenase (HO) with tin mesoporphyrin (SnMP) (10 μM) (n= 6) (A) and of nitric oxide synthase (NOS) with N^G-nitro-L-arginine methyl ester (L-NAME) (100 μM) (n= 6) (B) and of both HO and NOS (A-B) on the response to phenylephrine (PE) (-7.3 log mol) in the superior mesenteric vasculature (SMV) of cirrhotic rats. *= $p< 0.05$ vs. baseline; **= $p<0.05$ vs PE; ***= $p<0.05$ vs SnMP or L-NAME alone.

Figure 6. A) Western blot analysis of inducible heme-oxygenase (HO-1) and constitutive heme-oxygenase (HO-2) in the superior mesenteric vasculature (SMV) of normal (lanes 1-4) and cirrhotic (lanes 5-8) rats. The expression of β-actin is an index of the adequacy of sample loading. B) Quantitative densitometric evaluation of HO-1 and HO-2 in the SMV of control and cirrhotic rats. * = $p< 0.05$ vs. control rats.

Figure 7. HO activity in superior mesenteric vasculature (SMV) of control and cirrhotic rats. Results are the mean ± SE of the amount of C^{14} bilirubin formed (cpm/mg protein, n=4). * = $p< 0.01$ vs. control rats.

Figure 8. Human HO-1 (HHO-1) mRNA expression in the superior mesenteric vasculature (SMV) from rats injected with recombinant adenovirus expressing HHO-1 gene (Ad-HHO-1). A representative RT/PCR autoradiogram showing lane 1; positive control, lane 2; control adenovirus (Ad-control) treated SMV, and lanes 3-6; SMV from 4 different rats (R1-R4) treated with Ad-HHO-1. Rats were injected with Ad-HHO-1 viral particles (10^{12} PFU/300 g body weight, n=8). PCR analysis of HHO-1 specific product was done 7 days after injection. The lower panel shows equal amplification of GAPDH transcripts.

Figure 9. Effect of transfection of normal rats (n=6) with human HO-1 (HHO-1) gene on perfusion pressure in the superior mesenteric vasculature (SMV) and the response to phenylephrine (PE) (-8 log mol), before and after inhibition of heme-oxygenase (HO) with tin mesoporphyrin (SnMP) (10 μM). Normal rats were injected with 10^{12} pfu/300 g body weight of adenovirus containing the HHO-1 gene. After 1 week, the SMV was perfused as described in Figure. 1, and compared with rats treated with the empty virus. *= $p< 0.05$ vs. controls; **= $p< 0.05$ vs. PE controls; ***= $p< 0.05$ vs. PE + SnMP controls.

Fig. 1

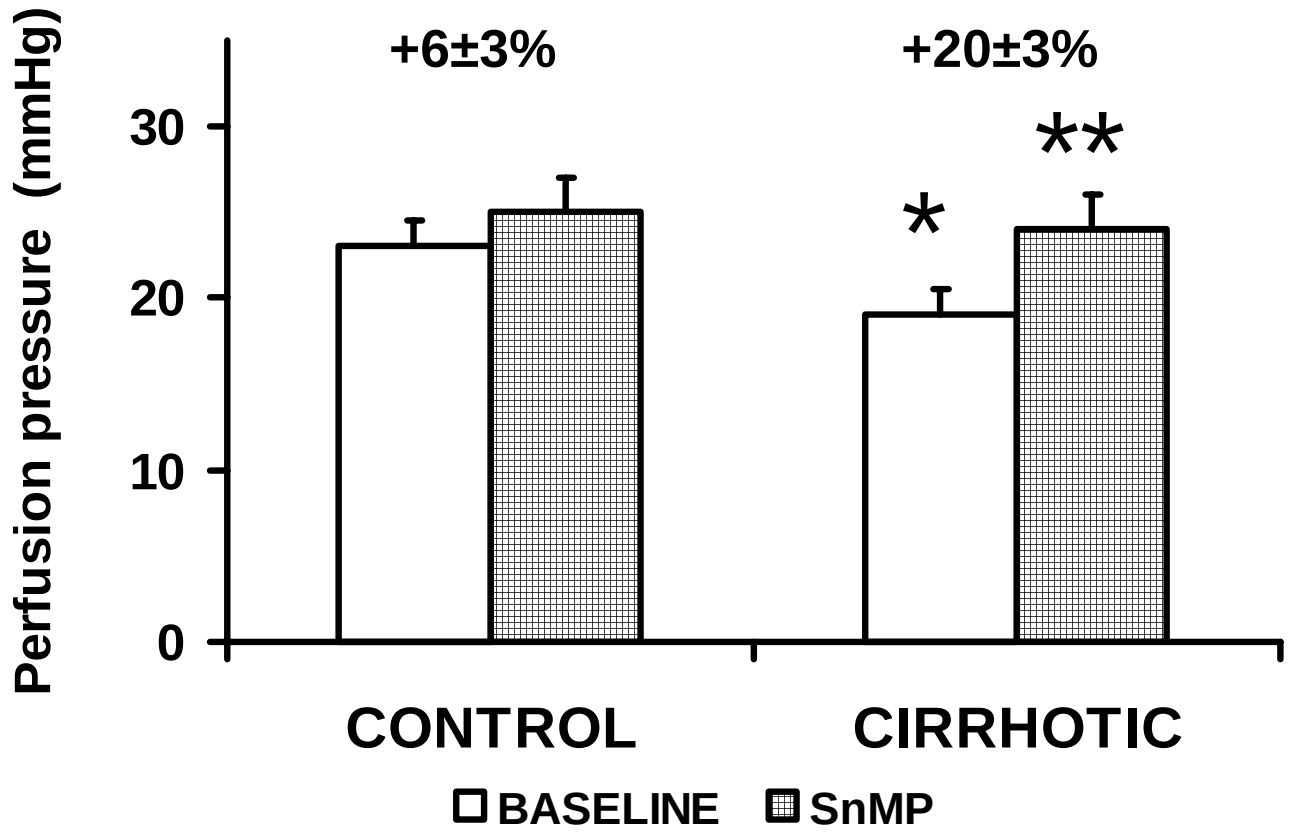
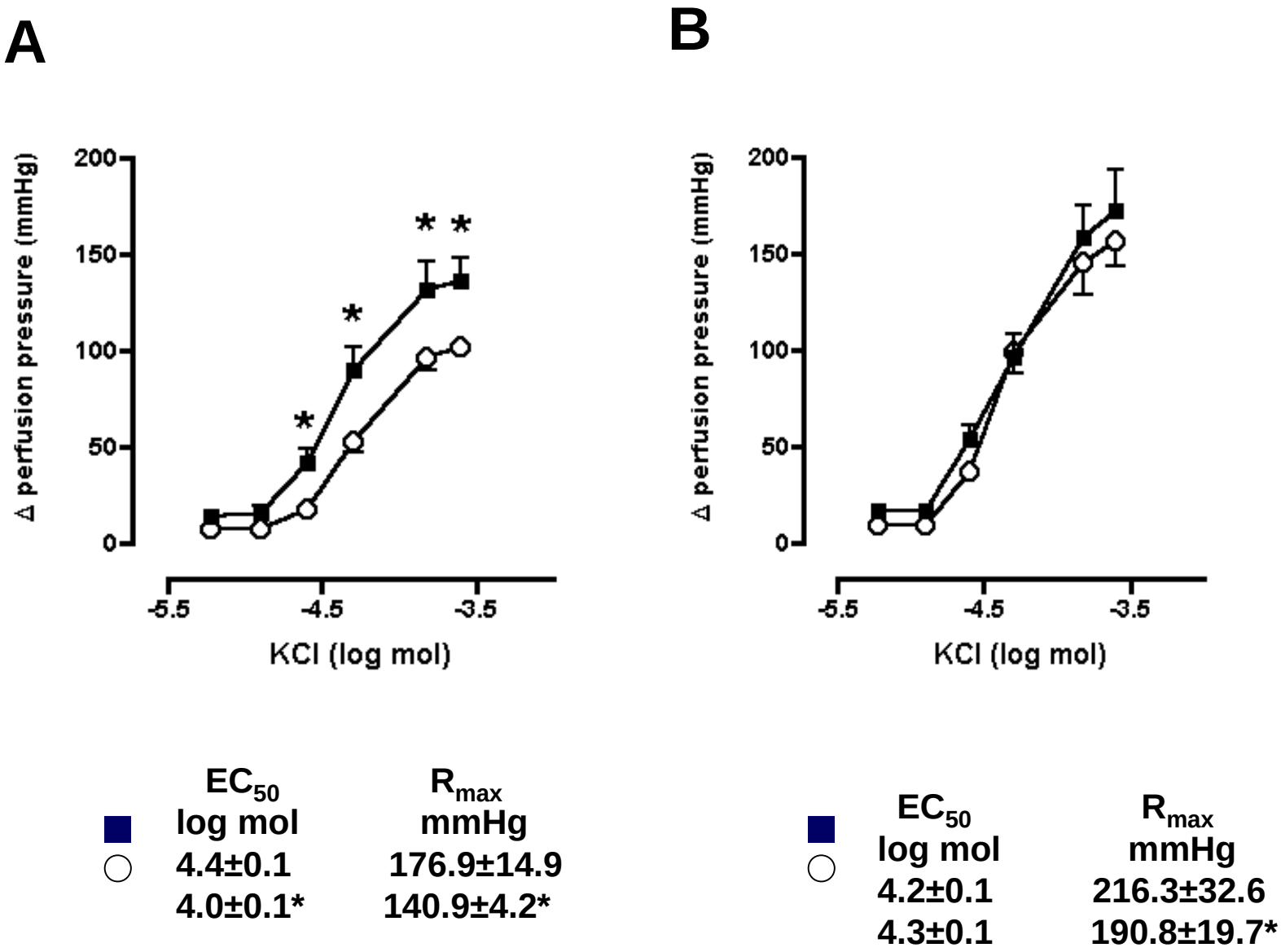


Fig. 2



	EC_{50} log mol	R_{max} mmHg
■	4.4±0.1	176.9±14.9
○	4.0±0.1*	140.9±4.2*

	EC_{50} log mol	R_{max} mmHg
■	4.2±0.1	216.3±32.6
○	4.3±0.1	190.8±19.7*

Fig. 3

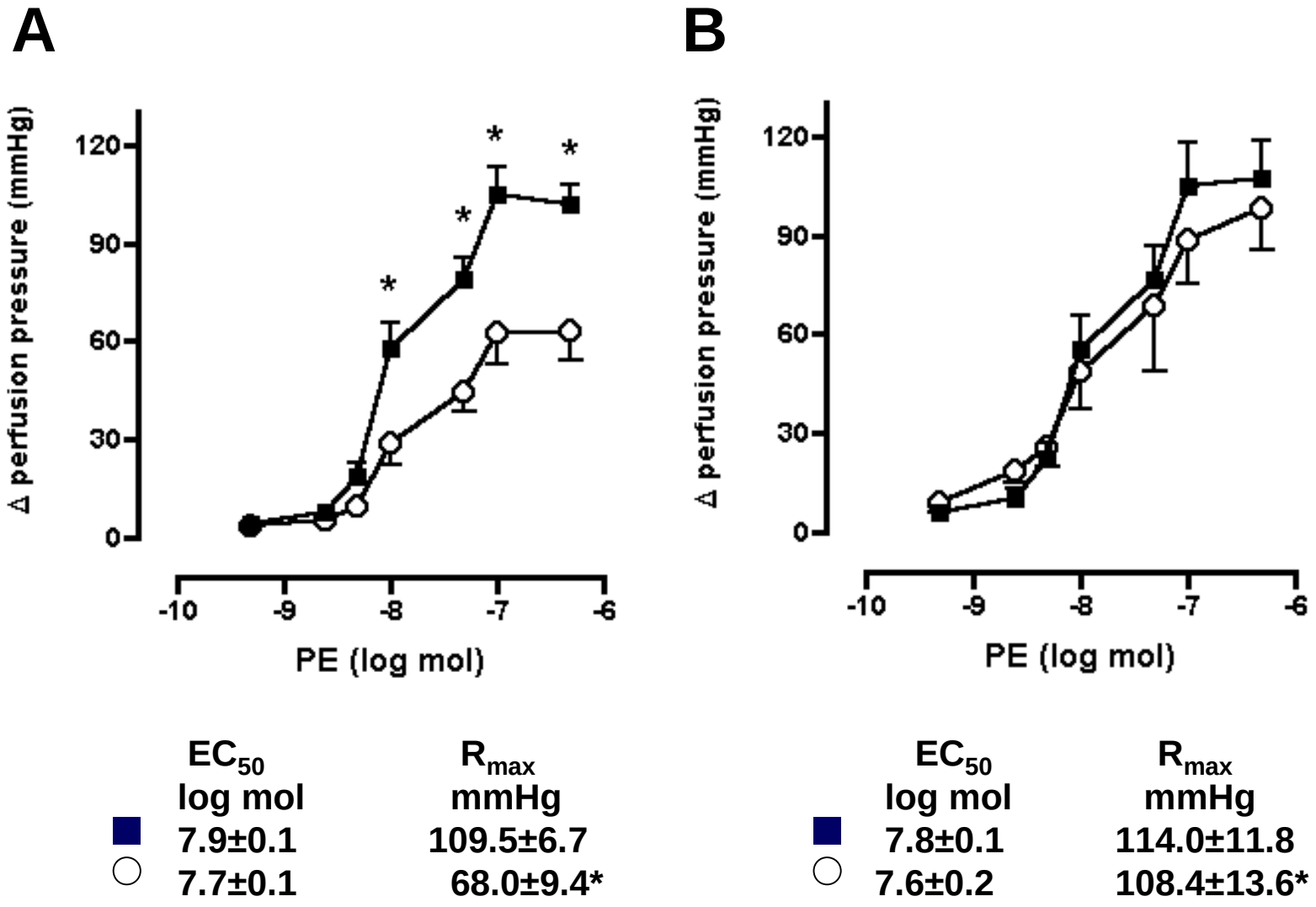


Fig. 4

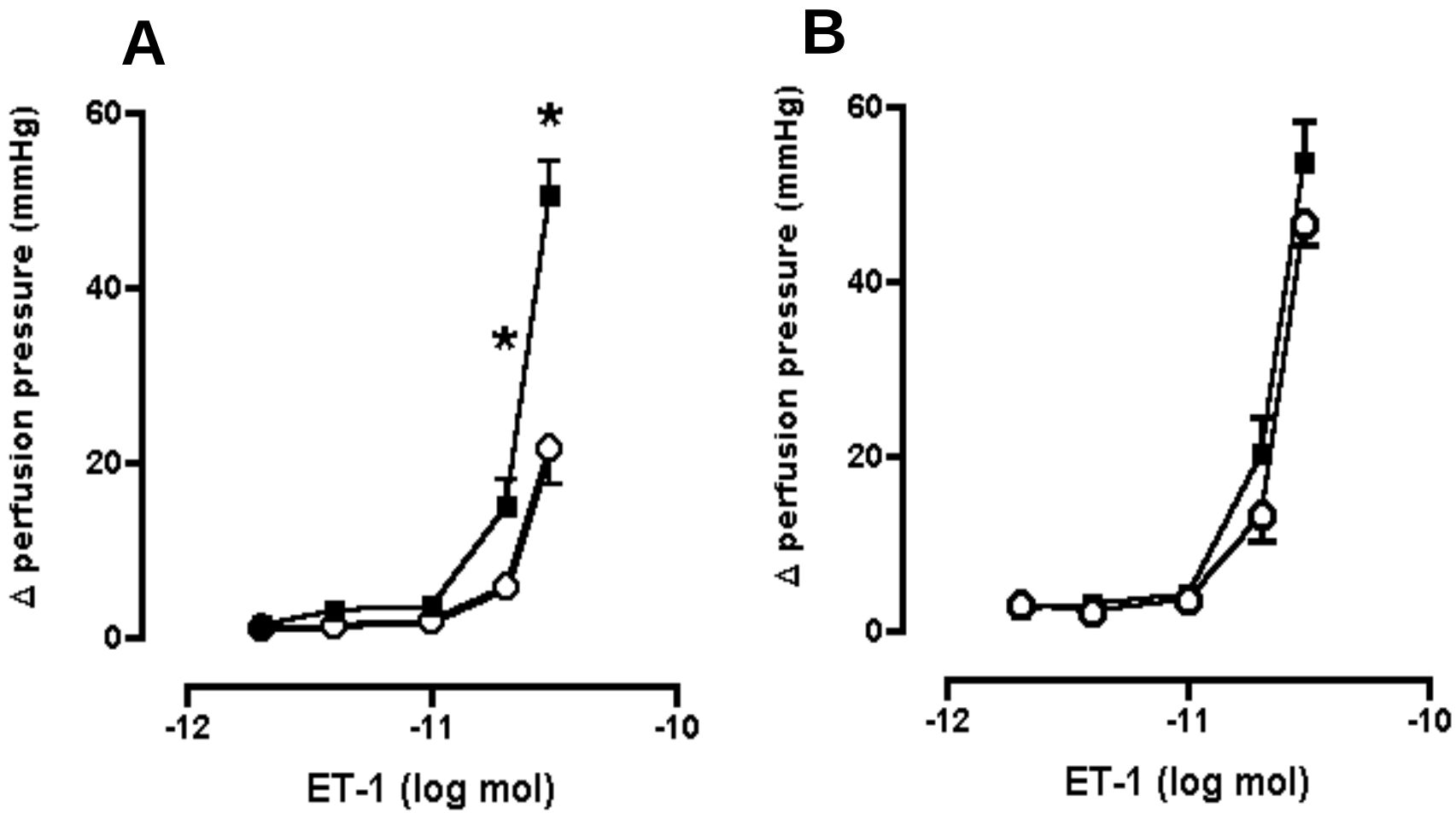


Fig. 5

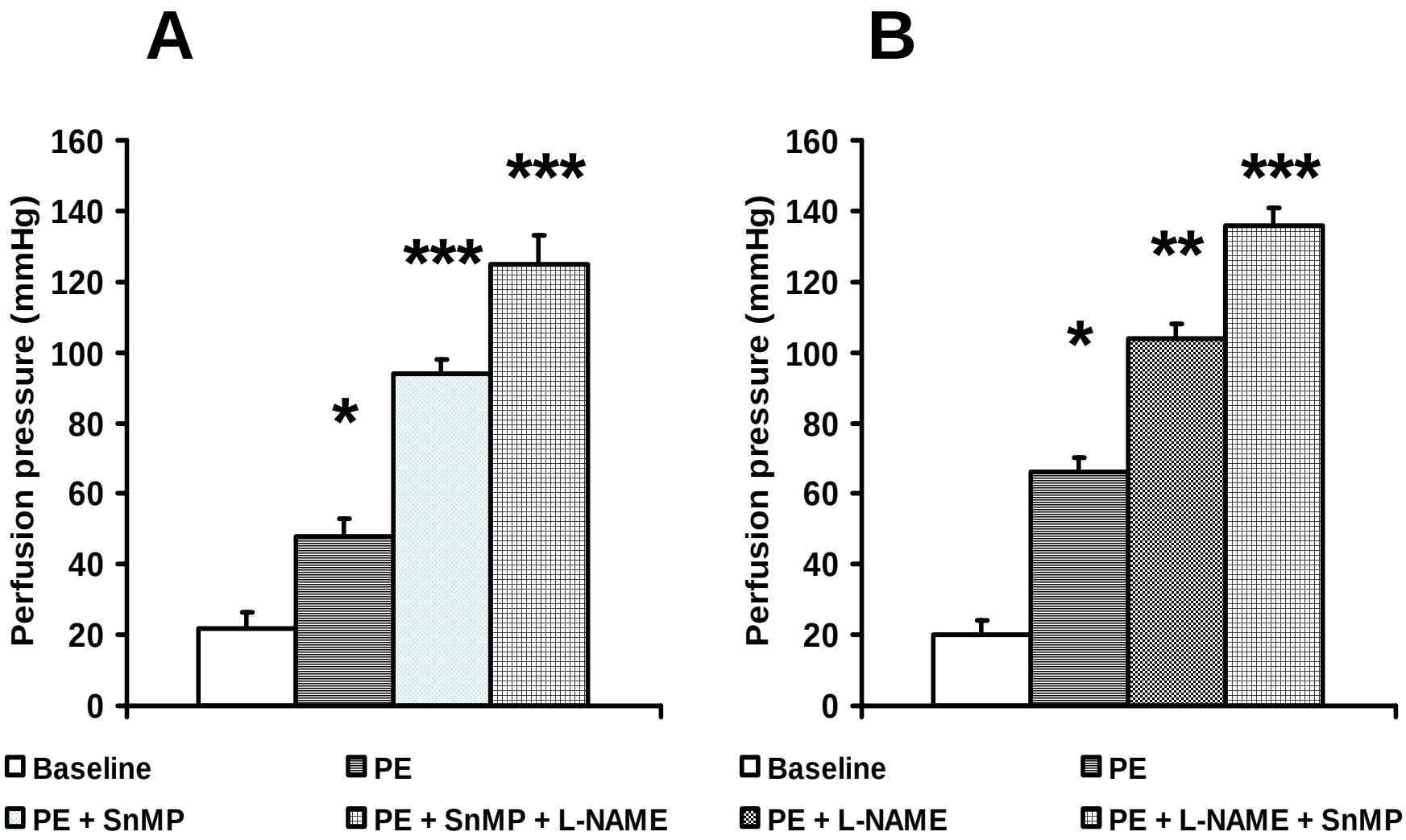


Fig. 6

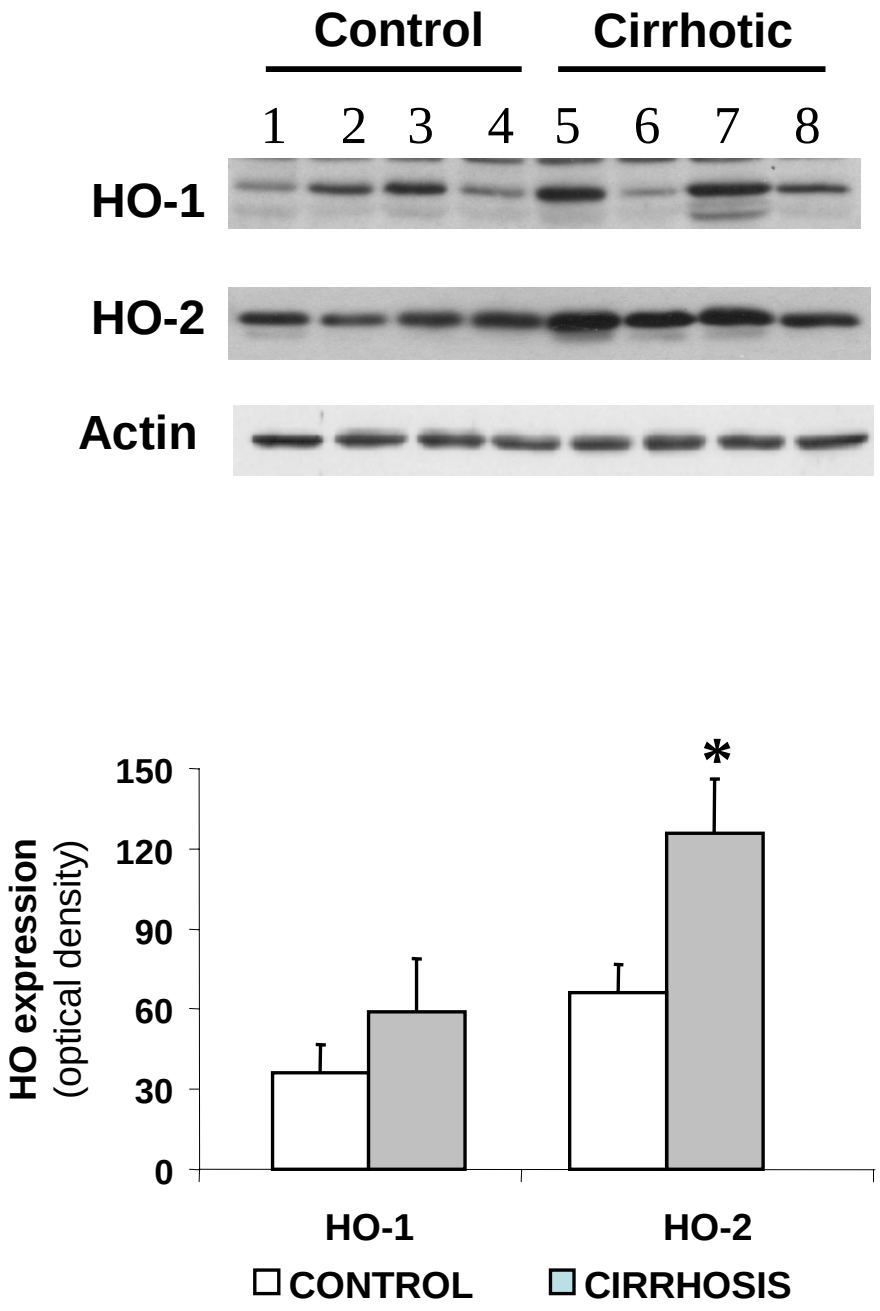


Fig. 7

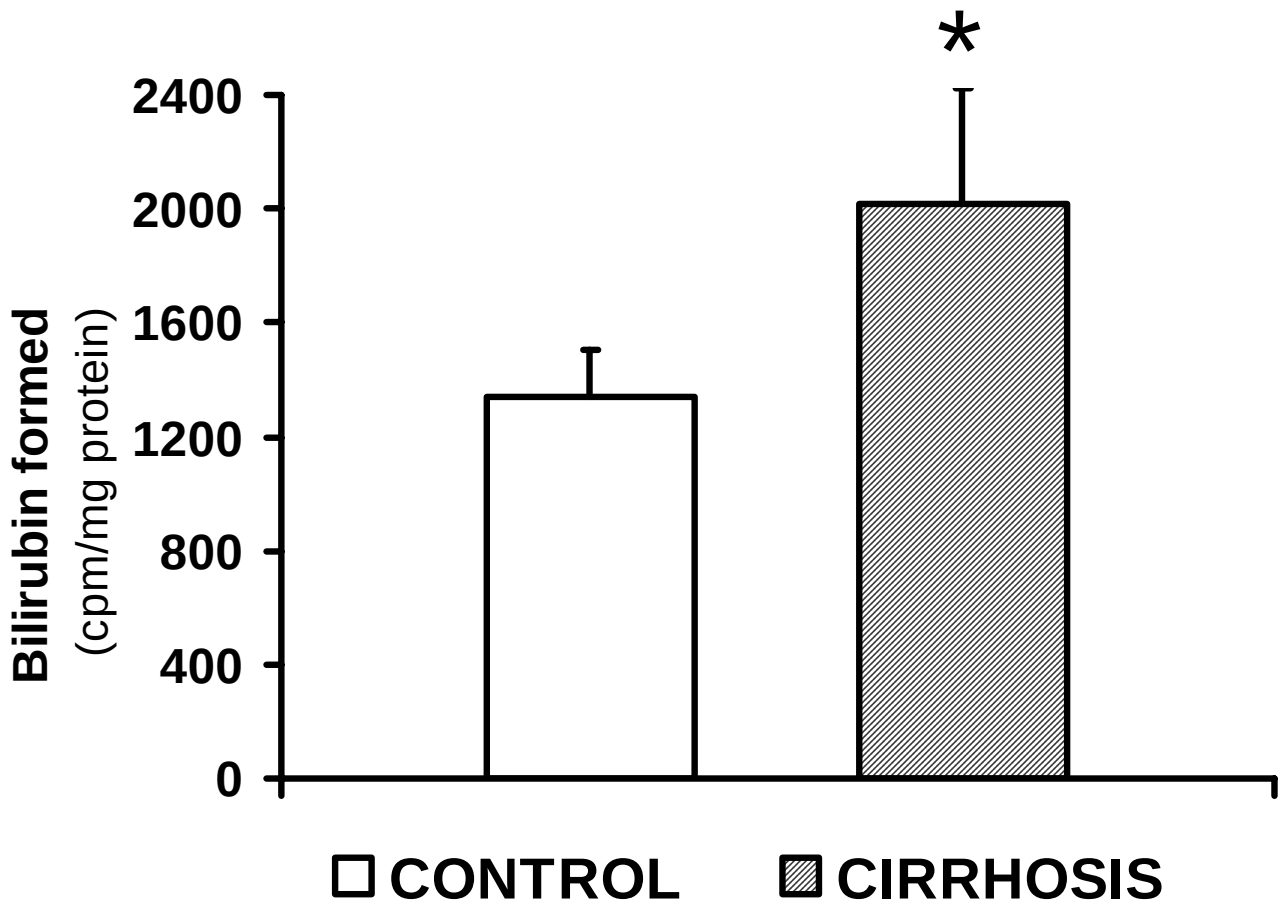


Fig. 8

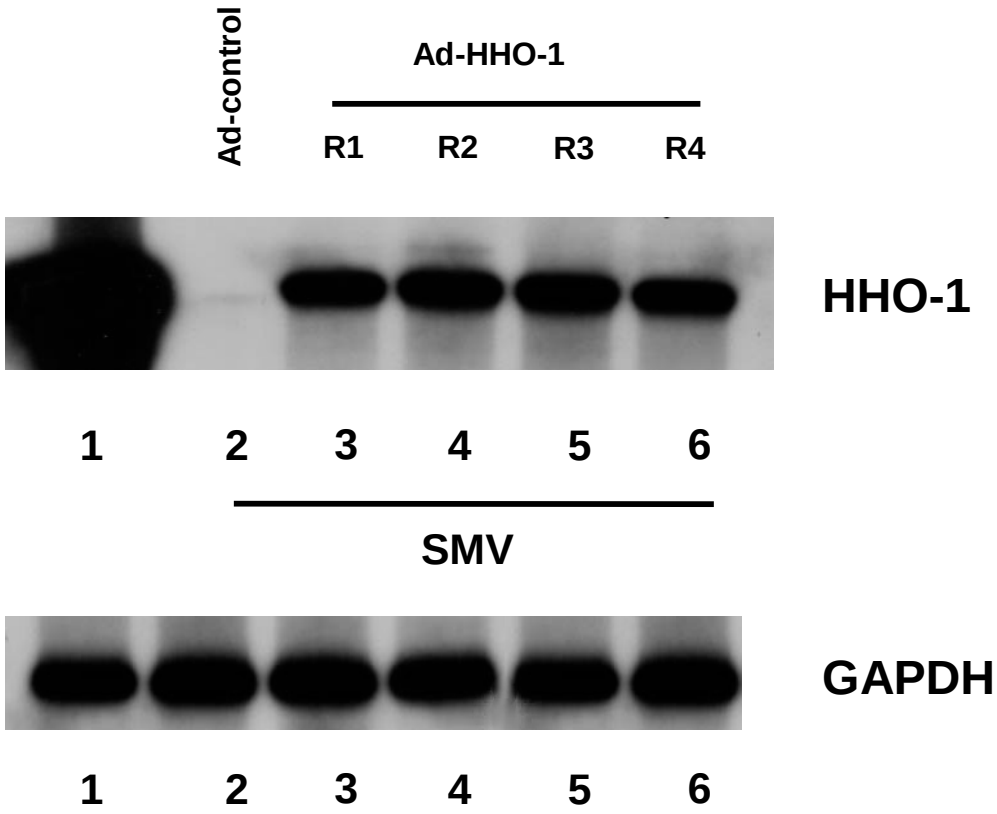


Fig. 9