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Nitric oxide inhibitor L-NAME potentiates induction of heme oxygenase-1 in kidney ischemia/reperfused model;

a novel mechanism for regulation of the oxygenase

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Running title: HO-1 regulation by NO

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abbreviations: L-NAME = N-nitro-L-arginine methyl/ester, HO = heme oxygenase, NOS = NO synthase, PBN = n-tert-butyl-α-phenyl nitrone

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ABSTRACT

The biological significance of the heme oxygenase (HO) system's response to stress reflects functions of its products -- CO and bile pigments. CO is a messenger molecule, while bile pigments are antioxidants and modulators of cell signaling.

Presently, an unexpected mechanism for sustained suprainduction of renal HO-1 following ischemia/reperfusion injury is described. Inhibition of NOS activity by L-NAME at resumption of reperfusion of rat kidney subjected to bilateral ischemia (30 min) was as effective as the most potent HO-1 inducer, the spin trap agent, PBN, in causing sustained suprainduction of HO-1 mRNA. PBN forms stable radicals of oxygen and nitrogen. 24h after reperfusion, HO-1 mRNA measured ~30-fold that of the control in the presence of L-NAME treatment; in its absence, the transcript increased to only ~5-fold. At 4h in the presence or absence of L-NAME, HO-1, mRNA was increased by ~30-fold. The transcript was translated to active protein as indicated by Western blotting, immunohistochemistry and activity analyses. L-NAME was not effective given 1h after resumption of reperfusion. Suprainduction was restricted to the kidney and not detected in the heart and aorta; and ferritin expression in the kidney was not effected.

It is reasoned that, in tissue directly insulted by ischemia/reperfusion increased production of NO radicals promotes the loss of HO-1 transcript. Because the absence of NO radicals and presence of PBN had a similar effect on HO-1. We propose that suprainduction of the gene is mainly caused by O_2 radicals formed on reperfusion. Inhibition of NOS is potentially useful for sustained induction of HO-1 in organs that will be subjected to oxidative-stress insult.

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Until recently, cellular degradation of the heme molecule (Fe-protoporphyrin IX, hemin) by the heme oxygenase (HO) system was viewed primarily in context of a mechanism for dispensing senescent heme compounds and recycling of iron. Other products formed in the course of catalytic activity of the HO system – CO and bile pigments – were solely considered in context of their toxicity. The view regarding the HO system dramatically changed when heme degradation products were identified as vital regulating factors in the cell. Compelling evidence has been presented showing that CO, in analogy with NO, is a signal molecule for the generation of cGMP, and plays a role in neuronal signaling, vascular tone relaxation as well as anti-apoptotic and apoptotic gene expression (Morita, 1995; Maines, 1997; Motterlini et al, 1998; Takeda et al, 2000; Panahian and Maines, 2001; Baranano and Snyder, 2001; Sato et al 2001, Brouard et al, 2002, Peyton 2002).

The system, as is known to date, consists of two catalytically active forms, we call them HO-1 and HO-2 (Maines et al, 1986), and an essentially inactive form, HO-3 (Huang et al, 1997). HO-1, also known as HSP32, is an oxidative stress responsive gene that rapidly responds to a variety of such forms of stress (Shibahara et al, 1987; reviewed in Maines, 1992). HO-2 is the constitutive cognate of the HSP32 family and is not induced by oxidative stress (Ewing and Maines, 1991).

Likewise, evidence that biliverdin and its reduced form, bilirubin, are associated with cellular defense mechanisms and are modulators of cell signaling pathways has been mounting (Stocker et al, 1987; McDonagh, 1990; Poss and Tonegawa, 1997; Dennery, 2000; Willis et al, 2000; reviewed in Maines, 2003). The significance of the catalytic activity of the HO system is accentuated by the fact that heme itself is an effector molecule that can regulate inflammatory response (Andersson, 2002), and activate molecular oxygen (Aust, 1982).

Collectively, the multidimensional functions of the system has lead to the current understanding that increase in cellular HO activity is a mechanism for protecting the cell against untoward stimuli such as ischemic/reperfusion insult to organs including the kidney (reviewed in Hill-Kapturczak et al, 2002). HO-1 expression is induced in the renal and cardiovascular system of rats subjected to ischemia/reperfusion along with increased cGMP and depletion of the cellular heme levels (Maines et al, 1993; Raju and Maines, 1996). Nitric oxide generated by inducible NOS and redox congeners are potent inducers of HO-1 in the normal tissue (Foresti et al, 1997; Doi et al, 1999; Hill-Kapturczak et al, 2002). The induction of HO-1 is considered a defense response to NO derived species, which can mediate tissue injury (Beckman and Koppenol, 1996; Hensley et al, 1997). The spin-trapping agent, PBN, is an effective scavenger for oxygen-, carbon-, and nitrogen-centered radicals with the interaction giving rise to relatively stable radicals (Phillis, 1997; Floyd, 1997). PBN, also mediates suprainduction of HO-1, which coincides with an essentially intact renal morphology and physiology (Pedraza-Chaverri et al, 1992; Maines et al, 1999). These findings further support the protective role of the HO system against oxidative injury.

In the course of ischemia/reperfusion both oxygen radicals and nitric oxide derived radicals are generated. The latter is mainly produced by inducible NOS of invading macrophages (Hensley, 1997). To investigate whether nitric oxide congeners are the stimuli responsible for the induction of HO-1 gene expression in the ischemic/reperfused kidney, the effect of inhibition of nitric oxide formation on HO-1 induction in this model of oxidative stress was examined. We present the surprising and unexpected observation that inhibition of nitric oxide formation, using NOS inhibitor, L-NAME, remarkably augments increase in HO-1 levels in the targeted organ of ischemic/reperfusion injury -- the kidney. We suggest oxygen radicals, rather than nitric oxide derived species, are likely inducers of HO-1 gene expression in kidney subjected to ischemia/

reperfusion. And, that nitric oxide congenes regulate HO-1 gene expression in this model of oxidative stress are detrimental to the sustained increase in HO-1 transcript.

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METHODS

Materials: Oligo (dT) cellulose, DNase I, Salmon testes DNA, N-nitro-L-arginine methyl ester (L-NAME), N-tert-butyl-α-phenyl nitrone (PBN), and cofactors were obtained from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes, Rediprime random-primer DNA labeling system, and Taq polymerase were purchased from USB Corporation (Cleveland, OH). Horseradish peroxidase conjugated goat anti-rabbit IgG was purchased from Organon Teknika Corporation (Westchester, PA). Nytran membranes and nitrocellulose membranes (0.2 μm pore size) were obtained from Schleicher & Schuell (Keene, NH). All chemicals used were of highest purity commercially available. [α-^{32}P] dCTP (3000 Ci/mmole) was purchased from NEN/Dupont (Boston, MA). Male Sprague-Dawley rats (200-250 g) were purchased from Harlan Industries (Madison, WI).

Induction of renal ischemia and tissue preparation: All *animal treatments were performed in strict accordance with the NIH guide for the care and use of Laboratory Animals as approved by the University Committee for Animal Resources.* Rats were subjected to pentobarbital anesthesia (40 mg/kg; ip). And, then subjected to 30 min renal ischemia followed by reperfusion and treated ip in the following manner: L-NAME 100 mg/kg or 100 mg/kg PBN) in 100 μl at the onset of reperfusion or L-NAME (100 mg/kg, ip) 1 h after reperfusion subsequent to 30 min of ischemia. Control rats were sham-operated with mobilization of kidney but no clamping of arteries. The dose of L-NAME was selected based on published reports which use up to 300 mg/kg of the inhibitor (Girchev et al, 2002). The duration of reperfusion was 4 or 24 h. All surgical manipulations were performed under normothermic conditions with rats kept on the homeothermic blanket with rectal temperature monitoring throughout surgery and the first 2 h after induction of

reperfusion. Renal ischemia was induced by means of occlusion of both renal arteries using Yasargil vascular clips (Aesculap, San Francisco, CA) with a closing force of 0.95 N. Reperfusion was confirmed in every case. After removal of the clips, immediate reperfusion was assessed by visual examination of the kidney surface, which recovered the usual color within 15-20 sec, as assessed with the help of an MZ-8 Leica stereomicroscope. Similar criteria for establishment of reperfusion was used previously (Maines et al, 1999). At time points indicated in appropriate figure legends, rats were killed and kidneys, heart and descending aorta were removed and frozen at -80°C for RNA isolation or for microsomal isolation. Rats were also subjected to protocols described below for immunohistochemical analyses. The number of animals used for biochemical experiments was 3-6 rats per group and 4 rats per group were used for histochemical analysis.

Microsomal isolation and measurement of heme oxygenase activity: Pooled kidneys of 2 rats were homogenized in five volumes of buffer containing 0.25 M sucrose and 0.01 M Tris-HCl (pH 7.4). The microsomes were prepared and used for HO activity measurement. The activity was measured in the presence of purified NADPH cytochrome P450 reductase and biliverdin reductase (Huang et al, 1989) as described before (Raju and Maines, 1996). The enzyme activity was assessed by the formation of bilirubin and was calculated as the amount of bilirubin produced per hour per milligram of protein. Protein was determined using a Biorad reagent. Statview® software package was used to evaluate the results using Anova and post hoc (Fisher's) tests, a paired t test on log transformed data. A p value of $p < 0.05$ was considered significant.

Western Blot Analysis: Analysis was carried out as before (Maines et al, 1999). Protein samples were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using an LKB2005 Transphor Apparatus (BioRad). Antigen-antibody complexes were immunochemically visualized using rabbit polyclonal antibody to rat HO-1 and

horseradish peroxidase-conjugated goat anti-rabbit antibody. GST-tagged *E.coli* expressed HO-1 was purified using a GST column as described before (Salim et al, 2001).

Immunocytochemical protocols: 24h after induction of reperfusion, rats were given an overdose of pentobarbital (100 mg/kg; ip) and perfused transcardially with heparinized saline, followed by a chilled solution of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Following overnight post fixation in 4% PFA at 4-6° C, the kidney was transferred into the cryoprotection solution of 30% ethylene glycol and 20% sucrose in 0.1M phosphate buffer (pH 7.4) at 4-6°C for 2-3 days. Both kidneys were then frozen in crushed dry ice and cut serially in 25 μm thick sections using a sliding microtome (Microm 400, Carl Zeiss). Staining of the kidney from the control and treated rats was carried out under identical conditions using the same reagents and solutions. For all immunocytochemical and histochemical protocols longitudinally cut specimens were used due to a larger surface area available for analysis.

HO-1 was detected using a 3B8C8 monoclonal antibody developed in collaboration with StressGen (Vancouver, Canada) as the primary antibody and 3'-3'-diaminobenzidine (DAB) as the chromagen. Detailed protocols are described elsewhere (Maines et al, 1999).

Northern Blot Analysis: The effect of renal ischemia/reperfusion on kidney, heart and descending aorta HO-1 mRNA levels was examined by Northern blot analysis. Poly(A)$^+$ RNA was isolated from pooled kidneys, hearts or descending aorta from 4 rats and subjected to blot hybridization as described before (Ewing and Maines, 1991). The blot was sequentially probed with α^{32}P-labeled probes: HO-1 cDNA and actin cDNA (loading control). As noted in the appropriate figure legends, some blots were also probed with HO-2 cDNA after HO-1. Additional blots were probed with heavy chain ferritin cDNA and actin. Each lane contained 4 μg of poly(A)$^+$ RNA.

An HO-1 cDNA corresponding to nucleotides +71 to +833 reported by Shibahara et al (1985) was generated using PCR and cloned into PBS(+) vector as described before (Ewing and Maines, 1991). A full length HO-2 cDNA (1300 base pairs) isolated from rat testis DNA library (Rotenberg and Maines, 1990) was used as an HO-2 hybridization probe. A PCR product consisting of full length ferritin cDNA was prepared and used. All probes were labeled with [γ^{32}-P]dCTP by the random primers DNA labeling system (U.S. Biochemical Corp.), according to the manufacturer's instructions, and further purified by spin column chromatography. Total RNA and poly(A)$^+$ RNA was isolated from pooled kidneys of three rats by oligo(dT)-cellulose chromatography, and the formaldehyde-denatured RNA was fractionated on a 1.2% (w/v) agarose gel and subsequently transferred to the Nytran membrane. Prehybridization and hybridization of the membranes with the appropriate ^{32}P-labeled cDNA were performed essentially as described previously (Sun et al, 1990).

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RESULTS

L-NAME-mediated suprainduction of HO-1 in ischemic reperfused kidney. The effect of L-NAME treatment immediately prior to the onset of reperfusion on HO-1 and HO-2 transcript levels in kidney subjected to 30 min of ischemia was examined by Northern blot analysis 24 h after resumption of reperfusion. The effect was compared with those of PBN-treated rats subjected to the same surgical manipulations. The intensity of the HO-1 mRNA signal was normalized to that of actin. As shown in Fig 1, top panel, at this time point the levels of the 1.8 kb HO-1 transcript were increased by ~5-fold in the ischemic/reperfused organ when compared with those of sham-operated rats treated with L-NAME. In contrast, there was a remarkable increase in HO-1 mRNA levels in rats given L-NAME before the onset of reperfusion, which measured nearly 30-fold that of the controls. Suprainduction of HO-1 was previously reported in ischemic/reperfused kidney treated with the spin-trapping agent, PBN (Maines et al, 1999). Therefore, the relative effectiveness of the NOS inhibitor and PBN in modulating levels of HO-1 transcript levels in ischemic/reperfused kidney was compared. As noted in the figure, both treatments were essentially comparable in their effectiveness. The relative values for HO-1 transcript levels, with a value of 1 assigned arbitrarily to the HO-1/actin signal ratio of the sham-operated +L-NAME control were: Ischemic/reperfused = 5; Ischemic/reperfused + L-NAME = 33; Ischemic/reperfused + PBN = 39.9. HO-2 transcription, which is the constitutive cognate of HSP32 family, does not increase in response to oxidative stress, (Maines, 1992) and as noted in the figure, levels of its two transcripts, 1.3 kb and 1.9 kb, did not increase either in response to L-NAME or PBN treatment. PBN when given to sham-operated rats did not effect HO-1 mRNA levels (lower panel), Again, ~5-fold increase in HO-1 mRNA levels was detected in ischemic/reperfused kidney. The finding suggest

that PBN, which modulates the level of free radicals is rather ineffective in the absence of free radicals in normal tissue. And, that its ability to modulate HO-1 mRNA levels requires presence of radicals. PBN interaction with O_2 radicals, which are generated in the course of reperfusion, stabilizes the radicals.

Inquiry was made into the basis for the augmentation by L-NAME treatment of HO-1 transcript increase caused by ischemia/reperfusion. One approach involved examining transcript level analysis at an early time point after resumption of reperfusion, for this 4h was selected. As shown in Fig 2, surprisingly, at 4h time point HO-1 transcript levels were comparable in presence or absence of L-NAME treatment of the kidney subjected to ischemia/reperfusion injury, and also were comparable to that noted with L-NAME treated ischemic/reperfused kidney at 24 h (Fig 1). In the absence of L-NAME treatment there was, however, ~ 3-fold difference in HO-1 transcript levels at 4h and 24h time points (Fig 2 vs Fig 1). It is notable that at the 4h time point, a moderate increase of ~3-fold in HO-1 mRNA levels was detected in sham-operated rats given L-NAME. Because the kidneys of sham-operated rats were physically manipulated as the experimental rats, findings suggest that NOS inhibition has a permissive effect on increase in HO-1 mRNA levels in a stressed organ. The relative intensity of HO-1 mRNA signals assessing the value of one to HO-1/actin ratio for sham-operated rat kidneys were: Ischemic/reperfused = 30; Sham + L-NAME = 3; Ischemic/reperfused + L-NAME = 28.

Next, inquiry was made into the basis for this observation by examining whether the sequence of L-NAME administration is of significance. For this, HO-1 mRNA levels in ischemic kidney of rats treated with L-NAME 1h after resumption of reperfusion was compared with that of L-NAME administered immediately before the onset of reperfusion (Fig 3). The timing of the treatment clearly had an impact on L-NAME effect. As noted, when assessed at 24h, a striking

difference in HO-1 mRNA levels were detected in the presence or absence of the NOS inhibitor, with L-NAME given 1h after reperfusion being essentially ineffective in modulating the outcome of response to ischemia. The intensity of HO-1 signals when expressed relative to that of actin and assigning the value of one to sham-operated rat kidney were: Ischemic/reperfused = 7; Ischemic/reperfused + 1h wait + L-NAME = 9; sham-operated + L-NAME = 2; Ischemic/reperfused + L-NAME = 33.

Suprainduction of HO-1 transcript by L-NAME in ischemia/reperfused kidney does not extend to heart and blood vessels. To further explore the physiological basis for permissive effect of L-NAME on sustained increase in HO-1 mRNA levels, the next series of experiments were conducted. Previous studies have shown that ischemia/reperfusion insult to the kidney resonates through the cardiovascular system and increases HO-1 expression in the heart and aorta (Maines et al, 1993; Raju and Maines, 1996). Because oxygen free radicals are produced in the kidney upon perfusion and also macrophages infiltrate the insulted organ, we examined whether augmentation of HO-1 mRNA induction response in ischemic/reperfused kidney by L-NAME treatment also extends to the heart and aorta.

Fig 4 shows the response of heart HO-1 in rats subjected to ischemia (30 min) and 4 h or 24 h reperfusion in the presence or absence of L-NAME treatment. As noted, at both time points, regardless of the presence or absence of L-NAME, heart HO-1 mRNA levels in rats subjected to ischemia/reperfusion were essentially indistinguishable. This finding suggests that L-NAME effect is localized to HO-1 response in the organ subjected directly to the insult. Also, it is of significance to note that the response of HO-1 in heart to ischemia/reperfusion insult to the kidney is delayed and the transcript level measures nearly 5-fold higher at 24h in heart of rats subjected to kidney ischemia/reperfusion when compared with appropriate control tissue at 4h. A rather similar

observation was made with the response of HO-1 in the descending aorta (Fig 5). Unlike the kidney, however, at 24 h when corrected for the actin loading standard, there was no detectable differences that could be assigned to L-NAME's presence.

Because iron bound to ferritin is not a catalyst for oxygen free radical formation, ferritin expression was analyzed to gain information on the disposition of iron released by increased activity in the ischemic/reperfused kidney. Figure 6 shows that in the kidney, ferritin mRNA in the presence of L-NAME treatment did not differ from that observed in the absence of the inhibitor when measured after 24h of reperfusion. The finding suggests that an increased sequestration of catalytic active iron and decrease in formation of oxygen radicals did not underlie the sustained increase in HO-1 mRNA levels.

Increased HO-1 mRNA in the presence of L-NAME is translated into active HO-1 protein. The following experiments were carried out to examine whether the effect of L-NAME on HO-1 mRNA levels is of consequence to the kidney. For this, 24 h after the start of reperfusion, kidney HO-1 protein levels and HO activity were assessed. Results are shown in Fig 7, panel A. As noted, the transcript was effectively translated into protein as assessed by Western blotting. For this analysis the amount of microsomal protein used for analysis of HO-1 in the presence of L-NAME treatment was reduced to 50% of that in the absence of such treatment and yet measured 2-fold higher than that in the absence of L-NAME treatment of the ischemic/reperfused kidney (Lanes 3 vs 4). As expected, the levels of HO-1 protein in sham-operated controls with or without L-NAME treatment was low. The immunoreactive bands seen in lanes 3 and 4 are HO-1 protein fragments produced in the process of manipulation of tissue for preparation of microsomes. The lower mobility of the standard HO-1 protein is caused by the added amino acids of the GST tag.

Increase in HO-1 protein could be also visualized by immunohistochemical analysis. As shown in panels B and C there is a pronounced increase in intensity of HO-1 immunostaining in the cortical region of the kidney in L-NAME treated rats (panel C) when compared with the ischemic/reperfused kidney not treated with L-NAME (panel B). It is noteworthy that the morphology of tissue was also different between the two treatment regions. Specifically, the preponderance of tubules with increased diameter and thinning of the tubule's lining that were observed in absence of L-NAME (panel B).

In addition, the HO-1 protein was active (Fig 7, panel D) as indicated by the rate of heme degradation of the microsomal preparations, with activity closely following the pattern of protein and transcript for HO-1 (Fig 7, panel D). A 3-fold difference in activity was detected in the presence and absence of L-NAME. The differential effectiveness of L-NAME to increase HO-1 protein and HO activity of the microsomal preparation may well reflect inactivation of HO-2 by NO radicals in the absence of L-NAME treatment (Ding et al, 1999).

DISCUSSION

Prior to this study PBN was identified as the most effective agent to increase HO-1 transcript levels leading to an unprecedented sustained increase of more than 30-fold in the transcript level in kidney subjected to ischemia/reperfusion (Maines et al, 1999). The present study has identified L-NAME, an NOS inhibitor, equally as effective as PBN, in this oxidative-stress model. The findings are highly unexpected and define a previously unknown interaction between the NO and CO generating systems, as it pertains to regulation of HO-1 by NO derived radicals. The significance of the suprainduction of HO-1 relates to the growing evidence that products of heme oxidation regulate physiologically important processes. For example, CO is considered to share with NO, neurotransmitter, anti-platelet and vasodilatory activities and to stimulate soluble guanylyl cyclase (Maines, 1997; Motterlini et al, 1998; Baranano and Snyder, 2001; Suematsu et al, 2001). On the other hand, the tetrapyrrole product of HO activity, biliverdin, and the product of biliverdin reductase activity, bilirubin, are potent antioxidants and effective modulators of cell signaling (Stocker et al, 1987; Phelan et al, 1998; Maines, 2003). The chelated heme iron released when the tetrapyrrole is cleaved is a physiological regulator of the ferritin (Ponka, 1998); when complexed with ferritin, iron does not activate molecular oxygen.

As depicted before, there is an intricate and intimate link between the CO and NO generating complex systems (Maines, 1997). CO could regulate NO generation at least two ways -- by controlling the level of heme for synthesis of NOS, and by binding to the heme moiety of the hemoprotein and inhibiting its activity. On the other hand, nitric oxide and its radical derivatives, in normal cells, can increase CO production by inducing HO-1 (Forresti et al, 1997; Ding et al, 1999, Naughton et al, 2002, Bouton and Demple, 2000). The finding that the remarkable increase in HO-1 mRNA was sustained 24h after reperfusion of the ischemic kidney when NO production

was inhibited (Fig 1 & 2), may suggest that: a) NO radicals generated in the course of reperfusion, are linked to the processes that mediate the rapid decline in the HO-1 transcript levels. Hence, by inhibiting their production, decline in HO-1 transcript is mitigated; or, b) that the observation reflects sustained local hypoxia and the absence of vasodilatory and anti-platelet activities of NO. However, considering that the product of HO activity, CO and NO, share similar activities on the vasculature and hemodynamics, the remarkable sustained increase in HO-1 would be predicted to compensate for inhibition of NOS. Therefore, rendering the second possibility less likely. This is in line with the report that activation of hypoxia-inducible factor 1 (HIF-1) in ischemic kidney persists only for 4h (Eickelberg et al, 2002). In the first case scenario, NO derivatives could: a) directly cause dissolution of the transcript; b) activate cellular factors that accelerate HO-1 mRNA degradation; or c) inactivate factors that stabilize the transcript. Production of nitric oxide is increased in an ischemic/reperfused organ largely due to inducible NOS of infiltrating macrophages (Hensleg et al, 1997). NO oxygen derivatives, such as peroxynitrite, are highly reactive and toxic compound that display destructive effects in the cell (Lipton et al, 1994; Beckman and Koppenol, 1996). Given that both HO-1 and inducible NOS are rapid-response inducible genes, the finding can be further extended to consider that the rapid decay in HO-1 transcript, subsequent to its induction that is detected in essentially every tissue (e.g Ewing and Maines, 1991) tested to date may involve NO radicals. This line of reasoning could be extended to envision NO as a “maker and a breaker” of HO-1, as such, it induces and stabilizes HO-1 mRNA (Bouton et al, 2000); yet, when complexed with oxygen radicals, it destroys the message.

Presently noted observations also make for a plausible explanation for the findings reported in the literature, which at a glance may seem to be contradictory; that, HO-1 is induced by nitric oxide and its induction and overexpression are effective in protecting against ischemia/reperfusion

injury (Maines et al, 1993; Hill-Kapturczak et al, 2002). Yet, it is also reported that inhibition of NOS is effective against such injury to not only the kidney but also the brain (Nagafuji et al, 1992). The presently noted observation that inhibition of NO generation by L-NAME circumvents rapid decline in HO-1 mRNA levels and maintains high levels of heme degradation activity links the reported phenomena in a reasonable way. Moreover, the present findings are consistent with why ischemia/reperfusion induced acute renal failure is ameliorated by PBN (Petraza-Chaverri et al, 1992). PBN, as was reported before and confirmed presently, promotes suprainduction of HO-1 in the ischemic/ reperfused kidney. PBN interacts with oxygen-, carbon-, and nitrogen-centered radicals (Phillis, 1997) and gives rise to relatively stable free radicals (Evans, 1979; Phillips, 1997).

We had considered previously that the sustained suprainduction of HO-1 in kidney of rats subjected to ischemia/reperfusion and PBN treatment was related to inactivation of NO or O_2 radicals, which are formed in the ischemic/reperfused organ. The other possibility we had considered was that free radicals upon conversion to stable radicals possess prolonged gene activating capability. The present findings that inhibition of NO production is as effective as treatment with PBN are consistent with the suggestion that NO derived radicals have deleterious effects on HO-1 mRNA. And, permit speculation that induction of HO-1 is primarily a response to oxygen radicals generated upon reperfusion of the ischemic kidney, rather than to the NO radical generated in the insulted organ. It can be reasoned that unchecked nitric oxide oxygen radicals cause inactivation/destruction of cellular constituents, including those vital to gene expression.

An additional contributing factor to the increase in tissue HO activity by inhibiting NOS activity is blocking inactivation of HO-2 by NO radicals. HO-2, unlike HO-1, is a hemoprotein and sulphydryl reactive protein (Rotenberg and Maines, 1990; McCoubrey et al, 1997). The

activity of this isozyme is inhibited by NO radicals (Ding et al, 2000). It follows, that the marked increased heme degrading activity in the presence of L-NAME, noted in Fig 7 is likely to, in part, reflect that of HO-2. And, the observation that in the absence of L-NAME the increase in heme degradation activity is rather modest, reflects inhibition of HO-2 activity by nitric oxide radicals. The assay used for measuring heme degradation by the microsomal fraction does not distinguish contribution of the HO isozymes.

Moreover, based on the finding that L-NAME-mediated suprainduction of HO-1 mRNA is dependent on the time interval for its administration relative to subjecting ischemic kidney to reperfusion is supportive of our interpretation of the interplay between HO-1 gene expression and nitric oxide radicals. It suggests that HO-1 mRNA expression is precipitately affected by nitric oxide and its derivatives and that the "damage", if one could use the term, is implemented as the tissue is exposed to oxygen radicals as reperfusion commences in the kidney.

The noted delayed response of heart HO-1 mRNA to renal ischemia/reperfusion and the absence of a discernable difference in response in the presence or absence of L-NAME suggest that NO radicals are not the effector molecules for altered HO-1 gene expression in heart of rats subjected to ischemia/reperfusion of kidneys. It also suggests that L-NAME is not a modulating factor in sustaining high levels of cardiac HO-1 mRNA. As noted in Fig 4, in heart, when compared with 4h post perfusion, HO-1 transcript levels were further increased at 24h time point. This finding is consistent with the forwarded concept that nitric oxide radicals generated locally in the ischemic/reperfused kidney are directly involved in decline in HO-1 transcript in kidney; and, that increased HO-1 gene expression is primarily a response to oxygen radicals, rather than to NO radicals. It follows, the delayed induction of HO-1 in the heart may well be a response to disruptions in systemic functions, such as change in cardiac load and endocrine and/or autocrine

hemostasis. Similarly, observation with the descending aorta supports the forwarded hypothesis regarding local effects and role of oxygen and NO radicals in regulation of HO-1 gene expression.

The apparent localization of L-NAME effect to the target organ of oxidative stress permits suggestion that inhibition of NOS is an effective approach for sustained induction of HO-1 in an organ that will become subjected to such stress. Specifically, this approach could be of utility in organ transplantation including the kidney. There are many reports indicating that the half-life of the transplanted organ is significantly prolonged by induction of HO-1, reflecting the anti inflammatory, antioxidants and vasodilatory activities of the heme degradation products (Hancock et al, 1998; Willis et al, 2000; Morse and Choi, 2002; Brouard et al, 2002).

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FIGURE LEGENDS

Figure 1. N-nitro-L-arginine methyl ester is as effective as the spin-trap, PBN, in mediating suprainduction of HO-1 mRNA in ischemic/ reperfused kidney. Male Sprague-Dawley rats (200-250g) were treated with 100 mg/kg (ip) L-NAME or 100mg/kg (ip) PBN at the onset of reperfusion following bilateral kidney ischemia. Control rats were sham-operated and were given the same doses of L-NAME or PBN, or were treated with saline. After 30 min of ischemia, reperfusion was resumed and rats were killed 24 h later. Poly(A)$^+$ RNA was isolated from kidneys and used for Northern blot analysis as described in the text. The blot was probed sequentially with ^{32}P-labeled HO-1 cDNA, HO-2 cDNA and actin, (top panel) or with HO-1 and actin (lower panel). Actin was used as the loading control. Each lane contained 4 μg of Poly(A)$^+$ mRNA.

Figure 2. HO-1 mRNA is rapidly increased in kidney following ischemia/reperfusion. Rats were treated (ip) with 100 mg/kg L-NAME at the onset of reperfusion following 30 min of bilateral kidney ischemia. Rats were killed 4 h later. Control rats were sham-operated and treated with saline or L-NAME. Northern blot analysis of mRNA was carried out as described in the text and

Figure 3. Inhibition of NOS activity at the onset of perfusion, but not subsequent to reperfusion, prolongs increase in HO-1 mRNA levels. Rats were subjected to ischemia/reperfusion (30 min) and treated with 100 mg/kg (ip) L-NAME immediately prior to the onset of reperfusion or 1h after resumption of reperfusion. Control rats were sham-operated and given L-NAME at the onset of reperfusion or subjected to ischemia/reperfusion. Rats were killed 24 h later. Northern blot analysis was carried out as described in the text.

Figure 4. Renal ischemia/reperfusion increaases HO-1 mRNA levels in heart; response is not modulated by inhibition of NOS. Male Sprague-Dawley rats (200-250g) were subjected to bilateral ischemia for 30 min and were administred L-NAME (100 mg/kg, ip) immediately at the

onset of reperfusion. Control rats were given L-NAME or were subjected to ischemia/reperfusion. Rats were killed 4 or 24h after reperfusion. Poly(A)$^+$ RNA was isolated from 3 pooled hearts and subjected to Northern blot hybridization as described in the text. The blot was sequentially probed with ^{32}P-labeled probes: HO-1 cDNA fragment and actin cDNA, the latter was used as a control for sample loading. Each lane contained 4 μg of poly(A)$^+$ RNA.

Figure 5. Effect of renal ischemia/reperfusion on descending aorta HO-1 mRNA levels. Male Sprague-Dawley rats were subjected to ischemia/reperfusion and L-NAME as detailed in Fig 4 and killed 24 h after reperfusion. Poly(A)$^+$ RNA was isolated by pooling descending aortic vessels of four rats subjected to 30 min bilateral kidney ischemia. Northern blot analysis for HO-1 transcript levels were performed as detailed in the text. Each lane contained 4 μg of poly(A)$^+$ RNA. The blot was probed with HO-1 and actin in that sequence.

Figure 6. Ferritin transcript levels are not modulated in the kidney of rats subjected to ischemia/reperfusion and treated with L-NAME. Male Sprague-Dawley rats were subjected to ischemia/reperfusion of the kidneys and L-NAME treatment as described in the legend to Fig 1. Ferritin transcript levels were analyzed by Northern blot procedures as described in the text. Each lane contained 4 μg poly(A)$^+$ RNA.

Figure 7. Western immunoblot and immunohistochemical analyses of HO-1 protein and measurement of heme oxygenase activity of ischemic/reperfused kidney in the presence or absence of N-nitro-L-arginine methyl ester treatment. Male Sprague-Dawley rats were given L-NAME (100mg/kg, ip) or saline immediately prior to the onset of reperfusion after being subjected to bilateral kidney ischemia (30 min). Rats were killed 24 h after reperfusion. Kidneys were removed and pooled from 2 rats and used for preparation of the microsomal fractions, which were utilized for Western blot analysis and activity measurement. Rats were also perfused and processed

for immunohistochemical analysis of HO-1 in kidney. Experimental details are provided in the text.

Panel A) Western blot analysis of HO-1 protein levels. Samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to the membrane. The blot was probed with rabbit anti rat HO-1 polyclonal antibodies. Each lane contained 250 mg microsomal samples except for ischemic/reperfusion plus L-NAME treated rat kidney samples which contained 50% less protein. The experimental details are described in the text. Lanes: 1 = sham-operated, L-NAME treated rat kidney sample, 2 = sham-operated sample; 3 = ischemic/reperfused kidney; 4 = ischemic/reperfused kidney of rats treated with L-NAME; ST = 50 ng of *E.coli* expressed purified rat HO-1. The differential mobility of the standard is due to the presence of a GST tag that was present in the construct used for affinity purification.

Panels B and C) Kidney sections from rats subjected to 30 min ischemia and 24 h reperfusion (panel B) or given L-NAME at the onset of reperfusion (panel C) were used for HO-1 immunostaining. Immunostaining procedures were performed as described in the text.

Panel D) Heme oxygenase activity measurement. Degradation of heme was assessed by formation of bilirubin as detailed in the text. 3 measurements were made using different pooled (2 kidneys) microsomal preparations. Data were analyzed by Anova and post hoc (Fisher's) tests. A p value of ≤ 0.05 denoted significance. *Significantly increased when compared with sham - operated controls + significantly different from ischemic/reperfused + L-NAME treated.

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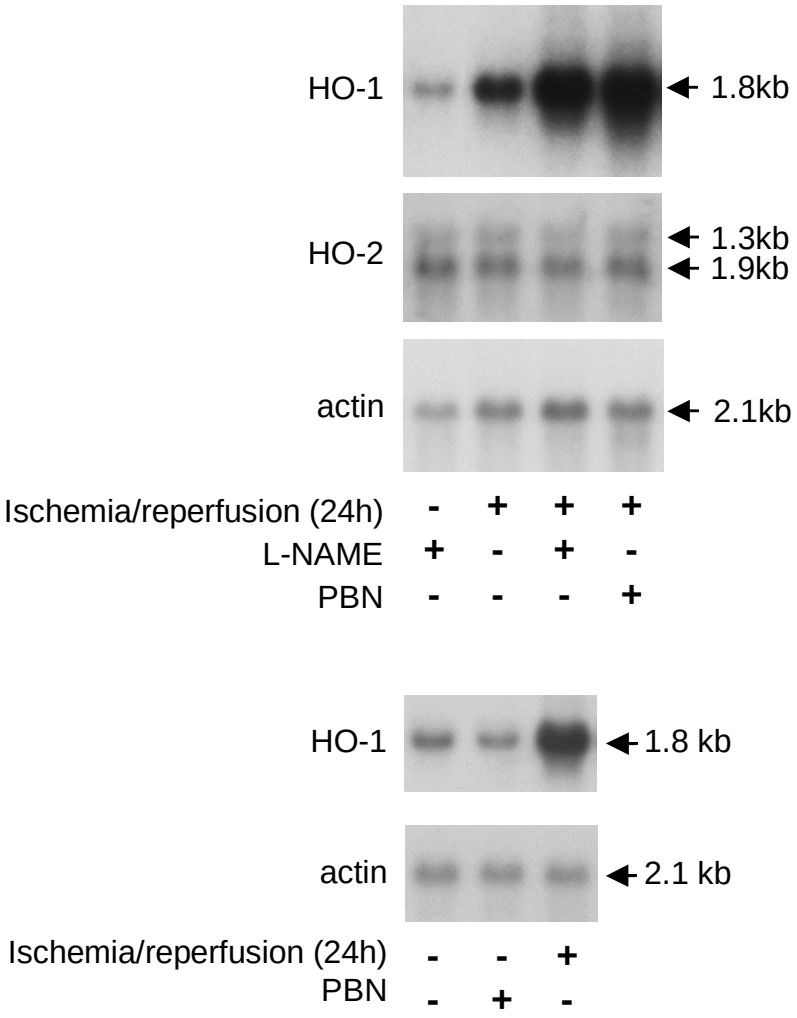

Figure 1

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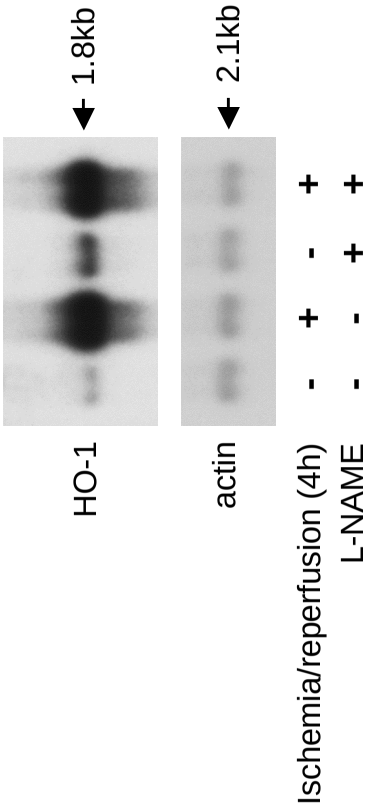

Figure 2

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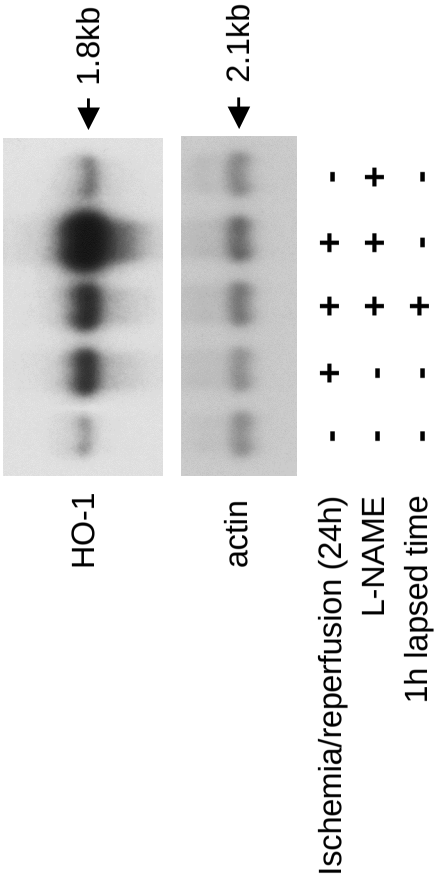

Figure 3

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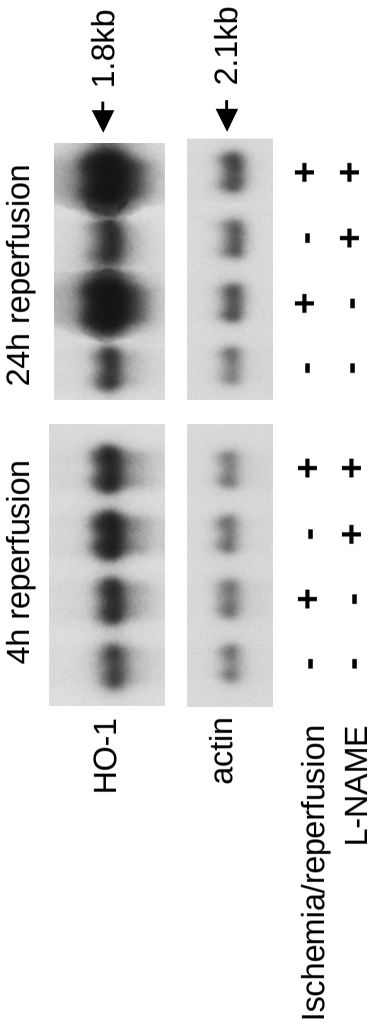

Figure 4

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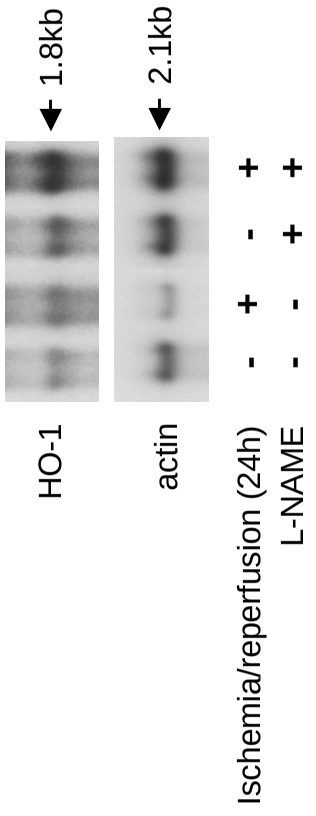

Figure 5

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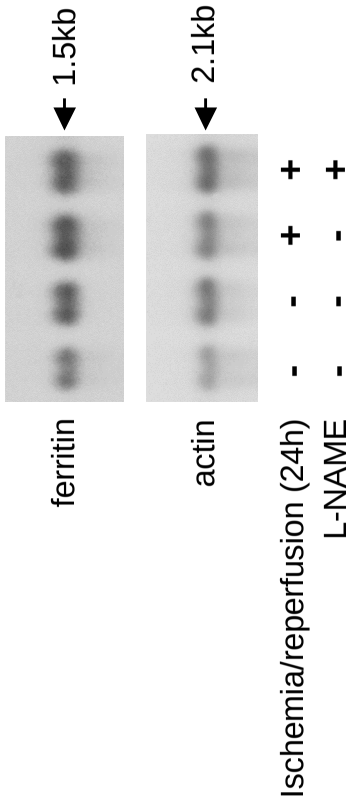

Figure 6

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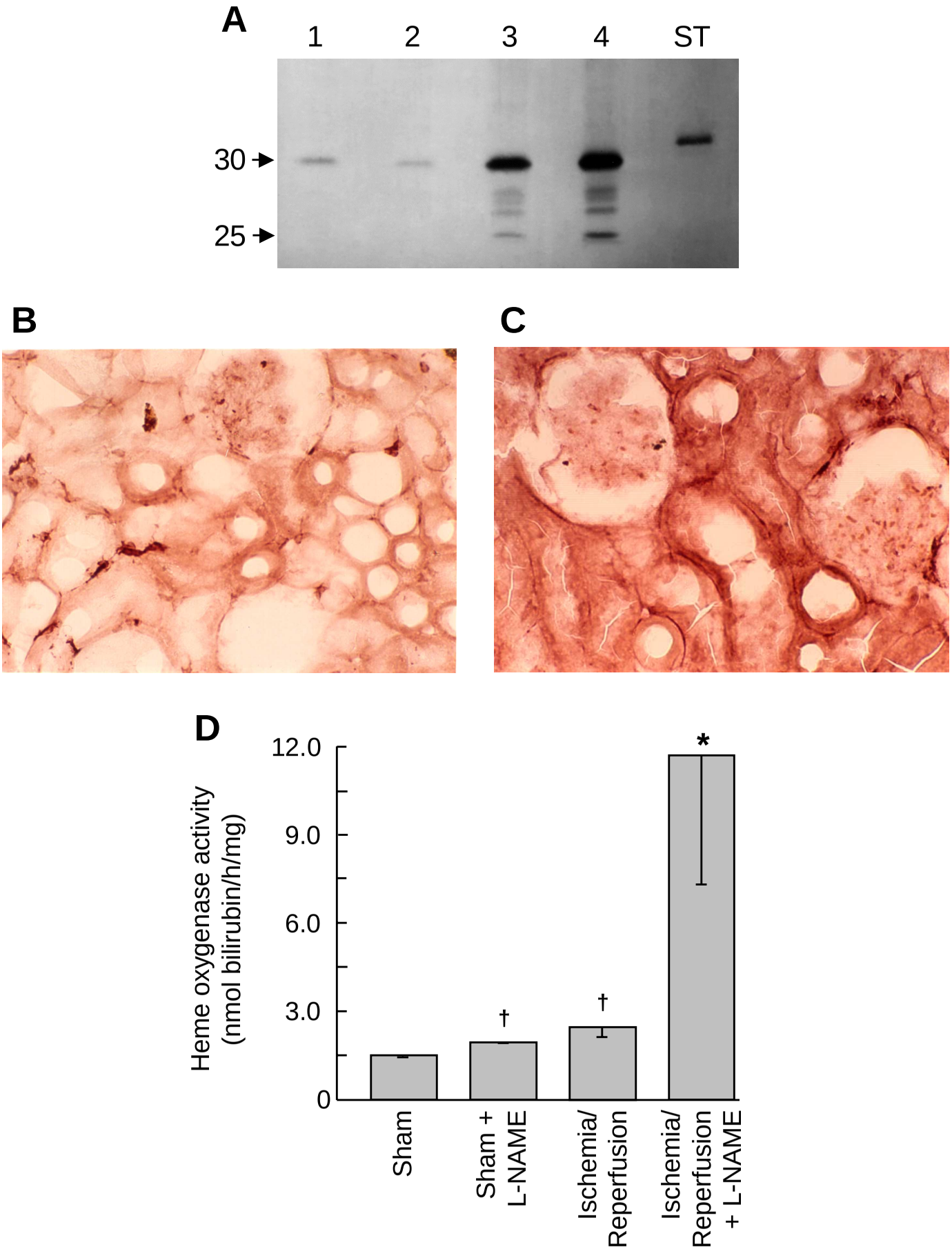

Figure 7