British Journal of Pharmacology (1998) 125, 1437–1444
http://www.stockton-press.co.uk/bjp

Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haeme oxygenase-1

[1]Ivan A. Sammut, [1]Roberta Foresti, [1]James E. Clark, [1]David J. Exon, [1]Martin J.J. Vesely, [1]Padmini Sarathchandra, [1]Colin J. Green & [1,2]Roberto Motterlini

[1]Vascular Biology Unit, Department of Surgical Research, Northwick Park Institute for Medical Research, Harrow, Middlesex HA1 3UJ

1 The contribution of haeme oxygenase-derived carbon monoxide (CO) to the regulation of vascular tone in thoracic aorta was investigated following induction of the inducible isoform of haeme oxygenase (HO-1).

2 Isometric smooth muscle contractions were recorded in isolated rat aortic ring preparations. Rings were incubated in the presence of the nitric oxide (NO) donor S-nitroso-N-acetyl penicillamine (SNAP, 500 μM) for 1 h, then repetitively washed and maintained for a further 4 h prior to producing a concentration-response curve to phenylephrine (PE, 1–3000 nM).

3 Treatment with SNAP resulted in increased mRNA and protein expression of aortic HO-1 and was associated with a significant suppression of the contractile response to PE ($P<0.05$ vs control). Immunohistochemical staining procedures revealed marked HO-1 expression in the endothelial layer and, to a lesser extent, in smooth muscle cells.

4 Induction of HO-1 in SNAP-treated rings was associated with a higher ^{14}CO release compared to control, as measured by scintillation counting after incubation of aortas with [2-^{14}C]-L-glycine, the precursor of haeme. Guanosine 3′,5′-monophosphate (cyclic GMP) content was also greatly enhanced in aortas expressing high levels of HO-1.

5 Incubation of aortic rings with the NO synthase inhibitor, N^G-monomethyl-L-arginine (100 μM), significantly ($P<0.05$) increased the contractile response to PE in controls but failed to restore PE-mediated contractility in SNAP-treated rings. In contrast, the selective inhibitor of haeme oxygenase, tin protoporphyrin IX (SnPP-IX, 10 μM), restored the pressor response to PE in SNAP-treated rings whilst markedly reducing CO and cyclic GMP production.

6 We conclude that up-regulation of the HO-1/CO pathway significantly contributes to the suppression of aortic contractility to PE. This effect appears to be mediated by the elevation of cyclic GMP levels and can be reversed by inhibition of the haeme oxygenase pathway.

Keywords: Haeme oxygenase-1; carbon monoxide; NO donors; aortic contractility; tin protoporphyrin IX; cyclic GMP

Introduction

The maintenance and local regulation of vascular tone is attributed to nitric oxide (NO), a free radical species identified as endothelium-derived relaxing factor (EDRF) and shown to have multiple biological functions (Ignarro *et al*., 1987; Palmer *et al*., 1987). This short-lived molecule is continuously generated by the constitutive isoform of endothelial NO synthase (cNOS) and activates a haeme-containing guanylate cyclase in adjacent smooth muscle cells to trigger vasorelaxation (Ignarro, 1989). There is however strong evidence to support a similar physiological role for carbon monoxide (CO), another endogenously produced signalling molecule which is gaining increasing attention in vascular biology. CO appears indeed to mimic many of the functions of NO, including inhibition of platelet aggregation (Brune & Ullrich, 1988) and activation of soluble guanylate cyclase (Kharitonov *et al*., 1995). Recent studies in the isolated perfused liver have also demonstrated the involvement of CO in the regulation of relaxation processes mediated by hepatic sinusoidal cells (Suematsu *et al*., 1995).

The major cellular source of CO is haeme oxygenase, a ubiquitously expressed protein that catalyzes the oxidative degradation of haeme to biliverdin, CO and iron (Abraham *et al*., 1988). Two isoforms of haeme oxygenase, expressed by two distinct genes, have so far been characterized (Maines *et al*., 1986). HO-2 is constitutively present at high levels in brain and testes (McCoubrey & Maines, 1994; Ewing & Maines, 1992). In contrast, the HO-1 isoform is an inducible enzyme which is regarded as a heat shock protein (HSP32) (Maines, 1988). The HO-1 gene is exquisitely sensitive to a large variety of stimuli and agents that cause oxidative stress, such as heat shock (Raju & Maines, 1994), ischaemia-reperfusion (Raju & Maines, 1996), hypoxia (Morita *et al*., 1995) and endotoxins (Rizzardini *et al*., 1994). Moreover, recent evidence revealed that increased haeme oxygenase activity and HO-1 expression occurs following exposure of cultured vascular endothelial and smooth muscle cells to NO donors (Motterlini *et al*., 1996; Foresti *et al*., 1997; Durante *et al*., 1997). Although the physiological significance of HO-1 induction still remains to be elucidated, activation of the haeme oxygenase pathway under various stress conditions may provide a defence mechanism in compromised tissues because of the potent antioxidant and vasoactive properties of biliverdin and CO, respectively (Abraham *et al*., 1996). The cardiovascular system has a high capacity to produce CO, since HO-2 is constitutively expressed in endothelial and smooth muscle cells (Zakhary *et al*., 1996; Werkstrom *et al*., 1997) and HO-1 can be greatly up-regulated by heat shock in the heart and blood vessels (Ewing *et al*., 1994). Moreover, the substrate of haeme oxygenase, haeme, is readily available for catalysis in both vascular and myocardial tissues (Maines, 1997). Previous reports suggested that CO generated from HO-2 is involved in vessel relaxation

[2]Author for correspondence. E-mail: r.motterlini@ic.ac.uk

(Suematsu *et al*., 1995; Zakhary *et al*., 1996) and that up-regulation of HO-1 is associated with reduction of vascular tone (Coceani *et al*., 1997; Yet *et al*., 1997). Accordingly, we have recently shown that the hypertensive response to haemoglobin or NO synthase inhibitors *in vivo* is virtually abolished following stress-mediated induction of the HO-1/CO pathway in vascular tissue (Motterlini, 1996; Motterlini *et al*., 1998). However, the specific role of HO-1-derived CO in the control of vessel contractility remains to be established.

The present study was designed to analyse whether the NO releasing agent S-nitroso-N-acetyl-D,L-penicillamine (SNAP) mediates an increase in HO-1 gene and protein expression in isolated rat aorta. Having verified induction of HO-1 in aortas treated with SNAP, we examined the effect of this treatment on vascular contractility to phenylephrine in the absence or presence of inhibitors of NO synthase and haeme oxygenase activities. Concomitantly, we have investigated the ability of haeme oxygenase to generate CO and its direct effect in regulating aortic cyclic GMP levels.

Methods

Experiments using isolated rat aortic rings

Transverse ring sections of aorta were isolated from male Lewis rats (350–450 g) and suspended under 2 g tension in an organ bath containing 9 ml of oxygenase (95% O_2/5% CO_2) Krebs Henseleit buffer (mM): (NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 22, glucose 11, K^+EDTA 0.03, $CaCl_2$ 2.5) using a method previously described (Scott *et al*., 1996). Indomethacin (10 μM) was present in the buffer to exclude prostaglandins as potential modulators of vascular tone. Before each protocol was carried out, rings were contracted with a standard dose of KCl (100 mM) in order to provide an internal reference and to control for variability in contractile responsiveness between tissues. All results were subsequently expressed as a percentage of the KCl-induced contraction. Relaxation responses to acetylcholine (1–1000 nM) in tissues pre-contracted with phenylephrine (3 μM) were used to confirm the integrity of the endothelium in this model. After the initial determination of vascular responsiveness to KCl (100 mM), aortic rings were incubated with SNAP (500 μM) for 1 h, then thoroughly washed and maintained for a further 4 h in buffer with intermittent washing. Control rings were similarly treated by adding an equal volume of DMSO (0.01% v/v, vehicle) to the organ bath. At the end of the 5 h incubation period, a cumulative concentration-response curve to phenylephrine (1–3000 nM) was obtained for each ring. In additional experiments, the NOS inhibitor L-NMMA (100 μM) or the haeme oxygenase inhibitor SnPP-IX (10 or 100 μM) was added to the organ bath of control and SNAP-treated rings during the last 30 min of incubation prior to producing a concentration-response curve to phenylephrine. In a final set of experiments, control and SNAP-treated vessels were incubated simultaneously with L-NMMA and SnPP-IX prior to assessing the vascular responsiveness to phenylephrine.

Reverse transcription polymerase chain reaction (RT–PCR)

Two aortas from each group were ground in a mortar under liquid nitrogen and suspended in guanidinium thiocyanate lysis buffer. Total mRNA was then extracted using a modification of the method described by Chomczynski & Sacchi (1987). RT–PCR was used to detect HO-1 gene expression. Aliquots of 1 μg RNA were reverse-transcribed using the Geneamp RNA PCR kit (Perkin-Elmer) and random hexamers to prime the reverse transcriptase. The cDNA was then amplified with 2.5 units of AmpliTAq DNA polymerase using a manual hot start, with primers for both HO-1 and β-actin (50 μM each in a total reaction volume of 100 μl) added to the same reaction tube. The sense and antisense primers for HO-1 were 5′-CTTTCAGAAGGGTCAGGTGTCCA-3′ and 5′-CTGAGAGGTCACCCAGGTAGCGG-3′, respectively, yielding a single fragment of 309 bp. As the internal control gene, β-actin was used with sense and antisense primer sequences of 5′-CGTGGGCCGCCCTAGGCACCA-3′ and 5′-CGGTTGCCTTAGGGTTCAGAGGGG-3′, respectively, yielding a fragment of 224 bp. After initial melting at 95°C, the PCR mixtures were amplified (Biometra Thermal Cycler) for a total of 34 cycles using a two step protocol of melting at 95°C for 1 min and annealing at 61°C for 1.5 min. Twenty microliters of the product was electrophoresed for 1 h on 2.5% agarose gel containing 0.5 μg ml^{-1} ethidium bromide in 1X-Tris-borate-EDTA buffer.

Northern blot analysis

Total RNA (10 μg lane^{-1}) was run on a 1.3% denaturing agarose gel containing 2.2 M formaldehyde and transferred onto a nylon membrane, according to the method described by Tyrrell & Basu-Modak (1994). The membrane was hybridized using α-^{32}P-dCTP-labelled cDNA probes to the rat HO-1 gene (Shibahara *et al*., 1985) and rat GAPDH gene and exposed to radiographic film.

Western blot analysis

Aortic tissues were homogenized in 1 ml of lysis buffer (50 mM HEPES, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, pH 7.5) containing Complete™ protease inhibitor (Boehringer Mannheim). Samples were kept on ice for 1 h and then centrifuged (4°C) for 30 min at 12,000 $\times g$. After the precipitated unsolubilized fraction was discarded, protein concentration was determined in the supernatant by the Lowry method and Western blot analysis was carried out using HO-1 antibody (Stressgen, Canada) as we previously described (Foresti *et al*., 1997).

Immunohistochemical staining

Sections of aortas (5 μm thickness) were treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. Immunohistochemical staining was performed using rabbit polyclonal antibody against HO-1 (1:1000 dilution) as previously described (Yet *et al*., 1997). The presence of HO-1 was indicated by the development of a brown colour. Counterstaining was performed using Cole's haematoxylin.

Measurement of CO and cyclic GMP production in aortic tissue

We specifically designed an appropriate device for measuring CO released from aortic tissue (Motterlini *et al*., 1998). Two plastic syringes were positioned vertically facing each other and connected by a 0.4 μm filter (Millipore, Watford, U.K.) to create two separate chambers. The lower chamber contained 5 μCi of [2-^{14}C]-L-glycine, a haeme precursor, and the upper chamber contained a solution of deoxyhaemoglobin (15 μmol l^{-1}), which is known to avidly bind CO. Aortas were transferred into the lower chamber containing [2-^{14}C]-L-

glycine in oxygenated Krebs Henseleit buffer and maintained at 37°C for 6 h to allow incorporation of [2-^{14}C]-L-glycine into the aortic tissue. At the end of the incubation period, produced CO was measured in the upper chamber as ^{14}CO bound to haemoglobin by scintillation counting (Beckman Liquid Scintillation Counter, Model LS6500). Levels of cyclic GMP were measured in freeze-clamped aortic tissue extracts using a commercial ELISA kit (Amersham). Duplicate measurements were performed on all samples ($n=4-5$ per group).

Drugs and materials

N^G-monomethyl-L-arginine (L-NMMA) was purchased from Alexis Corporation, Nottingham, U.K. S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and all other reagents were obtained from Sigma Chemicals (Poole, Dorset, U.K.) unless otherwise specified. The haeme oxygenase inhibitor, SnPP-IX, was purchased from Porphyrin Products Inc. (Logan, UT, U.S.A.). SnPP-IX was dissolved in 0.1 M NaOH and titrated with 0.1 M HCl to pH 7.4 immediately before use. All experiments were carried out in a darkened room because metalloporphyrins are light sensitive (Zygmunt *et al*., 1994). SNAP and indomethacin were dissolved in dimethylsulphoxide (DMSO, 0.01% v/v) at the desired concentration. Deoxyhaemoglobin was prepared from bovine haemoglobin using sodium dithionite as previously described (Martin *et al*., 1985). [2-^{14}C]-L-glycine was purchased from ICN (Oxfordshire, U.K.).

Statistics

All values are expressed as means ± s.e.mean of $n=4-6$ separate experiments per group. Differences in the data among the groups were analysed using one-way ANOVA combined with the Bonferroni test. A value of $P<0.05$ was considered significant.

Results

Effect of SNAP treatment on HO-1 induction and contractile responses to phenylephrine in aortic rings

A 1 h exposure of aortic tissue to SNAP (500 μM) followed by 4 h incubation in Krebs solution resulted in up-regulation of

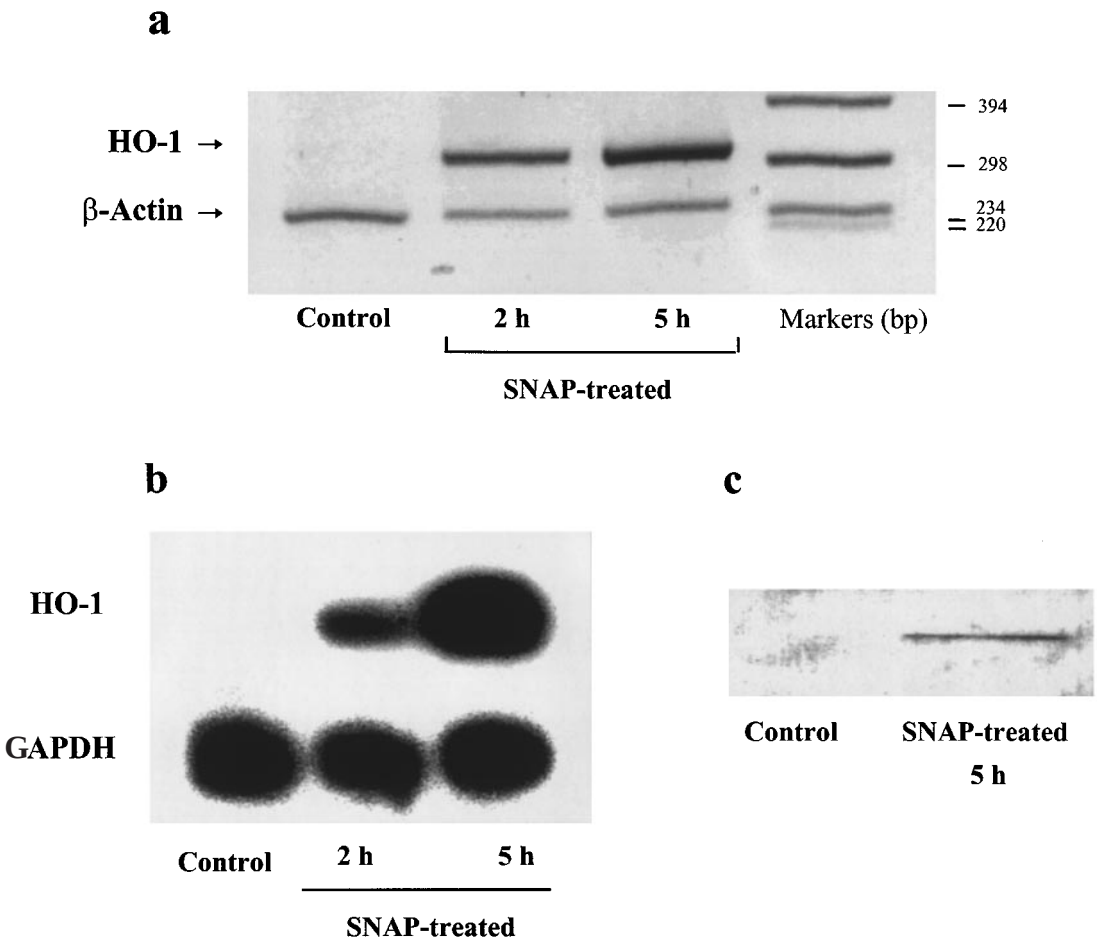

Figure 1 Effect of SNAP treatment on HO-1 mRNA and protein expression in isolated rat aorta. (a) Agarose gel showing the RT–PCR products in control and SNAP-treated isolated rat aortas. Aortas were treated with 500 μM SNAP for 1 h, washed and maintained for a further 1 h (2 h total) or 4 h (5 h total) in oxygenated Krebs Henseleit buffer (37°C). Control rings were incubated for 1 h with vehicle (0.01% DMSO), washed and maintained for a further 4 h (5 h total) in oxygenated Krebs Henseleit buffer at 37°C. (b) Northern blot analysis for HO-1 and GAPDH mRNA expression in control and SNAP-treated aortas. (c) Western blot analysis for HO-1 protein expression in control and SNAP-treated isolated aortas. Proteins (30 μg) from each sample were separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Non-specific antibody binding was blocked with 3% non-fat dried milk and membranes were probed with polyclonal rabbit anti-rat HO-1 antibodies. The analysis of HO-1 gene and protein expression is representative of two independent experiments.

the HO-1 gene. Densitometric analysis of the PCR products and Northern blot revealed a substantial and gradual increase in aortic mRNA HO-1 expression at 1 and 4 h post-treatment compared to control vessels (Figure 1a and b). Western blot analysis also demonstrated a significant increase in a 32 kDa band corresponding to HO-1 protein (Figure 1c). These data on HO-1 gene and protein expression were confirmed in two independent experiments. Immunohistochemical analysis showed an increased HO-1 staining which was localized primarily in the endothelial layer and to a lesser degree in the smooth muscle cells adjacent to the endothelium (Figure 2).

The increase in aortic HO-1 by SNAP treatment was associated with changes in the pressor response to the vasoconstrictor agent phenylephrine. Typical tracings of the vascular reactivity to phenylephrine are illustrated in Figure 3. Phenylephrine (1–3000 nM) produced a concentration-dependent increase in contractility in untreated rings (control, $n=5$); this contractile response was however significantly ($P<0.05$) suppressed in SNAP-treated rings (Figure 4a). At 300 nM phenylephrine, the contractile response of SNAP-treated rings was decreased to 8.7% of control (untreated vessels) (Table 1).

Effects of NO synthase and haeme oxygenase inhibitors on aortic contractile responses to phenylephrine

As shown in Table 1, the contractile response of untreated rings to phenylephrine (300 nM) in the presence of 100 μM L-NMMA was increased to 178.2% of control. However, L-NMMA failed to substantially restore the vascular responsiveness to the pressor agent in rings expressing high HO-1 levels (SNAP-treated aortas) (Table 1, Figure 4b). Since a correlation was found between HO-1 induction by SNAP treatment and decreased contractility, SnPP-IX was used to assess the direct contribution of HO-1 to the suppression of pressor responses to phenylephrine. Ten μM SnPP-IX had no effect on aortic contractility in untreated rings whereas, at higher concentrations (100 μM), SnPP-IX increased the phenylephrine-mediated response to 169.9% of control ($P<0.05$) (Table 1). Incubation of SNAP-treated rings with 10 μM SnPP-IX produced a marked ($P<0.05$) recovery of the aortic contractility (Figure 4b) which, at 300 nM phenylephrine, was 58.4% of control (Table 1). In the presence of a higher concentration of SnPP-IX (100 μM) the recovery was even

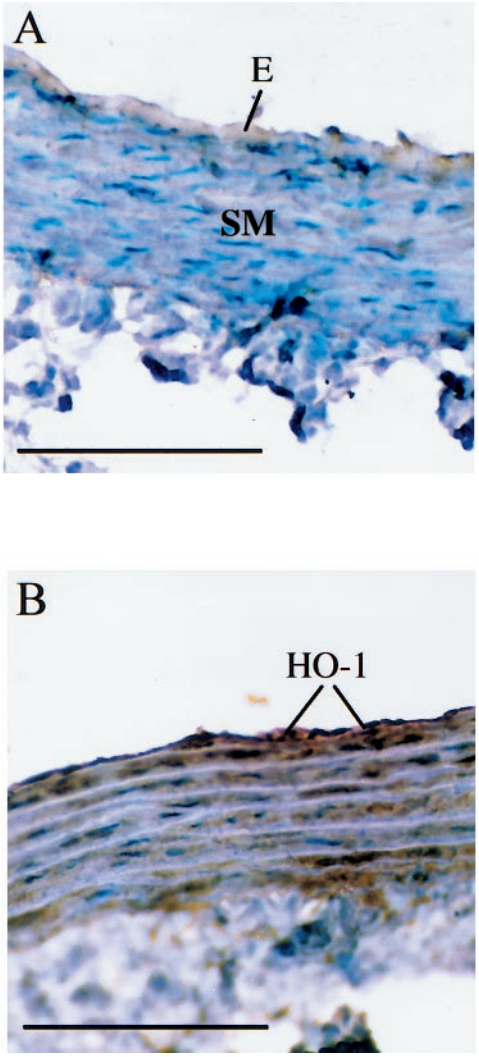

Figure 2 Localization of aortic HO-1 by immunohistochemistry. HO-1 immunoreactivity (brown staining) was assessed in sections of aortic tissue using the procedure described in Methods. While no HO-1 staining was observed in untreated aortas (control, A), SNAP-treated aortas (B) showed diffuse HO-1 protein expression (brown staining) localized primarily in the endothelium (E) and to a lesser extent in the smooth muscle (SM). Magnification bars = 100 μm.

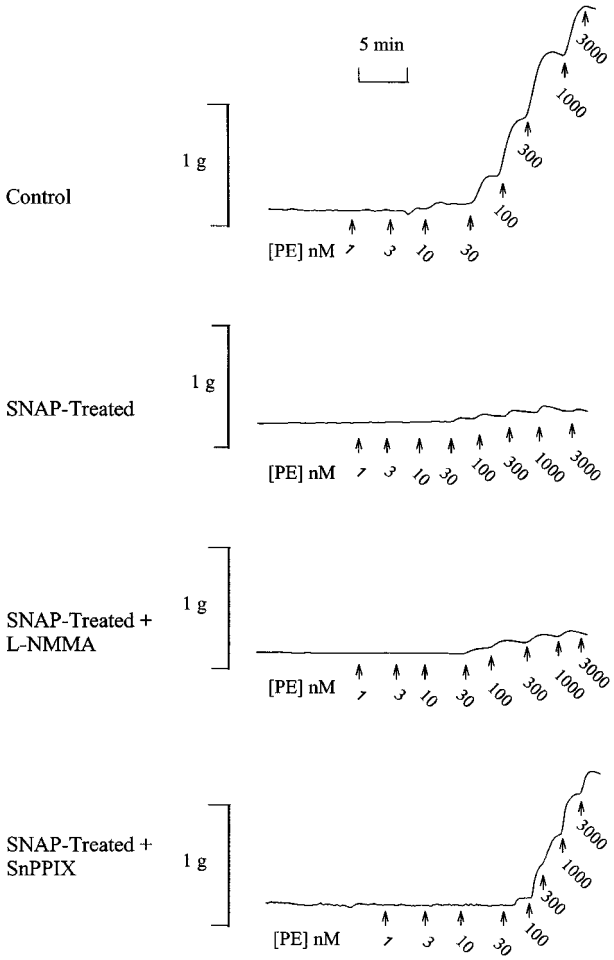

Figure 3 Typical isometric recordings of concentration-dependent responses to phenylephrine (PE; 1–3000 nM) in: control (untreated rings); SNAP-treated rings; SNAP-treated rings in the presence of L-NMMA (100 μM) or SnPP-IX (10 μM). Rings were exposed to SNAP for 1 h and allowed to recover in Krebs buffer for an additional 4 h prior to assessing the response curve to PE. L-NMMA or SnPP-IX were added to the organ bath of SNAP-treated rings during the last 30 min of incubation and then exposed to cumulative increasing PE concentrations indicated by the arrows.

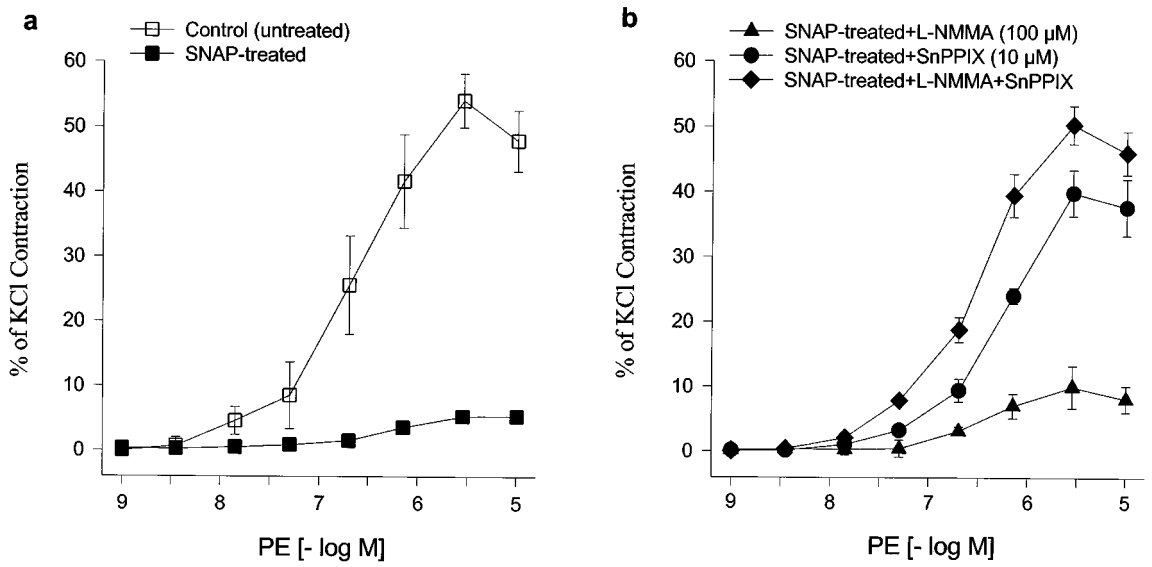

Figure 4 Effect of L-NMMA and SnPP-IX on the contractile response to phenylephrine (PE) in control and SNAP-treated rings. Rings were treated as described in Figure 3. All results are expressed as a percentage of the initial KCl-induced contraction. (a) Concentration-response curves to PE [−log M] in SNAP-treated rings compared to control (untreated). (b) Concentration-response curves to PE [−log M] in SNAP-treated rings incubated with 10 μM SnPP-IX, 100 μM L-NMMA or SnPP-IX plus L-NMMA. Each value represents the means±s.e.mean of 5–6 experiments.

Table 1 Contractile response of untreated and SNAP-treated aortic rings to 300 nM phenylephrine

	Untreated rings		*SNAP-treated rings*	
	(% of KCl)	(% of Control)	(% of KCl)	(% of Control)
Without inhibitors	41.7±7.2	100.0	3.6±0.5*	8.7
L-NMMA (100 μM)	73.3±8.1*	178.2	7.1±2.0*†	17.1
SnPP-IX (10 μM)	40.1±2.8	97.5	24.1±1.9*†	58.4
SnPP-IX (100 μM)	69.9±16*	169.9	34.6±4.0*†	84.0
SnPP-IX (10 μM) plus L-NMMA (100 μM)	72.9±13*	176.9	39.5±3.3†	95.9

Influence of haeme oxygenase and NO synthase inhibitors on the contractile response of aortic rings to phenylephrine. Phenylephrine was added cumulatively and the response to 300 nM is reported. Rings were exposed to SnPP-IX and L-NMMA individually or in combination as described in Figure 3. The contractility to phenylephrine was expressed as a percentage of the initial KCl (100 mM) contraction and normalized to control untreated vessels. Values represent the means±s.e.mean of 5–6 experiments. *$P<0.05$ compared to control; †$P<0.05$ compared to SNAP-treated rings without inhibitors.

more pronounced as the contractile response to phenylephrine increased to 84% of control (Table 1). Similarly, ZnPP-IX (100 μM) produced a recovery of the contractile response to phenylephrine, although it proved considerably less potent than SnPP-IX (10 or 100 μM) in reversing SNAP-mediated loss of contractility (data not shown). Co-incubation with SnPP-IX (10 μM) and L-NMMA (100 μM) almost completely restored the aortic contractility of SNAP-treated rings (Figure 4b) which, at 300 nM phenylephrine, was 95.9% of control (Table 1). This suggests that a residual cNOS activity exists in SNAP-treated rings only following blockade of the haeme oxygenase pathway. It is interesting to note that, in untreated rings, simultaneous incubation with SnPP-IX (10 μM) and L-NMMA (100 μM) did not cause any further change in the contractile response to phenylephrine compared to rings incubated with L-NMMA alone (Table 1).

Effect of HO-1 induction on CO and cyclic GMP production in aortic tissue

Up-regulation of HO-1 in SNAP-treated aortas resulted in an increased production of CO, one of the products of haeme degradation by haeme oxygenase. Following a 6 h incubation with the haeme precursor [2-^{14}C]-L-glycine, ^{14}CO release was significantly higher in SNAP-treated aortas compared to untreated aortas. This increase in ^{14}CO was markedly attenuated by 10 μM SnPP-IX (Figure 5a). Similarly, cyclic GMP was significantly elevated in SNAP-treated aortas. SnPP-IX (10 μM) but not L-NMMA (100 μM) considerably reduced the increase in cyclic GMP levels (Figure 5b). It is interesting to note that 10 μM SnPP-IX did not cause any change in the basal cyclic GMP level of untreated aortas. A substantial increase in cyclic GMP was also obtained by exposing untreated aortas to 1% CO gas for 30 s. This experiment was included to verify the direct effect of exogenously applied CO on aortic cyclic GMP formation.

Discussion

In this paper we present results concerning the specific role of the HO-1/CO system in the control of vascular contractility. The expression of HO-1, the inducible isoform of haeme oxygenase, can be enhanced *in vitro* and *in vivo* by a variety of stress-related agents (Abraham *et al*., 1996). Recent evidence provided by our own and other laboratories has shown the

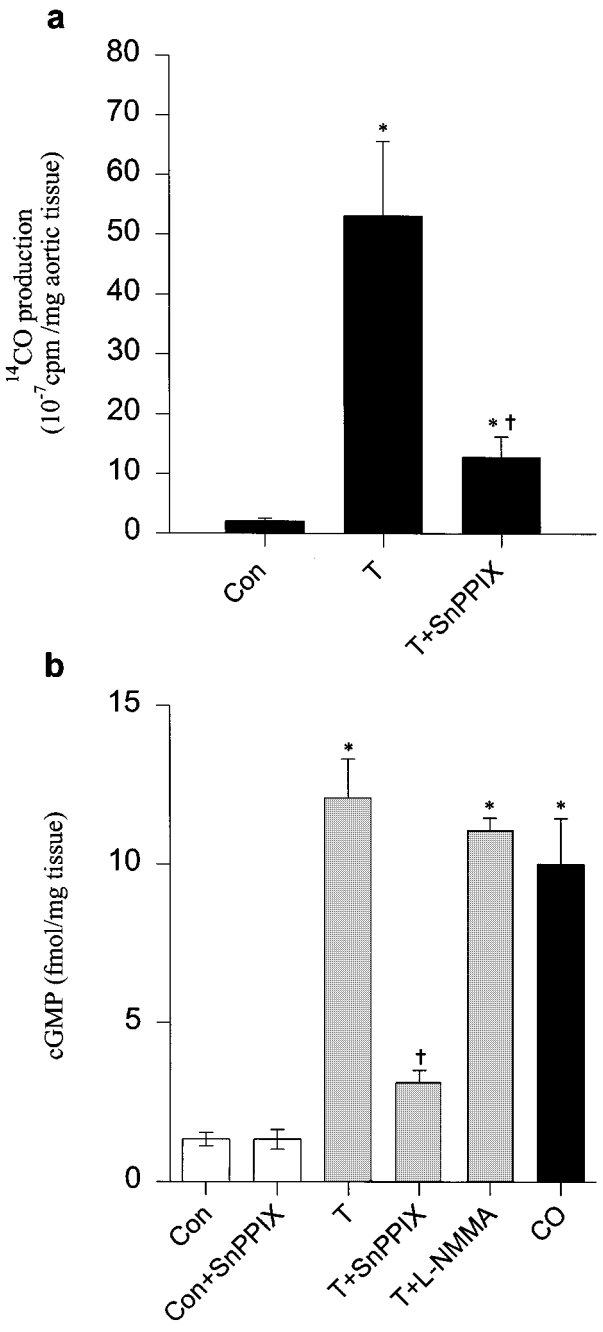

Figure 5 Effect of HO-1 induction on aortic CO production and cyclic GMP levels. (a) ^{14}CO release from aortas was measured by scintillation counting following a 6 h incubation with [2-^{14}C]-L-glycine. Control (Con); SNAP-treated aortas (T); SNAP-treated aortas in the presence of 10 μM SnPP-IX (T + SnPP-IX). (b) cyclic GMP levels measured in control (Con) and SNAP-treated (T) aortas. SnPP-IX (10 μM) and L-NMMA (100 μM) were added to the organ bath as reported in Figure 3. Exogenous CO (1% for 30 s) was also applied to untreated rings for comparison. Each value represents the means ± s.e.mean of 4–6 experiments. *$P<0.05$ compared to control, †$P<0.05$ compared to SNAP-treated rings.

ability of NO donors to up-regulate the HO-1 gene and consequently increase haeme oxygenase activity in aortic endothelial and smooth muscle cells (Motterlini *et al*., 1996; Foresti *et al*., 1997; Durante *et al*., 1997). In this study we found that treatment of aortic tissue with the NO donor, SNAP, resulted in a substantial increase in HO-1 mRNA and protein levels as shown by RT–PCR and Western blot, respectively. Based on this finding, we used SNAP-treated isolated aortic rings to investigate the contribution of HO-1 induction to the regulation of vascular tone.

Under physiological conditions, NO is the main intracellular regulator of vessel tone and blockade of NO synthase in aortic rings results in a potentiation of the contraction elicited by phenylephrine (Ignarro *et al*., 1987; Palmer *et al*., 1987; Kaneko & Sunano, 1993). Accordingly, we found that the contractile response of untreated rings to phenylephrine in the presence of the NO synthase inhibitor, L-NMMA, was significantly increased above control. Interestingly, when SNAP-treated rings were tested, phenylephrine did not elicit aortic contractions and L-NMMA failed to substantially restore the vascular responsiveness to the pressor. In addition, treatment of aortic tissue with SNAP did not result in changes in the protein expression of inducible NO synthase (iNOS, unpublished observations). Since increased levels of haeme oxygenase would be associated with higher haeme catabolism (Maines, 1988), we reasoned that up-regulation of HO-1 in SNAP-treated rings would result in increased CO production with possible consequent vascular effects.

To test the direct contribution of HO-1 induction to vessel tone regulation in our model, SNAP-treated rings were incubated with SnPP-IX, a potent competitive inhibitor of haeme oxygenase activity (Drummond & Kappas, 1981), prior to assessment of the responsiveness to phenylephrine. Vascular contractility to the pressor agent was, indeed, markedly restored by 10 μM SnPP-IX; this was even more pronounced at a higher concentration of SnPP-IX (100 μM). It has been suggested that SnPP-IX may also have a non-selective effect in inhibiting the activity of NO synthase and soluble guanylate cyclase, both haeme-dependent enzymes participating in vasorelaxation processes (Luo & Vincent, 1994; Grundemar & Ny, 1997). Nevertheless, and in agreement with previous reports (Zakhary *et al*., 1996; Maines, 1997), we found that 10 μM SnPP-IX had no effect on the pressor response to phenylephrine and cyclic GMP levels in untreated rings. Moreover, incubation of untreated rings with 10 μM SnPP-IX did not further increase the contractility to phenylephrine induced by 100 μM L-NMMA. These data confirm that maintenance and local regulation of vessel tone under normal conditions is primarily due to NO synthase, but emphasize a crucial role of the haeme oxygenase/CO pathway in conditions characterized by elevated HO-1.

Our data also showed that incubation of untreated rings with 100 μM SnPP-IX, unlike 10 μM SnPP-IX, resulted in an increased contractility to phenylephrine similar to that produced by 100 μM L-NMMA (see Table 1). This indicates that under normal conditions, 100 μM SnPP-IX may indeed have some inhibitory effect on NO synthase and/or guanylate cyclase as reported by others (Zakhary *et al*., 1996; Grundemar & Ny, 1997). However, the findings showing that 100 μM SnPP-IX did not completely restore the phenylephrine-mediated response in SNAP-treated rings (84% of control) may suggest that higher concentrations of the inhibitor are required to block haeme oxygenase activity following induction of the HO-1 gene. It has to be emphasized that both NO synthase and guanylate cyclase are haeme-containing proteins which implies that haeme is an integral part of the enzyme (Maines, 1997). In contrast, haeme oxygenase utilizes haeme as a substrate and metalloporphyrins, such as SnPP-IX, can exert their inhibitory action simply by competing with haeme for the catalytic site. Therefore, it can be inferred that by comparison with haeme-dependent enzymes, haeme oxygenase is the most likely target for metalloporphyrins, particularly when the level of HO-1 is enhanced. It is also interesting to note that, in SNAP-treated rings, the combina-

tion of NO synthase and haeme oxygenase inhibitors (100 μM L-NMMA plus 10 μM SnPP-IX) restored the contractile response to phenylephrine to 95.9% of control (see Table 1). These findings suggest that, in aortic tissue characterized by elevated HO-1 expression, suppression of the vascular contractility to pressor agents may be the result of reciprocal interactions between these two enzymatic pathways. Consistent with this idea, recent reports have shown that CO modulates the NO-cyclic GMP system in heart and cerebellar granule cells (Maulik *et al*., 1996; Ingi *et al*., 1996).

In the vascular tissue, constitutive HO-2 is the most abundant isoform of haeme oxygenase present under normal conditions (Maines, 1997) and provides the major enzymatic pathway for the degradation of haeme to CO and biliverdin. CO appears to mimic many of the functions of NO, including inhibition of platelet aggregation (Brune & Ullrich, 1988), activation of soluble guanylate cyclase (Kharitonov *et al*., 1995) and neurotransmission (Verma *et al*., 1993; Maines, 1993). A vasoregulatory role for CO produced by constitutive HO-2 has been previously postulated in distal pulmonary arteries (Zakhary *et al*., 1996), although no direct measurement of CO was provided. Increased cyclic GMP levels as a result of guanylate cyclase activation by exogenous CO have also been reported to play a crucial role in the relaxation of ileal smooth muscle (Utz & Ullrich, 1991) and in the maintenance of hepatic sinusoidal tone (Suematsu *et al*., 1995). In the present study we measured CO production by loading aortic tissues with [2-^{14}C]-L-glycine, the precursor of haeme, and then detecting the ^{14}CO released from aorta by scintillation counting. We found that, concomitant with increased HO-1 expression, ^{14}CO release and cyclic GMP levels in SNAP-treated aortas were indeed significantly elevated. Interestingly, we also found that both ^{14}CO and cyclic GMP production were markedly attenuated by incubation of SNAP-treated rings with 10 μM SnPP-IX. These results indicate that CO, endogenously released as a consequence of HO-1 induction, is directly associated with guanylate cyclase activation resulting in suppression of the aortic contractile response to phenylephrine. Consistent with the lack of effect of L-NMMA (100 μM) in restoring the phenylephrine-mediated pressor response in SNAP-treated aortas, we found that augmented cyclic GMP levels in this group were not significantly affected by the presence of the NO synthase inhibitor. Our results demonstrate a significant function of CO in the control of aortic contraction indicating that, under conditions of elevated HO-1, CO is a major contributor to this effect.

The findings presented here could have important clinical and pharmacological implications. Alteration of the local mechanisms controlling pressor responses in certain disease states, such as sepsis, is mainly attributed to overproduction of NO from iNOS (Thiemermann *et al*., 1993). However, recent reports have demonstrated that various pathophysiological conditions such as haemorrhagic shock (Bauer *et al*., 1996), endotoxic shock (Yet *et al*., 1997) and ischaemia/reperfusion injury (Maines *et al*., 1993; Raju & Maines, 1996; Geddes *et al*., 1996) are also characterized by elevated HO-1 expression in both parenchymal and vascular tissues. Therefore, it is plausible to suggest that NO-mediated stimulation of haeme oxygenase activity and CO production in certain disease states might be as crucial as the iNOS pathway in the control of vessel tone. In accordance with this hypothesis, our group has recently shown that CO generated in aortas following stress-mediated induction of HO-1 *in vivo* directly contributes to the suppression of acute hypertensive responses caused by haemoglobin and L-NAME, two agents commonly used to block NO function (Motterlini *et al*., 1998).

In summary, HO-1 induction in aortic tissue is correlated with an increased endogenous release of CO that suppresses vascular contractility by activation of the guanylate cyclase-cyclic GMP system. Our results emphasize the signalling properties of increased CO production in the control of vascular tone.

We are grateful to Prof S. Shibahara for providing the cDNA probe for rat HO-1 and to Sandra Shurey and Dr Saleem Mohammed for expert technical assistance. This work was supported by a Grant from the National Heart Research Fund, Leeds, United Kingdom (Roberto Motterlini).

References

ABRAHAM, N.G., LIN, J.H.C., SCHWARTZMAN, M.L., LEVERE, R.D. & SHIBAHARA, S. (1988). The physiological significance of heme oxygenase *Int. J. Biochem.,* **20,** 543 – 558.

ABRAHAM, N.G., DRUMMOND, G.S., LUTTON, J.D. & KAPPAS, A. (1996). The biological significance and physiological role of heme oxygenase. *Cell. Physiol. Biochem.,* **6,** 129 – 168.

BAUER, M., PANNEN, B.H.J., BAUER, I., HERZOG, C., WANNER, G.A., HANSELMANN, R., ZHANG, J.X., CLEMENS, M.G. & LARSEN, R. (1996). Evidence for a functional-link between stress-response and vascular control in hepatic portal circulation. *Am. J. Physiol.,* **271,** G929 – G935.

BRUNE, B. & ULLRICH, V. (1988). Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol. Pharmacol.,* **32,** 497 – 504.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* 156 – 159.

COCEANI, F., KELSEY, L., SEIDLITZ, E., MARKS, G.S., MCLAUGHLIN, B.E., VREMAN, H.J., STEVENSON, D.K., RABINOVITCH, M. & ACKERLEY, C. (1997). Carbon monoxide formation in the ductus arteriosus in the lamb: implications for the regulation of muscle tone. *Br. J. Pharmacol.,* **120,** 599 – 608.

DRUMMOND, G.S. & KAPPAS, A. (1981). Prevention of neonatal hyperbilirubinemia by tin protoporphyrin-IX, a potent competitive inhibitor of heme oxygenase. *Proc. Natl. Acad. Sci. U.S.A.,* **78,** 6466 – 6470.

DURANTE, W., KROLL, M.H., CHRISTODOULIDES, N., PEYTON, K.J. & SCHAFER, A.I. (1997). Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circ. Res.,* **80,** 557 – 564.

EWING, J.F. & MAINES, M.D. (1992). In situ hybridization and immunohistochemical localization of heme oxygenase-2 mRNA and protein in normal rat brain: differential distribution of isozyme 1 and 2. *Mol. Cell. Neurosci.,* **3,** 559 – 570.

EWING, J.F., RAJU, V.S. & MAINES, M.D. (1994). Induction of heart heme oxygenase-1 (HSP32) by hyperthermia: possible role in stress-mediated elevation of cyclic 3′:5′-guanosine monophosphate. *J. Pharmacol. Exp. Ther.,* **271,** 408 – 414.

FORESTI, R., CLARK, J.E., GREEN, C.J. & MOTTERLINI, R. (1997). Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxynitrite anions. *J. Biol. Chem.,* **272,** 18411 – 18417.

GEDDES, J.W., PETTIGREW, L.C., HOLTZ, M.L., CRADDOCK, S.D. & MAINES, M.D. (1996). Permanent focal and transient global cerebral-ischemia increase glial and neuronal expression of heme oxygenase-1, but not heme oxygenase-2, protein in rat-brain. *Neurosci. Lett.,* **210,** 205 – 208.

GRUNDEMAR, L. & NY, L. (1997). Pitfalls using metalloporphyrins in carbon monoxide research. *Trends. Pharmacol. Sci.,* **18,** 193 – 195.

IGNARRO, L.J. (1989). Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ. Res.,* **65,** 1–21.

IGNARRO, L.J., BUGA, G.M. & WOOD, K.S. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 9265–9269.

INGI, T., CHENG, J. & RONNETT, G.V. (1996). Carbon-monoxide: an endogenous modulator of the nitric oxide-cyclic GMP signaling system. *Neuron.,* **16,** 835–842.

KANEKO, K. & SUNANO, S. (1993). Involvement of a-adrenoceptors in the endothelium-dependent depression of noradrenaline-induced contraction in rat aorta. *Eur. J. Pharmacol.,* **240,** 195–200.

KHARITONOV, V.G., SHARMA, V.S., PILZ, R.B., MAGDE, D. & KOESLING, D. (1995). Basis of guanylate cyclase activation by carbon monoxide. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 2568–2571.

LUO, D. & VINCENT, S.R. (1994). Metalloporphyrins inhibit nitric oxide-dependent cGMP formation in vivo. *Eur. J. Pharmacol.,* **267,** 263–267.

MAINES, M.D. (1988). Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB, J.,* **2,** 2557–2568.

MAINES, M.D. (1993). Carbon monoxide: an emerging regulator of cGMP in the brain. *Mol. Cell. Neurosci.,* **4,** 389–397.

MAINES, M.D. (1997). The heme oxygenase system: a regulator of second messenger gases. *Annu. Rev. Pharmacol. Toxicol.,* **37,** 517–554.

MAINES, M.D., MAYER, R.D., EWING, J.F. & MCCOUBREY, W.K. (1993). Induction of kidney heme oxygenase-1 (HSP32) mRNA and protein by ischemia/reperfusion: possible role of heme as both promoter of tissue damage and regulator of HSP32. *J. Pharmacol. Exp. Ther.,* **264,** 457–462.

MAINES, M.D., TRAKSHEL, G.M. & KUTTY, R.K. (1986). Characterization of two constitutive forms of rat liver microsomal heme oxygenase: only one molecular species of the enzyme is inducible. *J. Biol. Chem.,* **261,** 411–419.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.,* **232,** 708–716

MAULIK, N., ENGELMAN, D.T., WATANABE, M., ENGELMAN, R.M., ROUSOU, J.A., FLACK, J.E., DEATON, D.W., GORBUNOV, N.V., ELSAYED, N.M., KAGAN, V.E. & DAS, D.K. (1996). Nitric oxide/carbon monoxide: a molecular switch for myocardial preservation during ischemia. *Circulation,* **94** (suppl II), II-398–II-406.

MCCOUBREY, W.M. & MAINES, M.D. (1994). The structure, organization and differential expression of the gene encoding rat heme oxygenase-2. *Gene,* **139,** 155–161.

MORITA, T., PERRELLA, M.A., LEE, M.E. & KOUREMBANAS, S. (1995). Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 1475–1479.

MOTTERLINI, R. (1996). Interaction of hemoglobin with nitric oxide and carbon monoxide: physiological implications. In *Blood Substitutes: New Challenges*. eds Vandegriff, K., Intaglietta, M. & Winslow, R.M. pp 74–98. Boston, MA: Birkauser.

MOTTERLINI, R., FORESTI, R., INTAGLIETTA, M. & WINSLOW, R.M. (1996). NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *Am. J. Physiol.,* **270,** H107–H114.

MOTTERLINI, R., GONZALES, A., FORESTI, R., CLARK, J.E., GREEN, C.J. & WINSLOW, R.M. (1998). Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses *in vivo*. *Circ. Res.,* **83,** 568–577.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature,* **327,** 524–526.

RAJU, V.S. & MAINES, M.D. (1994). Coordinated expression and mechanism of induction of HSP32 (heme oxygenase-1) mRNA by hyperthermia in rat organs. *Biochem. Biophys. Acta.,* **1217,** 273–280.

RAJU, V.S. & MAINES, M.D. (1996). Renal ischemia/reperfusion up-regulates heme oxygenase-1 (HSP32) expression and increases cGMP in rat heart. *J. Pharmacol. Exp. Ther.,* **277,** 1814–1822.

RIZZARDINI, M., CARELLI, M., CABELLO, PORRAS, M.R. & CANTONI, L. (1994). Mechanisms of endotoxin-induced haem oxygenase mRNA accumulation in mouse liver: synergism by glutathione depletion and protection by N-acetylcysteine. *Biochem. J.,* **304,** 477–483.

SCOTT, J.A., MACHOUN, M. & MCCORMACK, D.G. (1996). Inducible nitric oxide synthase and vascular reactivity in rat thoracic aorta: effect of aminoguanidine. *J. Appl. Physiol.,* **80,** 271–277.

SHIBAHARA, S., MULLER, R., TAGUCHI, H. & YOSHIDA, T. (1985). Cloning and expression of cDNA for rat heme oxygenase. *Proc. Natl. Acad. Sci. U.S.A.,* **82,** 7865–7869.

SUEMATSU, M., GODA, N., SANO, T., KASHIWAGI, S., EGAWA, T., SHINODA, Y. & ISHIMURA, Y. (1995). Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. *J. Clin. Invest.* **96,** 2431–2437.

THIEMERMANN, C., SZABO, C., MITCHELL, J.A. & VANE, J.R. (1993). Vascular hyporeactivity to vasoconstrictor agents and hemodynamic decompensation in hemorrhagic shock is mediated by nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 267–271.

TYRRELL, R.M. & BASU-MODAK, S. (1994). Transient enhancement of heme oxygenase 1 mRNA accumulation: a marker of oxidative stress to eukaryotic cells. *Methods Enzymol.,* **234,** 224–235.

UTZ, J. & ULLRICH, V. (1991). Carbon monoxide relaxes ileal smooth muscle through activation of guanylate cyclase. *Biochem. Pharmacol.,* **41,** 1195–1201.

VERMA, A., HIRSCH, D.J., GLATT, C.E., RONNETT, G.V. & SNYDER, S.H. (1993). Carbon monoxide: a putative neural messenger. *Science,* **259,** 381–384.

WERKSTROM. V., NY, L., PERSSON, K. & ANDERSSON, K.E. (1997). Carbon monoxide-induced relaxation and distribution of haem oxygenase isoenzymes in the pig urethra and lower oesophagogastric junction. *Br. J. Pharmacol.,* **120,** 312–318.

YET, S.F., PELLACANI, A., PATTERSON, C., TAN, L., FOLTA, S.C., FOSTER, L., LEE, W.S., HSIEH, C.M. & PERRELLA, M.A. (1997). Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. *J. Biol. Chem.,* **272,** 4295–4301.

ZAKHARY, R., GAINE, S.P., DINERMAN, J.L., RUAT, M., FLAVAHAN, N.A. & SNYDER, S.H. (1996). Heme oxygenase 2: endothelial and neuronal localization and role in endothelium-dependent relaxation. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 795–798.

ZYGMUNT, P.M., HOGESTATT, E.D. & GRUNDEMAR, L. (1994). Light-dependent effects of zinc protoporphyrin IX on endothelium-dependent relaxation resistant to Nω-nitro-L-arginine. *Acta. Physiol. Scand.,* **152,** 137–143.

(Received May 21, 1998
Revised September 1, 1998
Accepted September 7, 1998)