Haem and nitric oxide: synergism in the modulation of the endothelial haem oxygenase-1 pathway

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NO potently up-regulates vascular haem oxygenase-1 (HO-1), an inducible defensive protein that degrades haem to CO, iron and the antioxidant bilirubin. Since several pathological states are characterized by increased NO production and liberation of haem from haem-containing proteins, we examined how NO influences HO-1 induction mediated by haemin. Aortic endothelial cells treated with *S*-nitroso-*N*-acetylpenicillamine (SNAP), sodium nitroprusside (SNP) or diethylenetriamine-NONOate (DETA/NO) and haemin exhibited higher levels of haem oxygenase activity compared with cells exposed to NO donors or haemin alone. This was accompanied by a marked increase in bilirubin production and, notably, by a strong magnification of cellular haem uptake. A role for haem metabolites in modulating HO-1 expression by NO was assessed by exposing cells to SNAP, SNP or DETA/NO in medium derived from cells treated with haemin, which contained increased

bilirubin levels. This treatment considerably potentiated HO-1 expression and haem oxygenase activity mediated by NO and the use of a haem oxygenase inhibitor abolished this effect. Both iron liberated during haem breakdown and the formation of nitroxyl anion from NO appeared to partially contribute to the amplifying phenomenon; in addition, medium from haemin-treated cells significantly augmented the release of NO by NO donors. Thus we have identified novel mechanisms related to the induction of HO-1 by NO indicating that the signalling actions of NO vary significantly in the presence of haem and haem metabolites, ultimately increasing the defensive abilities of the endothelium to counteract oxidative and nitrosative stress.

Key words: bilirubin, haem catabolism, haem oxygenase-1 (HO-1) regulation, haem uptake, nitrosative stress.

INTRODUCTION

Haem oxygenase-1 (HO-1) is an inducible stress protein belonging to the family of haem oxygenases, a group of enzymes that degrades haem to biliverdin and CO (HO-1 and HO-2) [1] and is potentially involved in the control of intracellular haem levels (HO-2 and HO-3) [2,3]. Haem derived from oxidized haemoglobin can be readily taken up by endothelial cells and can serve both as inducer and substrate of the HO-1 pathway [4,5]. Indeed, HO-1 induction occurs during various pathological states such as haemorrhagic and subarachnoid shocks, which are characterized by increased haemolysis and the availability of free haem [6,7]. Interestingly, these and other circulatory shocks are also accompanied by over-production of NO with prominent vascular effects [8]. Several other compounds and conditions that cause oxidative stress stimulate the induction of HO-1 in different tissues and relevant experimental work has established a strong role for this protein in protection against oxidative-stress-mediated damage [1,9]. This defence appears to be related to the potent antioxidant properties of biliverdin and its reduction product bilirubin [10,11] as well as to CO, a signalling molecule with important vasoactive and anti-inflammatory characteristics [12]. In addition, iron induces synthesis of the iron-sequestering protein ferritin, thereby limiting the free metal's participation in perpetuating oxidative injury [13].

NO and NO-releasing agents distinguish themselves from other inducers of HO-1 because, apart from producing oxidation of biological targets, they may potentially impose an additional cellular threat, termed nitrosative stress [10,14–16]. Damage in these circumstances arises from excessive nitrosation of nucleophilic centres, leading to protein modification and loss

of function [17]. Thus if a rational explanation for oxidative-stress-mediated up-regulation of HO-1 can be offered on the basis of the antioxidant activities of biliverdin and bilirubin and the removal of the pro-oxidant haem, less obvious is the physiological significance of HO-1 stimulation by NO. We have postulated that NO serves as an intracellular signal to modulate the tissue stress response and that the haem oxygenase pathway interacts with NO to control cellular homeostasis [18,19]. Specifically, HO-1 could act as a feedback inhibitor of NO when the concentrations of the gaseous molecule exceed a critical threshold and the products of haem degradation might play a role in this process. In support of this hypothesis, *in vitro* studies recently conducted in our laboratory have shown that bilirubin and biliverdin interact efficiently with NO and its congeners, revealing novel 'anti-nitrosative' features of bile pigments [20]. Interesting aspects on the interrelation of NO with the haem oxygenase system have been reported by Juckett and colleagues [21]. These authors found that NO nitrosylates intracellular free haem and prevents its degradation by haem oxygenase, suggesting that NO could buffer excessive intracellular haem by reducing its redox activity. We were intrigued by these findings, which are apparently in contrast with the evidence that NO donors potently induce HO-1 in various cell types and this indicated that NO might regulate haem oxygenase's functions at multiple diverse levels. Indeed, it has been shown that NO induces loss of catalytic activity of HO-2, the constitutive isoform of haem oxygenase, by combining with haem bound to HO-2 haem motifs [22]. However, the enzymic activity of purified HO-1 is virtually unaffected by NO donors, presumably because of lack of haem-binding motifs in the protein structure of the enzyme [22]. In the present study, we aimed to investigate further these issues and examine the biological effect

Abbreviations used: HO-1, haem oxygenase-1; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; DETA/NO, diethylenetriamine-NONOate; SIN-1, 3-morpholininosydnomine; SnPIX, tin protoporphyrin IX; CM, conditioned medium.

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of haem and haem metabolites on NO-mediated induction of endothelial HO-1.

EXPERIMENTAL

Reagents

Bovine aortic endothelial cells ('BAECs') were from Coriell Cell Repositories (Camden, NJ, U.S.A.). Haemin and tin protoporphyrin IX (SnPPIX) were from Porphyrin Products (Logan, UT, U.S.A.). 3-Morpholininosydnomine (SIN-1) and diethylenetriamine-NONOate (DETA/NO) were obtained from Alexis Corp. (Bingham, Nottingham, U.K.). *S*-Nitroso-*N*-penicillamine (SNAP), sodium nitroprusside (SNP), ferrozene and all other chemicals were purchased from Sigma.

Cell culture and experimental protocol

Endothelial cells were grown in Iscove's modified Dulbecco's medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. To examine the effects of haem on NO-mediated HO-1 induction, experiments were performed by initially pre-treating cells with a combination of haemin (50 μ M) and NO donors for 2 h, followed by incubation with medium alone. Cells were also treated with 10–50 μ M haemin in complete medium for 2 h followed by 22 h incubation with complete medium alone to allow for accumulation of haem-degradation products. The medium from these haemin-treated cells (conditioned medium, CM) was then used for incubation of new cells with various NO-releasing agents (500 μ M SNAP, 500 μ M SNP, 500 μ M DETA/NO or 300 μ M SIN-1). CM plus SnPPIX was obtained by incubating cells with 50 μ M haemin and 100 μ M SnPPIX for 2 h followed by 22 h incubation with complete medium containing 100 μ M SnPPIX. Cells were also exposed to CM plus SnPPIX in the presence of DETA/NO. The amount of NO released in culture medium supplemented with SNAP, SNP or DETA/NO was measured over time (2 h) using an NO-sensitive electrode (ISO-NO Mark II; World Precision Instruments, Sarasota, FL, U.S.A.). Steady-state concentrations of 1–2 μ M, 25–50 nM and 8–10 nM NO were achieved during the degradation of SNAP, SNP and DETA/NO (500 μ M each), respectively. All experiments were performed in the dark because of the sensitivity to light exhibited by biliverdin and bilirubin. In some experiments, cells were exposed to SNAP or SNP in the presence of exogenous bilirubin (0.1–1 μ M). In another set of experiments, endothelial cells were incubated with SNAP and SNP in CM supplemented with $K_3Fe(CN)_6$ (10 μ M), a one-electron oxidant that converts nitroxyl anion (NO^-) into NO, or CM containing 100 μ M ferrozene, a specific chelator of Fe^{2+} [23].

Determination of bilirubin in culture medium

Cells were incubated with haemin or a combination of haemin and NO donors (SNP, SNAP or DETA/NO) for 2 h and bilirubin was determined in the culture supernatant at 0, 3, 6, 12 and 24 h [10,11,24]. Briefly, 0.5 ml of culture supernatant was added to 250 mg of barium chlorate monohydrate and vortex-mixed very well. Benzene (0.75 ml) was then added to the mixture and tubes were vigorously vortex-mixed again. The benzene phase containing the extracted bilirubin was separated from the aqueous phase by centrifugation at 13 000 *g* for 30 min. Bilirubin was measured spectrophotometrically as a difference

in absorbance between 450 and 600 nm ($\epsilon = 27.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) and was expressed as μ M/mg of protein.

Assay for endothelial haem oxygenase activity

Haem oxygenase activity was determined in endothelial cells 6 h after various treatments as described previously by our group [14]. Briefly, harvested cells were subjected to three cycles of freeze/thawing and sonicated before addition to a reaction mixture containing NADPH, rat liver cytosol as a source of biliverdin reductase, and the substrate haem. The reaction was conducted at 37 °C in the dark for 1 h, terminated by the addition of 1 ml of chloroform, and the extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm ($\epsilon = 40 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

Western-blot techniques for detection of HO-1 protein expression

Samples of endothelial cells were also analysed by the Western immunoblot technique as already reported [14]. Briefly, an equal amount of protein (30 μ g) for each sample was separated by SDS/PAGE, transferred overnight to nitrocellulose membranes, and the non-specific binding of antibodies was blocked with 3 % non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, Canada; 1:1000 dilution in Tris-buffered saline, pH 7.4). After three washes with PBS containing 0.05 % (v/v) Tween 20, blots were visualized using an amplified alkaline phosphatase kit from Sigma (Extra-3A). To verify equal loading, samples were also probed with β -actin monoclonal antibodies (Abcam, Cambridge, U.K.) and the densitometric ratio analyses are reported in the Results section.

RNA extraction and Northern-blot analysis

Total RNA was isolated by phenol/chloroform using the method described by Chomczynski and Sacchi [25]. Total RNA was run on a 1.3 % denaturing agarose gel containing 2.2 M formaldehyde and transferred on to a nylon membrane. The membrane was hybridized using [α - 32 P]dCTP-labelled cDNA probes to the rat *HO-1* gene as described previously [15], and staining of the 18 S rRNA band was used to confirm integrity and equal loading of RNA. The hybridized membrane was exposed to radiographic film to visualize bands.

Assay for determination of cellular haem content

Cells were incubated for 2 h with 50 μ M haemin or haemin in the presence of NO donors (500 μ M). After the initial 2 h treatment, the haemin-containing medium was replaced with fresh medium for the rest of the experiment. Haem content was determined at 0, 2, 4 and 6 h according to a method already published [5]. Briefly, at the indicated times, cells were washed twice with PBS and the endothelial layer was solubilized by adding 1 ml of concentrated formic acid (Sigma). The haem concentration of the formic acid solution was determined spectrophotometrically at 398 nm ($\epsilon = 1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Haem content was expressed as pmol/well.

Detection of NO released from NO donors

NO was measured amperometrically using an NO-sensitive electrode (ISO-NO Mark II). The calibration of the electrode was performed daily by measuring known amounts of NO in solution

according to the manufacturer's instructions. This consisted of generating NO by cumulative additions of KNO_2 to an acidic solution (0.1 M H_2SO_4) containing 0.1 M KI. Experiments were performed by using a Nitric Oxide Chamber (NOCHM; World Precision Instruments) maintained at 37 °C. The electrode was immersed in 1 ml of complete medium or medium from cells pre-treated with haemin. The NO-releasing agents, SNAP or SNP (500 μM final concentrations), were then added to the chamber and the NO concentration monitored continuously under constant stirring. The amount of NO released over time was expressed as the area under the curve calculated after 15 min of reading ($\mu\text{M} \times \text{s}$).

Statistical analysis

Differences in the data among the groups were analysed by using one-way ANOVA combined with the Bonferroni test. Values were expressed as means $\pm$ S.E.M. and differences between groups were considered to be significant at $P < 0.05$. The number of independent experiments conducted for each protocol is reported in the Figure legends.

RESULTS

Haem and NO donors act synergistically in the induction of HO-1

We first examined how haem oxygenase is affected by co-incubation of endothelial cells with haemin (50 μM) and SNAP (500 μM) for 2 h. Interestingly, this treatment resulted in a considerable increase in haem oxygenase activity at 6 h, which was significantly ($P < 0.05$) higher than that observed in cells exposed to haemin or SNAP alone (Figure 1A). Similarly, haem oxygenase activity was also enhanced by treatment of cells with a combination of haemin and SNP (Figure 1A). Incubation of haemin with DETA/NO, a slow releaser of NO, resulted in a slight increase in haem oxygenase activity compared with haemin alone (Figure 2A). These data indicate that the simultaneous presence of haem and NO donors, which alone already have a high capacity to up-regulate HO-1, can synergize and augment haem oxygenase activity to very high levels. It is worth noting that steady-state concentrations of 1–2 μM , 25–50 nM and 8–10 nM NO were achieved in the culture medium during the degradation of 500 μM SNAP (see Figure 7A below), SNP and DETA/NO (results not shown), respectively. These concentrations of NO are well within the range of physiological/pathophysiological production [26].

We wondered whether bilirubin production would occur in our experimental setting, since Juckett and colleagues have shown that NO inhibits haem degradation by haem oxygenase [21]. Measurements of bilirubin present in the culture medium at different times (0, 3, 6, 12 and 24 h) showed a time-dependent increase in the generation of the bile pigment in cells incubated with haemin. Notably, and in contrast with Juckett's results, the simultaneous presence of haem and NO donors significantly ($P < 0.01$) intensified bilirubin production, and this effect was noticeable as early as 3 h in the case of SNAP and became evident at 6 h with SNP and DETA/NO (Figures 1B and 2B). Despite the known increase in haem oxygenase activity and HO-1 expression, NO donors alone did not produce any change in bilirubin accumulation (see Table 1). Thus the synergistic effect obtained with haem and NO donors on up-regulation of HO-1 was reflected by a magnified production of bilirubin. To avoid a direct interaction of haemin with NO donors, addition of SNAP and SNP was also carried out 4 h after the initial exposure of

Figure 1 Synergistic effect of haemin and NO donors in stimulating endothelial haem oxygenase activity, bilirubin production and haem uptake

(A) Endothelial cells were exposed for 2 h to complete medium alone (CON) or medium supplemented with 50 μM haemin (H), 500 μM NO donors (SNP or SNAP) or haemin in the presence of SNP or SNAP (H+SNP and H+SNAP, respectively). At the end of the initial 2 h period, the medium was removed and replaced with complete fresh medium for further 4 h (total incubation time, 6 h). Each bar represents the mean $\pm$ S.E.M. from five or six different cell cultures. * $P < 0.05$ versus CON; † $P < 0.05$ versus SNP or SNAP. (B) Endothelial cells were subjected to the same treatments as in (A) and the production of bilirubin was determined in the culture medium at different time points. Each time point represents the mean $\pm$ S.E.M. from five different cell cultures. * $P < 0.05$ versus control; † $P < 0.05$ versus haemin. (C) Endothelial cells were exposed for 2 h to complete medium (control), medium supplemented with 50 μM haemin or haemin in the presence of 500 μM SNP or SNAP. Incubation in fresh complete medium was subsequently carried out for different times. Each time point represents the mean $\pm$ S.E.M. from five different cell cultures. * $P < 0.05$ versus control; † $P < 0.05$ versus haemin alone.

cells to haemin. Interestingly, this treatment still resulted in a potentiated bilirubin generation (Table 1), suggesting that NO could somehow accelerate haem oxygenase activity and therefore the rate of production of bilirubin.

NO donors increase the cellular uptake of haem

We wanted to determine the mechanisms by which bilirubin production was intensified to such an extent by the simultaneous

Figure 2 Effect of DETA/NO on haem oxygenase activity, bilirubin production and haem uptake

(A) Endothelial cells were exposed for 2 h to complete medium (CON), medium supplemented with 50 μ M haemin (H), 500 μ M DETA/NO or haemin in the presence of DETA/NO (H+DETA/NO). At the end of the initial 2 h period, the medium was removed and replaced with complete fresh medium for further 4 h (total incubation time, 6 h). Each bar represents the mean $\pm$ S.E.M. from five different cell cultures. * P < 0.05 versus CON. (B) Endothelial cells were subjected to the same treatments as in (A) and the production of bilirubin was determined in the culture medium at different time points. Each time point represents the mean $\pm$ S.E.M. from five different cell cultures. * P < 0.05 versus control; † P < 0.05 versus haemin. (C) Endothelial cells were subjected to the same treatments as in (A) and haem uptake was measured at different time points. Each time point represents the mean $\pm$ S.E.M. from six different cell cultures. * P < 0.05 versus control; † P < 0.05 versus haemin.

Figure 3 Bilirubin production following stimulation of endothelial cells with haemin

An initial exposure of cells for 2 h to increasing concentrations of haemin (10–50 μ M) was followed by 22 h incubation in complete fresh medium to allow accumulation of haem oxygenase-derived products. Cells were also incubated for 2 h with 50 μ M haemin and 100 μ M SnPPIX, followed by 22 h incubation with medium containing 100 μ M SnPPIX (haemin+SnPPIX). Control cells (CON) were incubated with complete medium alone. Each bar represents the mean $\pm$ S.E.M. from five different cell cultures. * P < 0.05 versus control. † P < 0.05 versus 50 μ M haemin.

incubation of cells with haemin and NO donors. What intrigued us most was the fact that cells had been exposed to equal amounts of haemin in both the absence and presence of NO donors, but generated considerably different levels of bilirubin. Hence we analysed the intracellular haem content at various time points after the treatments. As shown in Figures 1(C) and 2(C), incubation of cells with 50 μ M haemin produced a significant increase in haem uptake at 2 h; however, haem content started to decrease at 4 and 6 h, indicating that haem was being metabolized as a consequence of HO-1 induction. Notably, endothelial cells exposed to haemin and NO donors exhibited a strongly enhanced uptake of haem at 2 h (P < 0.01), followed by a substantial decrement at 4 and 6 h, again suggesting intervention of HO-1 in removal of endogenous haem. Interestingly, SNAP caused a more pronounced augmentation of intracellular haem content compared with SNP or DETA/NO (Figures 1C and 2C). Altogether these findings reveal that NO donors possess the unique ability of stimulating and increasing the uptake of haem, which results in potentiation of both HO-1 up-regulation and bilirubin generation.

Bilirubin production in endothelial cells incubated with haemin

Having discovered that a synergism between haem and NO donors profoundly affects haem uptake, haem oxygenase activity and bilirubin production, we also wanted to examine the effect of haem metabolites on NO-mediated induction of endothelial HO-1. We first determined the generation of bilirubin upon treatment of cells with haemin. Endothelial cells were exposed to haemin (10–50 μ M) or medium alone (control) for 2 h, followed by 22 h incubation with fresh medium to allow for accumulation of products of haem oxygenase activity. As illustrated in Figure 3, exposure of cells to haemin increased bilirubin production in a concentration-dependent manner. Similar experiments conducted by incubating haemin with SnPPIX show a complete inhibition of bilirubin generation (Figure 3). It needs to be emphasized that experiments were conducted in complete medium containing 10% serum and that serum contains proteins, such as haemopexin, that tightly bind free haem [4]. Therefore, the actual amount of haemin available to be taken up by cells was greatly reduced in our experiments by the presence of serum in the culture medium. These data confirm that endothelial haem oxygenase activity

Table 1 Bilirubin production in endothelial cells exposed to haemin and NO donors

Bovine aortic endothelial cells were initially exposed for 2 h to complete medium (CON) or medium supplemented with haemin (50 μ M), SNAP (500 μ M) or SNP (500 μ M), followed by incubation with medium alone for different times. In experiments indicated with an asterisk, SNAP or SNP were added to cells 4 h after the initial pre-treatment with haemin (50 μ M for 2 h) to avoid direct interactions of haem with NO donors. Bilirubin production (expressed as μ M/mg of protein) in the cell culture medium was measured at 6 and 24 h as described in the Experimental section. N.D., not detectable.

	CON	Haemin (24 h)	SNAP (6 h)	SNAP (24 h)	SNP (6 h)	SNP (24 h)	Haemin + SNAP* (24 h)	Haemin + SNP* (24 h)
Bilirubin production	0.58 $\pm$ 0.09	2.99 $\pm$ 0.3†	0.14 $\pm$ 0.09	0.11 $\pm$ 0.1	0.29 $\pm$ 0.09	N.D.	4.85 $\pm$ 0.7†‡	3.98 $\pm$ 0.5†‡

†*P* < 0.05 versus control (CON).
‡*P* < 0.05 versus haemin.

can be appropriately stimulated to consume haem and produce bilirubin, which is released and accumulated in the culture supernatant. Because haem oxygenase generates stoichiometric amounts of CO and iron during haem breakdown, our results also imply the formation of these two additional catalytic products.

Effect of CM on NO-donor-mediated HO-1 induction

We next examined the effect of the products generated during the degradation of haem on NO-mediated HO-1 induction. For this purpose, we exposed endothelial cells to medium originated from other cells previously stimulated to induce HO-1, where we had directly verified that accumulation of haem catalytic products indeed occurred (see above). This approach was chosen to reproduce more closely a physiological situation of haem catalysis. Endothelial cells exposed to 500 μ M of the NO donors SNAP, SNP or DETA/NO in CM (CM₅₀, medium from cells pre-treated with 50 μ M haemin) exhibited a strong amplification of haem oxygenase activity compared with cells incubated with SNAP, SNP or DETA/NO in fresh medium (Figures 4A and 4C). A potentiation of 95 % in cellular haem oxygenase activity was also obtained when cells were exposed to CM in the presence of SIN-1, another NO-releasing agent (results not shown). The amplification effect appeared to be strictly dependent on the amount of haem-degradation products present in the culture supernatant, since haem oxygenase activity was more pronounced when NO donors were added to CM from cells treated with 50 μ M haemin compared with 10 μ M (CM₁₀; Figure 4B). The fact that the amplifying effect could not be reproduced in cells exposed to DETA/NO in CM in the presence of SnPPIX, where no bilirubin production could be detected (see Figure 3), strongly supports the idea that haem metabolites are involved in this phenomenon (Figure 4C).

We also performed Western- and Northern-blot analyses to determine whether the amplifying phenomenon was a consequence of increased HO-1 protein expression as opposed to enhancement of haem oxygenase catalytic activity. Quantification of three independent Western blots expressed as the HO-1/ β -actin ratio showed that in the presence of CM₅₀, SNAP and SNAP + CM₅₀, HO-1 protein levels increased 1.3 $\pm$ 0.1-, 4.3 $\pm$ 0.01- and 7.2 $\pm$ 0.1-fold, respectively. Similarly, HO-1 protein expression increased 5.7 $\pm$ 0.01- and 7.9 $\pm$ 0.01-fold in cells treated with SNP and SNP + CM₅₀, respectively. Representative blots are reported in Figure 5(A). We also found that cells treated with NO donors in CM₅₀ displayed higher levels of HO-1 mRNA (Figure 5B).

Search for factors involved in the amplification phenomenon

We wanted to investigate further the amplifying effect by searching for the potential elements that contributed to the potentiation of HO-1 expression in the presence of CM₅₀. We

initially incubated cells with SNAP or SNP in the presence of bilirubin (0.1–1 μ M). As illustrated in Figure 6(A), bilirubin reduced the increase in haem oxygenase activity produced by SNAP. In contrast, the bile pigment enhanced haem oxygenase activity obtained with SNP (Figure 6B), supporting the concept that the different chemical properties of SNAP and SNP dictate diverse reactivities towards haem catabolites and, in this specific case, bilirubin. Bubbling of the culture medium for 30 s with 1 % CO before addition of NO donors did not produce any difference in haem oxygenase activity compared with incubation of cells with NO donors only (results not shown). These results were not unexpected, since treatment with CO gas for a short period of time certainly does not reproduce a situation of continuous CO production by haem oxygenase, considering that it is difficult to quantify the exact amount of CO generated over time. In addition, the possibility that CO will not accumulate in the culture medium at any time point could not be excluded. The use of CO traps, such as haemoglobin or myoglobin, was not considered feasible as these scavengers induce HO-1 *per se*.

During haem breakdown catalysed by haem oxygenase, iron is released from the tetrapyrrole ring in the form of Fe²⁺ [23], which is catalytically active in promoting oxidant injury. The cellular adaptation to increased endogenous iron concentration is to produce the iron-binding protein ferritin, which rapidly sequesters iron and prevents exacerbation of oxidative damage [27]. It appears that HO-1 promotes iron efflux [28], possibly through association with an iron ATPase transporter [29]. Notably, NO can react with iron to form dinitrosyl-iron complexes [30] and, in view of the possibility that some of the iron released from haem by haem oxygenase could be transported extracellularly and participate in the amplifying phenomenon, we incubated cells with haemin in the presence of ferrozene (100 μ M). This compound specifically chelates ferrous (Fe²⁺), as opposed to ferric (Fe³⁺), iron and has been shown to form a Fe²⁺-ferrozene complex in studies *in vitro* analysing the reaction steps of haem oxidation by purified HO-1 [23]. Interestingly, exposure of cells to SNAP in CM₅₀ containing ferrozene significantly decreased the amplification effect, indicating that iron liberated during haem degradation is partially responsible for the potentiation of HO-1 induction by CM in the presence of NO (Figure 7A). Endothelial cells were also exposed to SNAP or SNP in CM₅₀ supplemented with K₃Fe(CN)₆ (10 μ M), a one-electron oxidant that converts NO⁻ into NO. This treatment reduced approximately by 20 % the amplifying effect of CM₅₀ on SNAP- and SNP-induced HO-1 expression (Figure 7B), suggesting that NO⁻, possibly derived from a reaction between NO donors and haem metabolites, is one of the factors contributing to the CM-mediated effect.

Effect of CM on the release of NO from NO donors

To investigate further the amplifying phenomenon, we wondered whether CM₅₀ affected the reactivity of SNAP and SNP and

Figure 4 Haem-degradation products potentiate NO-mediated increase in haem oxygenase activity

(A) Endothelial cells were exposed for 6 h to NO donors (SNAP or SNP; 500 μ M) in complete medium or medium derived from cells treated with 50 μ M haemin (CM₅₀). At the end of the incubation, cells were collected for the haem oxygenase assay. Each bar represents the mean $\pm$ S.E.M. from five or six different cell cultures. * P < 0.05 versus control (CON); † P < 0.05 versus NO donors in fresh complete medium. (B) Haem oxygenase activity was also measured in cells exposed for 6 h to 500 μ M SNAP in complete medium, CM₁₀ or CM₅₀ (medium derived from cells treated with 10 or 50 μ M haemin, respectively). Each bar represents the mean $\pm$ S.E.M. from five or six different cell cultures. * P < 0.05 versus control (CON). (C) Endothelial cells were exposed for 6 h to 500 μ M DETA/NO in complete medium or in medium derived from cells treated with 50 μ M haemin (CM₅₀) or CM₅₀ plus 100 μ M SnPPiX (CM₅₀ + SnPPiX). At the end of the incubation cells were collected for the haem oxygenase assay. Each bar represents the mean $\pm$ S.E.M. from five different cell cultures. * P < 0.05 versus control (CON); † P < 0.05 versus DETA/NO in fresh complete medium.

measured NO released from NO donors in complete medium or CM₅₀. Notably, CM₅₀ markedly increased the amount of NO liberated from SNAP compared with medium alone (Figure 8). Although SNP releases much less NO compared with SNAP

Figure 5 Haem-degradation products potentiate NO-mediated increase in HO-1 protein and gene expression

(A) HO-1 protein expression was analysed by Western blotting in cells exposed for 6 h to NO donors (SNAP or SNP; 500 μ M) in complete medium or medium derived from cells treated with 50 μ M haemin (CM₅₀). The housekeeping protein β -actin was used to verify equal loading. (B) HO-1 gene expression was analysed by Northern blot at 3 or 6 h after exposure of cells to various treatments: lane 1, control; lane 2, CM₅₀; lane 3, 0.5 μ M bilirubin; lane 4, 500 μ M SNP (3 h); lane 5, 500 μ M SNP (6 h); lane 6, SNP in CM₅₀ (3 h); lane 7, SNP in CM₅₀ (6 h); lane 8, SNP in the presence of 0.5 μ M bilirubin (3 h); lane 9, SNP in the presence of 0.5 μ M bilirubin (6 h); lane 10, positive control. The 18 S band is shown to confirm integrity and equal loading of RNA.

under these conditions [14,31], CM₅₀ still promoted an increase in NO liberated from this iron-nitrosyl compound (results not shown). These results indicate that haem-degradation products stimulate an enhanced NO release from SNAP or SNP, which results in a potentiation of HO-1 induction by NO donors.

DISCUSSION

NO is a signalling molecule with a broad spectrum of activities and its capability to induce the stress protein HO-1 in the vasculature has been demonstrated extensively [18,19]. The significance of this adaptive response in mammalian cells is still obscure, although postulations on the potential role of HO-1 to control and confer resistance to the deleterious actions of NO have been advanced by our group and others [1,18]. Indeed, several pathogenic micro-organisms have been shown to express constitutive and inducible systems for NO metabolism and detoxification [32,33], providing ground for the existence of similar defence mechanisms in mammals. NO affects the haem oxygenase pathway at various levels, highlighting the complexity of the interaction between these two systems [18,19] (see the Introduction). We report here novel findings on the effect of NO donors on haem-mediated increase in haem oxygenase activity. Specifically, we observed that SNAP, SNP and DETA/NO act synergistically with haemin to augment endothelial haem oxygenase activity to very high levels. This is accompanied by a marked elevation in bilirubin production and, more importantly, by a strong magnification of the cellular haem uptake in the presence of NO-releasing agents. These data emphasize a

Figure 6 Effect of bilirubin on SNAP- and SNP-mediated increase in haem oxygenase activity

(A) Cells were incubated for 6 h in complete medium alone (CON), 500 μ M SNAP or 500 μ M SNP in the presence of different concentrations of bilirubin (BIL). (B) Cells were incubated for 6 h in complete medium alone (CON), 500 μ M SNP or 500 μ M SNP in the presence of different concentrations of bilirubin. At the end of the experiments, cells were collected for the haem oxygenase activity assay. Each bar represents the mean $\pm$ S.E.M. from five or six different cell cultures. * P < 0.05 versus control (CON); † P < 0.05 versus NO donors alone.

previously unknown role for NO in modulating the incorporation of haem into cellular compartments, consequently also affecting the expression of HO-1. The results also point to the importance of NO in affecting the availability of haem as a substrate for haem oxygenase enzymic activity; in line with this concept we have recently reported that haem is required for the cytoprotective action afforded by HO-1 against hypoxia/reoxygenation damage [24]. Considering that pathophysiological conditions such as atherosclerosis, haemorrhagic and endotoxic shock result in increased circulating haem and overproduction of NO [6–8,34,35], our findings reveal a potential interaction between haem and NO in mediating protection against vascular-related dysfunctions.

Figure 7 Ferrous iron (Fe²⁺) and nitroxyl anion (NO⁻) contribute to the amplifying phenomenon

(A) Cells were exposed for 6 h to 500 μ M SNAP in CM₅₀ or CM₅₀ supplemented with ferrozene (FeZ, 100 μ M), an iron chelator. (B) Cells were exposed for 6 h to 500 μ M SNAP or SNP in CM₅₀ or CM₅₀ supplemented with K₃Fe(CN)₆ (FeCN; 10 μ M). At the end of the incubation, cells were collected for the haem oxygenase assay. Each bar represents the mean $\pm$ S.E.M. from five or six different cell cultures. * P < 0.05 versus NO donors in CM₅₀.

The fact that SNAP releases more NO than SNP and DETA/NO [14,31] probably explains why the S-nitrosothiol produced a more significant rise in haem uptake compared with SNP, implying that the different chemical properties of NO donors influence their reactivity with haem and/or a potential haem receptor. It is interesting to note that conclusive evidence for the presence of a haem receptor in mammalian cells is still lacking, although a receptor for the haemopexin–haem complex appears to exist in pig liver cells [36] and mouse hepatoma cells [37] and some authors have reported that transport of haem across membranes requires a specific carrier in murine erythroleukaemia cells [38]. If such a receptor is present in bovine endothelial cells, our data suggest that NO could regulate its function leading to increased passage of haem from the extracellular to the intracellular environment.

Figure 8 Medium from haemin-treated cells increases the amount of NO released from SNAP

(A) An NO electrode was used to detect the amount of NO released from SNAP (500 μM) in complete medium (control) or medium derived from cells treated with haemin (CM₅₀). Representative traces of NO detection over time are shown. (B) Quantification of NO release from SNAP in control or CM₅₀. The amount of NO released over time is expressed as the area under the curve (AUC) calculated after 15 min of reading (μM × s). Bars represent the mean ± S.E.M. from four different experiments. **P* < 0.05 versus control (CON).

NO might also increase directly the permeability of membranes to haem. Alternatively, the formation of nitrosyl–haem adducts following nitrosylation of haem by NO [21,39] may significantly facilitate the crossing of membranes, despite the notion that haem, by virtue of its lipophilic characteristics, is already expected to intercalate into lipid membranes easily. In this respect, the data showing enhanced bilirubin generation when cells were incubated with haem and NO donors also indicate that a potential nitrosyl–haem complex might accelerate the degradation of haem by haem oxygenase; that is, nitrosyl–haem could be a more potent inducer of HO-1 and a better substrate for haem oxygenase enzymic activity compared with haem alone. In support of this idea are the findings that the production of bilirubin was still potentiated when SNAP or SNP were added to culture medium 4 h after exposure of cells to haem, to prevent direct interaction of haem with NO before entry of the tetrapyrrole ring into the cell. In this situation one might expect that if nitrosyl–haem adducts should form, they would do so endogenously, possibly in the proximity

of the haem oxygenase catalytic site. This could potentially occur, since it has been reported that NO binds to the ferrous haem–haem oxygenase complex [39]. In contrast with the findings presented here, Juckett and colleagues [21] reported that NO inhibits haem oxygenase activity and, therefore, haem degradation to biliverdin and bilirubin; they also observed that NO donors prevent the induction of HO-1 by haemin. We cannot offer at present any plausible explanation about these contradictory results, apart from the fact that different NO-releasing agents were used in the two studies. However, it is interesting to consider the effect of NO on the purified proteins HO-2 and HO-1. In these experiments, Ding and co-workers [22] have shown that HO-2 catalytic activity is significantly inhibited by NO donors, in a mechanism involving interaction of NO with the haem regulatory motifs present on the protein. Conversely, HO-1 enzymic activity is virtually refractory to treatment with NO donors, as assessed by measuring bilirubin formation in an *in vitro* assay. We believe that the different susceptibility of HO-2 and HO-1 to NO donors should be taken into consideration when interpreting both Juckett's and our results.

Having analysed the effects of NO on the induction of haem oxygenase by haemin, we were also interested in investigating what influence haem-degradation products could exert on the induction of HO-1 by NO donors. We have discovered recently that bilirubin and biliverdin can interact with NO *in vitro* [20], suggesting that the two bile pigments might possess anti-nitrosative features besides their established antioxidant properties [10,11]. Based on these findings, we were expecting that cells incubated in medium containing products of haem degradation (CM), where the presence of bilirubin had been verified, would exhibit a reduction of HO-1 up-regulation by NO donors. On the contrary, the treatment resulted in an amplification of HO-1 expression and increased haem oxygenase activity. The phenomenon was highly reproducible and, once again, more evident for SNAP than for SNP. Furthermore, it appeared to be strictly dependent on the amount of haem metabolites present in CM, since the amplification was more prominent when SNAP was added to CM from cells treated with 50 μM (CM₅₀) compared with 10 μM haemin (CM₁₀). CM containing SnPPIX virtually abolished the amplification, supporting the important involvement of haem metabolites in this effect. We reasoned that CM did not contain only bilirubin, but eventually a combination of haem-degradation products; these metabolites could all affect the cellular inducibility of HO-1 by NO donors and contribute to some extent to the amplifying effect. For instance, opposite results in relation to SNAP or SNP were obtained when bilirubin was examined individually; in fact, the bile pigment decreased haem oxygenase activity stimulated by SNAP but potentiated the action of SNP. These data can be explained by considering the outcome of our experiments *in vitro* on the interaction of NO donors with bilirubin [20]: in this context we observed that SNAP reacts with bilirubin and causes its partial degradation, whereas SNP was not reactive and did not promote any degree of disappearance of the bile pigment even at very high concentrations (5 mM). Thus bilirubin could partly prevent the chemical reactivity of SNAP, in terms of NO release, but could not exert any evident scavenging activity with respect to SNP. Interestingly, we also found that iron liberated during the opening of the haem moiety participated in potentiating haem oxygenase activity since ferrozene, a chelating compound specific for Fe²⁺, reduced by approx. 20% the amplifying effect obtained with SNAP and CM₅₀. In addition, a similar decrease in the amplifying phenomenon was observed with K₃Fe(CN)₆, a one-electron oxidant that converts NO⁻ to NO, suggesting that NO⁻ contributed to magnify haem oxygenase activity of cells incubated

in CM₅₀ in the presence of NO donors. These data are in line with recent evidence from our laboratory showing that NO⁻ induces HO-1 expression in cardiomyocytes [40]. As already reported in the Results section, a role for CO was more difficult to ascertain in our experimental setting. Finally, by directly assessing the amount of NO liberated from NO donors, we were able to establish that a determining factor in the occurrence of amplifying phenomenon was the enhanced release of NO from SNAP and SNP in CM₅₀, indicating that haem-degradation products could substantially modulate the chemical stability of NO donors and accelerate their decomposition rate.

In conclusion, we have identified novel mechanisms related to the induction of the stress protein HO-1 by NO in the endothelium, which comprise the following. (i) A synergism between haem and NO that strongly stimulates HO-1 by considerably enhancing the intracellular haem uptake; the most relevant subsequent effect is the magnified production of haem metabolites with important physiological functions. (ii) An amplification of NO-mediated HO-1 up-regulation by haem-degradation products, which appear to modulate the action of NO donors by accelerating and increasing the release of NO. The present results shed new light on the interaction of the haem/haem oxygenase/NO pathways and emphasize a potential co-operation between NO and HO-1 to augment the generation of antioxidant (bilirubin and biliverdin), signalling (iron and CO) and anti-inflammatory (CO) molecules during pathophysiological situations affecting vascular functions. Our data also indicate that the reactivity and signalling activities of NO can vary significantly in the presence of haem and haem metabolites.

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