Dynamics of haem oxygenase-1 expression and bilirubin production in cellular protection against oxidative stress

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The inducible isoform of haem oxygenase (HO-1) has been proposed as an effective system to counteract oxidant-induced cell injury. In several circumstances, this cytoprotective effect has been attributed to increased generation of the antioxidant bilirubin during haem degradation by HO-1. However, a direct implication for HO-1-derived bilirubin in protection against oxidative stress remains to be established. In the present study, we examined the dynamics of HO-1 expression and bilirubin production after stimulation of vascular smooth-muscle cells with hemin, a potent inducer of the HO-1 gene. We found that hemin-mediated increase in HO-1 protein expression and haem oxygenase activity is associated with augmented bilirubin levels. The majority of bilirubin production occurred early after exposure of cells to hemin. Hemin pre-treatment also resulted in high resistance to cell injury caused by an oxidant-generating system. Interestingly, this protective effect was manifest only when cells were actively producing bilirubin as a consequence of increased haem availability and utilization by HO-1. Tin protoporphyrin IX, an inhibitor of haem oxygenase activity, significantly reduced bilirubin generation and reversed cellular protection afforded by hemin treatment. Furthermore, addition of bilirubin to the culture medium markedly reduced the cytotoxicity produced by oxidants. Our findings provide direct evidence that bilirubin generated after up-regulation of the HO-1 pathway is cytoprotective against oxidative stress.

Key words: carbon monoxide, glucose oxidase, hydrogen peroxide , smooth-muscle cells, tin protoporphyrin IX.

INTRODUCTION

Oxidative stress is the result of excessive production of oxidant species and/or depletion of intracellular antioxidant defences, leading to an imbalance in the redox status of the cell. Because of its anatomical location, the vascular tissue is particularly exposed to oxidative stress-mediated injury and vascular wall dysfunction is associated with the pathology of a number of common cardiovascular diseases. Similarly to other cellular systems, endothelium and smooth muscle possess the intrinsic ability to induce a series of protective enzymes once the production of oxidants overcomes the primary endogenous defences [1].

In recent years, the inducible isoform of haem oxygenase (HO-1) has been suggested to function as an effective system to counteract the oxidative threat [2]. Haem oxygenase is the rate-limiting enzyme in haem degradation to CO, iron and biliverdin, the latter being converted into bilirubin by biliverdin reductase [3]. The proposed antioxidant role for HO-1 is based on some crucial experimental observations: (i) HO-1 gene expression is extremely sensitive to up-regulation by oxidative stress in a variety of mammalian tissues [2,4–6]; (ii) induction of HO-1 protein or transfection of cells with the HO-1 gene protect tissues against oxidant-mediated injury [7–10]; and (iii) HO-1 knock-out mice exhibit reduced stress defences when exposed to oxidative challenge [11]. Since biliverdin and bilirubin have been shown to possess potent antioxidant properties [12] and up-regulation of HO-1 is usually accompanied by increased levels of ferritin, a protein which sequesters intracellular catalytic iron [13], HO-1 appears to be an excellent candidate for cytoprotection. Stocker and co-workers [12] originally showed that micromolar concentrations of bilirubin efficiently scavenge peroxyl radicals *in vitro* and that, at low oxygen tension, the antioxidant activity of bilirubin surpasses that of α-tocopherol, the best-known antioxidant against lipid peroxidation [12]. In previous studies we have reported that exogenously applied bilirubin attenuates hydrogen peroxide-induced damage in vascular endothelial cells [14]. More recently, we have implicated HO-1-derived bilirubin in reduction of endothelial apoptosis mediated by peroxynitrite [15] and amelioration of post-ischaemic myocardial dysfunction in the isolated perfused rat heart [16]. Doré and co-workers [17] have also demonstrated that enhanced bilirubin production following activation of haem oxygenase-2 (HO-2), the constitutive isoform of haem oxygenase, is protective against the neurotoxicity elicited by hydrogen peroxide [17]. However, further studies on the physiological importance of bilirubin generated by HO-1 are required to fully account for the cytoprotection conferred by HO-1 induction.

Here we examined the dynamics of HO-1 expression and bilirubin production after stimulation of vascular smooth-muscle cells with hemin, a potent inducer of the HO-1 gene. The response to an oxidant-generating system was then evaluated in control cells and cells producing increased amounts of bilirubin.

EXPERIMENTAL PROCEDURES

Materials

Bovine vascular smooth-muscle cells were obtained from the Coriell Cell Repository (Camden, NJ, U.S.A.) and grown in Dulbecco's minimal essential medium (MEM) supplemented with 20 % fetal calf serum, 2 × MEM vitamins, 2 × MEM non-essential and essential amino acids, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Hemin and tin protoporphyrin IX (SnPPIX) were from Porphyrin Products (Logan, UT, U.S.A.). All other chemicals were obtained from Sigma (Poole, Dorset, U.K.).

Abbreviations used: HO-1, haem oxygenase-1; SnPPIX, tin protoporphyrin IX; GOX, glucose oxidase.

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Western-blot analysis for HO-1 expression and haem oxygenase activity

Confluent cells were exposed to increasing concentrations of hemin (50–400 μM) for 2 h followed by incubation with medium alone for various times. At the end of the incubation, cells were collected and analysed for HO-1 protein expression and haem oxygenase activity. Western-blot technique was performed using specific antibodies against rat HO-1 [18,19] and haem oxygenase activity was determined spectrophotometrically as described previously [18].

Determination of bilirubin in cell-culture medium

Haem oxygenase-derived bilirubin was determined as described recently by Turcanu and co-workers [20]. Cells were pre-treated with hemin (100 and 200 μM) for 2 h and exposed to medium alone or medium containing an inhibitor of haem oxygenase activity (SnPPIX, 40 μM) for an additional 4 or 22 h. Each culture supernatant (500 μl) was then collected in a tube and 250 mg of barium chloride was added. After vortexing, 0.75 ml of benzene was added and the tubes mixed again. The benzene phase containing the extracted bilirubin was separated from the aqueous phase by centrifugation for 30 min at 13000 ***g***. Bilirubin was determined spectrophotometrically as a difference in absorbance between 450 and 600 nm ($\epsilon_{450} = 27.3\ \mathrm{mM^{-1} \cdot cm^{-1}}$).

Cytotoxicity assay

Cells were pre-treated with hemin (25–400 μM) for 2 h followed by 4 or 22 h incubation with medium alone or medium containing SnPPIX (40 μM). In another set of experiments, different concentrations of exogenous bilirubin were applied to cells for 2 h. At the end of the incubation, cells were washed and exposed for 2 h to fresh culture medium containing various concentrations (10–120 m-units/ml) of glucose oxidase (GOX), an enzyme that catalyses the conversion of D-glucose to D-gluconolactone, producing hydrogen peroxide at constant rate. This oxidant-generating system has been used previously in cell culture *in vitro* to promote oxidative injury [21]. Cell viability was assessed using a colorimetric assay kit from Promega (Madison, WI, U.S.A.) according to manufacturers' instructions.

Statistical analysis

Results were expressed as means ± S.E.M. of six independent experiments per group. Statistical analysis was performed using ANOVA combined with Student's two-tailed *t* test. Differences among the groups were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Vascular smooth-muscle cells exposed to hemin for 2 h exhibited considerable changes in HO-1 expression and haem oxygenase activity in a time- and concentration-dependent manner. Maximal protein levels and activity were detected 4 h after treatment with hemin at 100 μM (Figure 1). Although HO-1 protein expression was more prominent at 200 μM compared with 100 μM hemin, haem oxygenase activity peaked at 100 μM and started to decrease at higher concentrations of hemin. This effect is presumably a consequence of the cytotoxic nature of the hemin moiety. In fact, by virtue of its iron content, hemin can act as a promoter of reactive-oxygen-species formation and thus mediate cell injury [8,22,23]. This concept is also sustained by our findings showing that at 200 μM hemin-stimulated cells started to exhibit increased levels of inducible heat-shock protein 70 (hsp70; Figure 1C), a chaperone involved in refolding of damaged and denatured proteins. It is interesting to note that HO-1 expression and haem oxygenase activity were considerably diminished 22 h after exposure to hemin at all concentrations used (Figure 1A), indicating a gradual return of cells to basal conditions after the initial response to hemin.

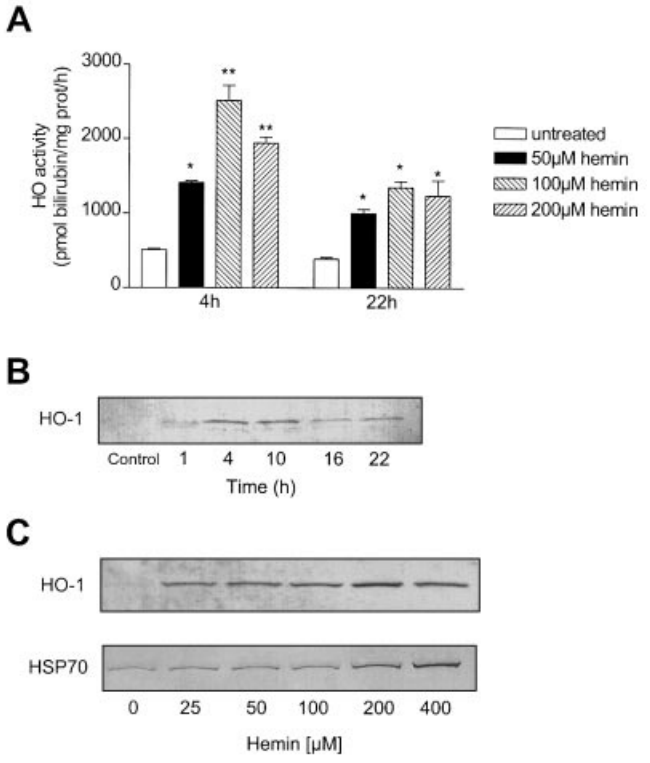

Figure 1 Effect of hemin on haem oxygenase activity, and HO-1 and hsp70 protein expression in vascular smooth-muscle cells

(**A**) Cells were exposed to hemin (0–200 μM) for 2 h and haem oxygenase activity was measured at 4 and 22 h after hemin treatment as reported in the Experimental procedures section. Each bar represents the mean ± S.E.M. of six experiments performed independently. $^{*}P < 0.05$ versus untreated; $^{**}P < 0.01$ versus untreated. (**B**) HO-1 protein expression in smooth-muscle cells was detected by Western blotting at different time points after treatment with 100 μM hemin. (**C**) HO-1 and hsp70 protein expression were determined 4 h after exposure of cells to various concentrations of hemin (0–400 μM).

Increased HO-1 expression following treatment of cells with hemin was accompanied by augmented bilirubin production, as shown in Table 1. However, and in accordance with the haem oxygenase activity results, we observed that the majority of the bile pigment (86 and 68 %) was generated within the first 4 h after exposure to 100 and 200 μM hemin, respectively. Notably, after this initial period of incubation, cells treated with 100 μM hemin did not exhibit a significant further elevation in bilirubin levels. This suggests that most of the hemin taken up by cells is consumed by haem oxygenase in a relatively short period of time and that the tetrapyrrole ring subsequently becomes the limiting factor in the generation of bilirubin despite HO-1 protein expression remaining elevated. This hypothesis is substantiated by the fact that smooth-muscle cells exposed to higher concentrations of hemin (200 μM) continued to release bilirubin into the medium during both the earlier and later times of incubation.

Having established that HO-1 up-regulation is accompanied by increased bilirubin production, we wanted to verify whether this inducible pathway could provide protection against oxidative

Table 1 Production of bilirubin in control and hemin-treated cells

Cells were exposed for 2 h to medium alone (control) or medium supplemented with hemin (100 or 200 μM). Bilirubin released into the culture medium was measured 4 or 22 h after hemin treatment, as described in the Experimental procedures section. Bilirubin was also measured in the medium of cells treated with 100 μM hemin for 2 h followed by incubation with 40 μM SnPPIX for 4 h. Results represent the means $\pm$ S.E.M. of six experiments for each group. $^*P < 0.05$ versus control; †$P < 0.05$ versus 4 h.

	Bilirubin (nM/well)	
	4 h	22 h
Control	375 $\pm$ 38	311 $\pm$ 33
100 μM Hemin	540 $\pm$ 33*	622 $\pm$ 111*
100 μM Hemin + 40 μM SnPPIX	302 $\pm$ 35	–
200 μM Hemin	769 $\pm$ 54*	1117 $\pm$ 174*†

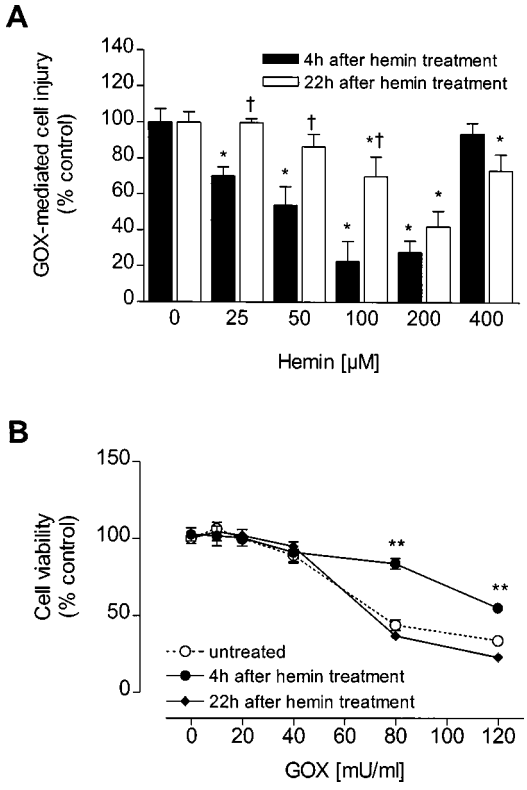

Figure 2 Effect of hemin pre-treatment on GOX-mediated cell injury

(**A**) Cells were pre-treated for 2 h with increasing concentrations of hemin (0–400 μM) and were then incubated with fresh medium for 4 or 22 h prior to exposure to 80 m-units/ml GOX for 2 h. Cell injury was assessed as reported in the Experimental procedures section. (**B**) Cells were pre-treated for 2 h with 100 μM hemin, incubated with fresh medium for 4 or 22 h and then exposed to increasing concentrations of GOX (20–120 m-units/ml) for 2 h. Control experiments (untreated) were performed by exposing cells to medium alone prior to GOX challenge. Each bar represents the mean $\pm$ S.E.M. of six experiments performed independently. $^*P < 0.05$ versus 0 μM hemin; †$P < 0.05$ versus 4 h; $^{**}P < 0.01$ versus untreated.

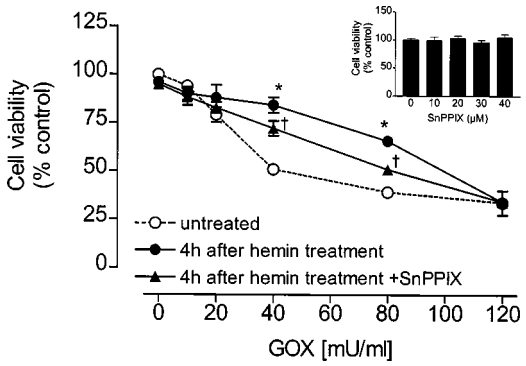

Figure 3 SnPPIX attenuates hemin-mediated cytoprotection against oxidative stress

Cells were pre-treated with 100 μM hemin for 2 h and then incubated for 4 h in medium alone or medium containing 40 μM SnPPIX. After incubation, cells were exposed to increasing concentrations of GOX for 2 h and cell viability was assessed as described in the Experimental procedures section. Control experiments (untreated) were performed by exposing cells to medium alone prior to GOX challenge. Inset: effect of various concentrations of SnPPIX on cell viability. Cells were incubated for 4 h with 0–40 μM SnPPIX and cell viability was assessed. Results represent the means $\pm$ S.E.M. of six experiments for each group. $^*P < 0.05$ versus untreated; †$P < 0.05$ versus hemin treatment.

stress. Several previous reports have revealed a direct correlation between HO-1 induction and high resistance to oxidant-mediated damage [5,9,24,25]. This cytoprotective effect has always been attributed to augmented levels of the antioxidants biliverdin and bilirubin although actual measurements of any of these HO-1 products were lacking. We believe this is an important point to address because (i) up-regulation of the HO-1 gene does not necessarily imply up-regulation of the products and (ii) specific investigations on the availability of haem as a substrate for increased HO-1 expression have yet to be performed. In the present study, cells exposed to an oxidant-generating system (GOX, 80 m-units/ml) for 2 h showed a considerable decrease in viability (Figure 2). In contrast, pre-treatment with hemin resulted in a concentration-dependent reduction in GOX-mediated cell injury. Highest protection was seen 4 h after treatment with 100 μM hemin, the concentration that resulted in maximal HO activity. A similar decrease in cell injury was also observed with 200 μM hemin. Significantly less protection was found 22 h following exposure to hemin ($P < 0.05$ versus 4 h); however, pre-treatment with 200 μM hemin still resulted in considerable reduction of cell damage caused by oxidative stress. These data are best explained when considering the experimental protocol employed. Following exposure of smooth-muscle cells to hemin (4 or 22 h), the medium was replaced with fresh medium containing GOX for 2 h prior to assessment of cell viability. Therefore, the medium in which bilirubin accumulated was removed before the addition of GOX. In view of this finding and the bilirubin data presented in Table 1, cytoprotection afforded 4 h after treatment with 100 μM hemin was probably a result of endogenous bilirubin actively generated by HO-1 at the time of oxidant challenge. Accordingly, production of less bilirubin can account for the decreased ability of hemin (100 μM) pre-treatment to protect cells at 22 h. Also at 22 h, the effect of 200 μM hemin could reflect sustained bilirubin generation over a longer period of time with a possible contribution of other inducible defensive systems such as hsp70.

Injury following exposure to increasing concentrations of GOX (10–120 m-units/ml) was also significantly diminished in cells pre-treated with 100 μM hemin; once again this protection was evident at 4 but not at 22 h (Figure 2B) and is consistent with the dynamics of HO-1 expression, haem oxygenase activity and bilirubin production in relation to haem availability. A direct contribution of the haem oxygenase/bilirubin pathway in minimizing oxidative damage was ascertained in our study by using

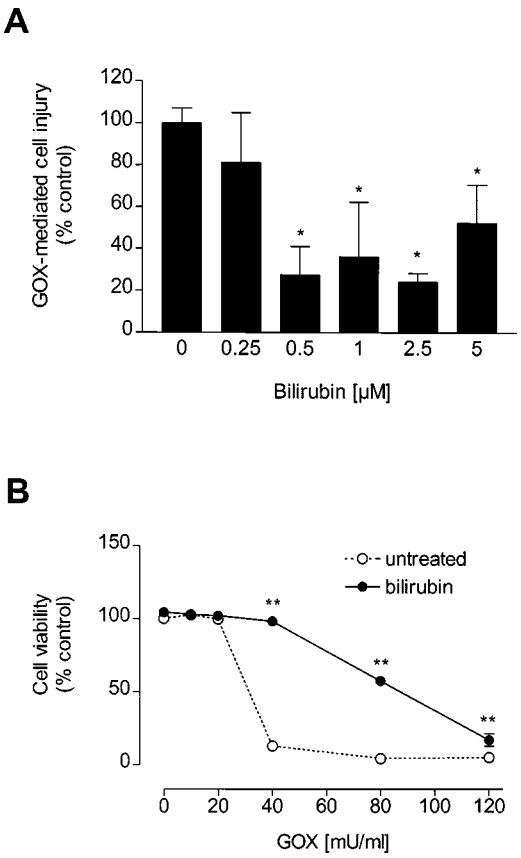

Figure 4 Effect of exogenous bilirubin on GOX-mediated cell injury in smooth-muscle cells

(**A**) Cells were incubated for 2 h with increasing concentrations of bilirubin (0–5 μM) and cell injury was assessed 2 h after exposure to 80 m-units/ml GOX. (**B**) Cells were incubated for 2 h with medium alone (untreated) or medium containing bilirubin (1 μM) and cell viability was assessed 2 h after exposure to increasing concentrations of GOX (0–120 m-units/ml). $^{*}P < 0.05$ versus 0 μM bilirubin; $^{**}P < 0.05$ versus untreated.

an inhibitor of its activity, SnPPIX. As shown in Table 1 and Figure 3, SnPPIX (40 μM) reduced bilirubin formation (302 ± 35 nM/well) to levels comparable with the control (375 ± 38 nM/well) and attenuated the cytoprotection conferred by hemin treatment against GOX-induced cell injury. It is important to note that SnPPIX itself did not cause any evident cytotoxic effect at the concentrations tested (Figure 3, inset). The participation of additional defence mechanisms cannot be excluded since SnPPIX did not completely reverse the protective effect mediated by hemin. To confirm the important role of bilirubin in protection against oxidative stress, cells were incubated with various concentrations of the bile pigment prior to GOX challenge. A marked decrease in cell damage was observed with bilirubin at 0.5 μM and above (Figure 4A). Similarly, 1 μM bilirubin significantly reduced the toxic actions of increasing concentrations of GOX (Figure 4B). Although our data are in agreement with recent published observations by Doré and co-workers in cultured neurons [17], the concentrations of bilirubin that provided cytoprotection in the present work were higher. However, differences in cell types and experimental conditions used can explain differences in results. For instance, we found that perfusion of isolated rat hearts with serum-free buffer containing bilirubin at concentrations as low as 100 nM reduces infarct size and improves myocardial function following ischaemia–reperfusion [16].

Thus, this study demonstrates that increased bilirubin as a consequence of HO-1 induction contributes to cytoprotection of smooth-muscle cells against oxidant-mediated damage. In addition, our results show how the dynamics of haem oxygenase activity and bilirubin production determine the adaptive response of cells to oxidative stress. The important issues concerning the availability of haem as a substrate for augmented HO-1 levels and the generation of bilirubin in sufficient amounts to provide resistance to stressful stimuli were also raised. The present findings strongly support a role for bilirubin derived from HO-1 in cytoprotection; nevertheless, the participation of the other products of haem degradation by HO-1 is attainable since CO, biliverdin and iron have all been shown to modulate biological processes [12,13,26–28]. Accordingly, Otterbein and co-workers have reported recently that exposure of rats to low concentrations of CO increases tolerance to hyperoxic lung injury [29]. The fact that bilirubin can counteract oxidative injury caused by hydrogen peroxide [14,17], peroxynitrite [15,30] and peroxyl radicals [12] indicates a wide spectrum of antioxidant activities for this bile pigment. It is tempting to suggest that these properties of bilirubin can be extended to nitric oxide and other reactive nitrogen species [31,32].

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