Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: a link to apoptosis

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Peroxynitrite ($ONOO^-$) is a potent oxidizing agent generated by the interaction of nitric oxide (NO) and the superoxide anion. In physiological solution, $ONOO^-$ rapidly decomposes to a hydroxyl radical, one of the most reactive free radicals, and nitrogen dioxide, another species able to cause oxidative damage. In the present study we investigated the effect of $ONOO^-$ on the expression of haem oxygenase-1 (HO-1), an inducible protein that is highly up-regulated by oxidative stress. Exposure of bovine aortic endothelial cells to $ONOO^-$ (250–1000 μM) produced a concentration-dependent increase in haem oxygenase activity and HO-1 protein expression. This effect was completely abolished by the $ONOO^-$ scavengers uric acid and *N*-acetylcysteine, and partly attenuated by 1,3-dimethyl-2-thiourea, a scavenger of hydroxyl radicals. $ONOO^-$ also produced a concentration-dependent increase in apoptosis and cytotoxicity, which were considerably decreased by uric acid and *N*-acetylcysteine. A 70% decrease in apoptosis was observed when cells were exposed to $ONOO^-$ in the presence of 10 μM tin protoporphyrin IX (SnPPIX), an inhibitor of haem oxygenase activity. When SnPPIX was added 5 min after $ONOO^-$, apoptosis decreased by only 40%, which suggests that an interaction between $ONOO^-$ and the protoporphyrin occurs in our system. Increased haem oxygenase activity by pretreatment of cells with haemin resulted in elevated bilirubin production and was associated with a substantial decrease (35%) in $ONOO^-$-mediated apoptosis. These results indicate the ability of $ONOO^-$ to modulate the expression of the stress protein HO-1 and suggest that the haem oxygenase pathway contributes to protection against the cytotoxic action of $ONOO^-$.

Key words: bilirubin, HSP32, stress response, tin protoporphyrin IX.

INTRODUCTION

Haem oxygenase is a widely distributed enzyme in mammalian tissues; its main function is associated with the degradation of haem to iron, CO and biliverdin [1]. The subsequent step in haem breakdown is accomplished by biliverdin reductase, which converts biliverdin into bilirubin. Two distinct isoforms of haem oxygenase have been extensively studied: HO-2 is the constitutive isoenzyme and HO-1 (heat shock protein 32, HSP 32), the inducible form, is a stress protein [2]. The expression of HO-1 is elicited by many conditions and agents that produce an imbalance in cellular homoeostasis. A variety of factors, including heavy metal ions [1,3], various haemoglobins [4–6], endotoxins [7] and oxidative stress [8–10], are capable of up-regulating the gene for HO-1 in different tissues. The induction of HO-1 by stress situations might have physiological importance because the end products of haem catabolism, biliverdin and bilirubin, possess antioxidant properties [11] and HO-1-derived CO has been implicated in vasoregulation [12,13] and signal transduction [14]. Recent experimental evidence showed that HO-1-deficient cells and mice are susceptible to the accumulation of free radicals and to oxidative injury after endotoxin administration and established that HO-1 is an important enzymic antioxidant system [15].

Among oxidant molecules, peroxynitrite ($ONOO^-$) is very powerful. It derives from the equimolar reaction of NO and superoxide ($O_2^{-\bullet}$) and decomposes at physiological pH to generate a strong oxidant with a reactivity similar to the hydroxyl radical, and nitrogen dioxide [16,17]. $ONOO^-$ has recently been considered to be a mediator of cellular injury in many systems and its production has been implicated in the pathophysiology of diseases such as acute endotoxaemia, neurological disorders, atherosclerosis and ischaemia/reperfusion [18,19]. Furthermore, in studies *in vitro*, $ONOO^-$ has been shown to be highly bactericidal toward *Escherichia coli*, to cause thiol oxidation and tyrosine nitration, as well as inducing apoptosis in tumour cell lines and human endothelial cells [19–21].

Apoptosis is a highly regulated process of cell death and seems to be an essential and critical mechanism used by biological systems to maintain homoeostasis. Severe oxidative stress is thought to be a major mediator of apoptosis in several cellular systems. For example, H_2O_2 can induce apoptosis in murine peritoneal macrophages when applied extracellularly [22]. Increased $O_2^{-\bullet}$ production due to the down-regulation of Cu/Zn superoxide dismutase has also been shown to induce apoptosis [23]. Accordingly, apoptosis can be prevented by compounds with antioxidant properties [24].

We have shown that NO donors induce HO-1 expression in vascular endothelial cells and that thiol compounds diminish the increase in haem oxygenase activity and HO-1 protein expression mediated by NO [25,26]. Moreover, we suggested that both $O_2^{-\bullet}$ and $ONOO^-$ partly contribute to the increased activity of this protein [26]. To extend the findings of our previous work, we sought in the present study to investigate whether $ONOO^-$ affects HO-1 expression and causes apoptosis in bovine aortic endothelial cells. In addition, we attempted to define the specific role of HO-1 induction in $ONOO^-$-mediated apoptosis.

MATERIALS AND METHODS

Materials

Bovine aortic endothelial cells were obtained from the European Collection of Animal Cell Culture (Salisbury, Wilts., U.K.). $ONOO^-$ was purchased from Alexis Corp. (Bingham, Notting-

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

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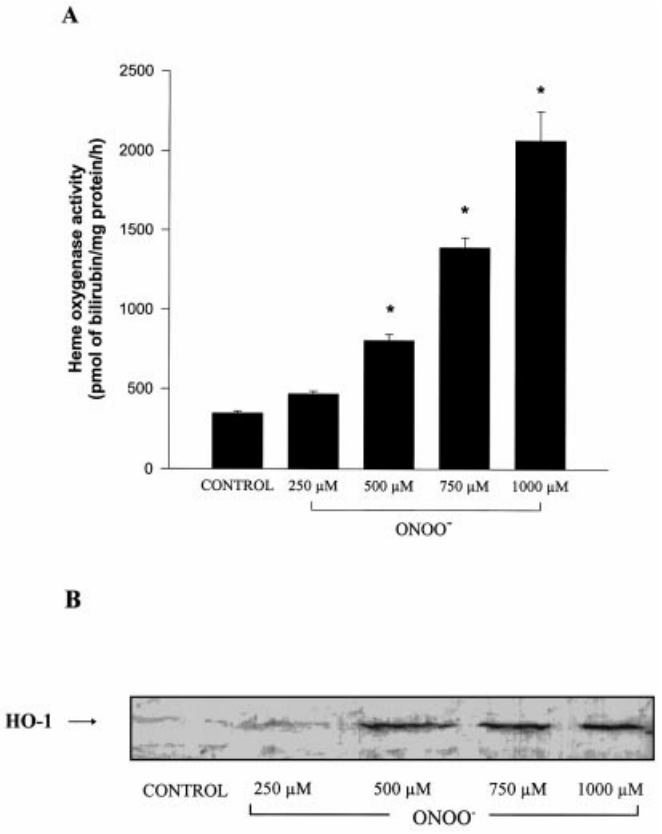

Figure 1 Effect of $ONOO^-$ on haem oxygenase activity and HO-1 expression in endothelial cells

(**A**) Haem oxygenase activity was measured 6 h after exposure of endothelial cells to various concentrations of $ONOO^-$ (250–1000 μM) added as a bolus to the culture medium. Control experiments were performed by exposing cells to complete medium alone. Each bar represents the mean $\pm$ S.E.M. for five or six independent experiments. $^*P < 0.05$ compared with control. (**B**) HO-1 protein expression was analysed by the Western immunoblot technique (as described in the Materials and methods section) in samples of cells treated as above. The antibody complex was detected with an ExtrAvidin alkaline phosphatase kit. Results are representative of three independent experiments.

ham, Notts., U.K.). $ONOO^-$ was supplied in a 0.3 M NaOH solution; on arrival it was immediately stored at -80 °C. The $ONOO^-$ concentration was monitored each time before the experiment by measuring the absorbance at 302 nm (molar absorption coefficient 1670 $M^{-1} \cdot cm^{-1}$) after the addition of 25 μl of $ONOO^-$ stock solution to 975 μl of ice-cold 0.3 M NaOH. $ONOO^-$ was found to be stable for at least 1 month. Decomposed $ONOO^-$ was prepared by reacting fresh $ONOO^-$ with an equal volume of 0.3 M HCl; 1 h after the reaction, the absorbance was read at 302 nm and it was verified that the $ONOO^-$ concentration had decreased to less than 5% of that in the original stock solution. All chemicals used for the fixation and preparation of cells for electron microscopy studies were purchased from Agar Scientific (Stansted, Essex, U.K.). Tin protoporphyrin IX (SnPPIX) and haemin were from Porphyrin Products (Logan, UT, U.S.A.). *N*-Acetylcysteine, uric acid, 1,3-dimethyl-2-thiourea and all other chemicals were obtained from Sigma.

Culture of endothelial cells

Bovine aortic endothelial cells were grown in 75 cm^2 flasks with Iscove's modified Dulbecco's medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 i.u./ml penicillin and 0.1 mg/ml streptomycin. For the haem oxygenase activity assay and Western blot analysis for HO-1, cells were cultured in 75 cm^2 flasks to reach confluence. For electron microscopy studies, cells were grown in 25 cm^2 flasks or in tissue-culture Petri dishes (60 mm in diameter); for the cytotoxicity assay, cells were subcultured in 24-well tissue culture plates.

Experimental protocol

Confluent endothelial cells were washed with phosphate buffer and fresh medium was added to the cultures. A single bolus of $ONOO^-$ (250, 500, 750 or 1000 μM final concentration) was rapidly added against the edge of the culture dish (or flask) and mixed for few seconds to distribute $ONOO^-$ across the dish. In some experiments, 1 mM uric acid or *N*-acetylcysteine, two agents that directly scavenge $ONOO^-$, were added to the medium before the addition of $ONOO^-$ (750 μM). Because $ONOO^-$ decomposes at physiological pH to generate nitrogen dioxide and the harmful hydroxyl radical, in another group of experiments $ONOO^-$ (750 μM) was added to culture medium already supplemented with 1 mM 1,3-dimethyl-2-thiourea, a compound that scavenges hydroxyl radicals. Endothelial cells were also incubated for 2, 5 and 10 min in phosphate buffer to which 750 μM $ONOO^-$ was added as a bolus; at the end of $ONOO^-$ exposure, the buffer was removed and replaced with fresh complete medium for 6 h. Additional experiments were performed by exposing cells to $ONOO^-$-treated medium, which was obtained by mixing $ONOO^-$ (750 μM final concentration) for 5 min in a tube containing complete medium. SnPPIX (10, 25 or 50 μM), an inhibitor of haem oxygenase activity, was added to the cell culture medium either before or 5 min after $ONOO^-$ addition. Finally, endothelial cells were pretreated with haemin (100 μM) in complete medium for 2 h followed by 16 h of incubation in medium alone. The haemin-preconditioned cells were then exposed to 1000 μM $ONOO^-$ added as a bolus to the medium.

Assay for endothelial haem oxygenase activity

Endothelial haem oxygenase activity was measured 6 h after exposure to $ONOO^-$ or 16 h after haem treatment as described previously [25,26]. In brief, microsomes from cells exposed to various treatments were added to a reaction mixture containing the following: NADPH (0.8 mM), glucose 6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 unit), 3 mg of rat liver cytosol prepared from a 105000 ***g*** supernatant fraction as a source of biliverdin reductase, $MgCl_2$ (0.2 mM), PBS (100 mM, pH 7.4) and haemin (20 μM). The reaction was conducted in the dark for 1 h and terminated by the addition of 1 ml of chloroform; the extracted bilirubin was then calculated by the difference in absorbance between 464 and 530 nm (ϵ 40 $mM^{-1} \cdot cm^{-1}$). Haem oxygenase activity was expressed in pmol of bilirubin/h per mg of cell protein. The total protein content of confluent cells was determined with a Bio-Rad DC protein assay (Bio-Rad, Hemel Hempstead, Herts., U.K.) by comparison with a standard curve obtained with BSA.

Western blot analysis for HO-1

Samples of endothelial cells treated for the haem oxygenase assay were also analysed by the Western immunoblot technique, as described previously [26]. In brief, cells were lysed and an equal amount of protein (30 μg) from each sample was boiled in Laemmli buffer and separated by SDS/PAGE. Proteins were

Table 1 Haem oxygenase activity and bilirubin production in endothelial cells treated with haemin or $ONOO^-$

Endothelial cells were exposed to medium alone (control) or medium containing 100 μM haemin for 2 h followed by an additional 16 h of incubation in complete medium. $ONOO^-$ (750 μM) was added as a single bolus to endothelial cells in PBS for 2, 5 or 10 min followed by exposure of cells to complete medium for a total incubation period of 6 h. Additional experiments were performed by exposing cells to $ONOO^-$-treated medium, which was obtained by mixing $ONOO^-$ (750 μM final concentration) for 5 min in a tube containing complete medium. SnPPIX (10 μM), an inhibitor of haem oxygenase activity, was added to cells in culture medium 5 min after the addition of $ONOO^-$ (750 μM) as a single bolus. Haem oxygenase activity was determined by using a spectrophotometric assay that measures the formation of bilirubin as described in the Materials and methods section. Bilirubin released into the culture medium was determined after extraction with benzene as described in the Materials and methods section. The results are expressed as means $\pm$ S.E.M. for three to five independent experiments. $^*P < 0.05$ compared with control.

Treatment	Haem oxygenase activity (pmol of bilirubin/h per mg of protein)	Bilirubin production (nmol/h per flask)
Control	347 ± 13	9 ± 0.7
Haemin	$2738 \pm 124^*$	$18 \pm 1.4^*$
$ONOO^-$ in PBS for 2 min	$751 \pm 13^*$	–
$ONOO^-$ in PBS for 5 min	$893 \pm 17^*$	–
$ONOO^-$ in PBS for 10 min	$886 \pm 13^*$	–
$ONOO^-$-treated medium	$456 \pm 19^*$	–
$ONOO^-$ + SnPPIX added 5 min later	$117 \pm 5^*$	–

then transferred to nitrocellulose membrane and probed with polyclonal rabbit anti-(HO-1) antibodies (Stressgen, Victoria, Canada). The antibody complex was detected with an ExtrAvidin alkaline phosphatase kit (Sigma).

Determination of apoptosis in endothelial cells by morphological analysis

Although different methods for detecting apoptosis have been developed, such as the detection of DNA fragmentation by electrophoresis and assays *in vitro* for endonucleases and the cytoplasmic release of histones, electron microscopy still provides the most reliable method for recognizing apoptotic cells [27]. Apoptosis was assessed 6 h after each treatment as described above. Cells were washed with 0.1 M phosphate buffer and fixed in 3 % (v/v) glutaraldehyde in 0.1 M phosphate buffer for 2 h. Samples were washed twice with phosphate buffer and the secondary fixation was performed with 1 % (w/v) osmium tetroxide in 0.1 M phosphate buffer for 1 h at room temperature. Cell monolayers were then washed twice with distilled water, block-stained with 4 % (w/v) aqueous uranyl acetate for 1 h in the dark, washed with distilled water and scraped into Eppendorf tubes. Specimens were dehydrated through increasing acetone series, and infiltrated with acetone/Araldite CY212 resin (1:1, v/v) overnight. Samples were finally embedded in Araldite CY212 resin and blocks were polymerized at 60 °C for 18 h. Sections were cut with a microtome (Reichert-Jung Ultracut E), floated on distilled water, collected on Formvar-coated copper grids and stained with 2 % (w/v) uranyl acetate for 30 min and in 0.13 M lead citrate for 5 min. Stained sections were examined with a 1200CX electron microscope (Jeol, Tokyo, Japan). The apoptotic index was calculated by considering the maximal apoptosis caused by 1000 μM $ONOO^-$ as 100 % and expressing all results from the various protocols as a percentage of this value.

Cytotoxicity assay

A colorimetric assay kit from Promega (Madison, WI, U.S.A.) was used to assess cell viability 6 h after the various treatments. The reaction is based on the reduction of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium salt] (MTS) into a coloured formazan product that is soluble in tissue culture medium. The conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. At the end of the incubation, cells were washed and the assay was performed by adding new medium containing the tetrazolium compound (at a ratio of 20 μl of reagent/100 μl of medium) and incubating for 2 h at 37 °C. The amount of formazan product was measured with a microplate reader (MR 7000; Dynatech) at 490 nm. The measured absorbance is directly proportional to the number of living cells in culture.

Determination of bilirubin in cell culture medium

Haem oxygenase-derived bilirubin was measured in the cell culture medium by using a modification of a method described recently [28]. In brief, endothelial cells pretreated with haemin for 2 h were exposed to fresh culture medium for an additional 16 h. Control experiments were represented by cells exposed to medium alone for 18 h. At the end of the incubation period, 0.5 ml of culture supernatant was collected and 250 mg of $BaCl_2$ was added. After vortex-mixing (10–15 s), 0.75 ml of benzene was added and tubes were vortex-mixed again vigorously for 60 s. The benzene phase containing the extracted bilirubin was separated from the aqueous phase by centrifugation at 13000 ***g*** for 30 min. Bilirubin was measured spectrophotometrically as a difference in absorbance between 464 and 530 nm (ϵ 27.3 $mM^{-1} \cdot cm^{-1}$) and the rate of bilirubin excreted into the culture medium was expressed as nM/h per flask.

Statistical analysis

Differences in the data between the groups were analysed by using one-way analysis of variance combined with the Bonferroni test. Values were expressed as means $\pm$ S.E.M. and differences between groups were considered to be significant at $P < 0.05$.

RESULTS

Effect of $ONOO^-$ on haem oxygenase activity and HO-1 expression in endothelial cells

When endothelial cells were treated with increasing concentrations of $ONOO^-$ added as a single bolus to the cell culture medium, a concentration-dependent increase in haem oxygenase activity, as well as HO-1 expression, was observed at 6 h (Figure

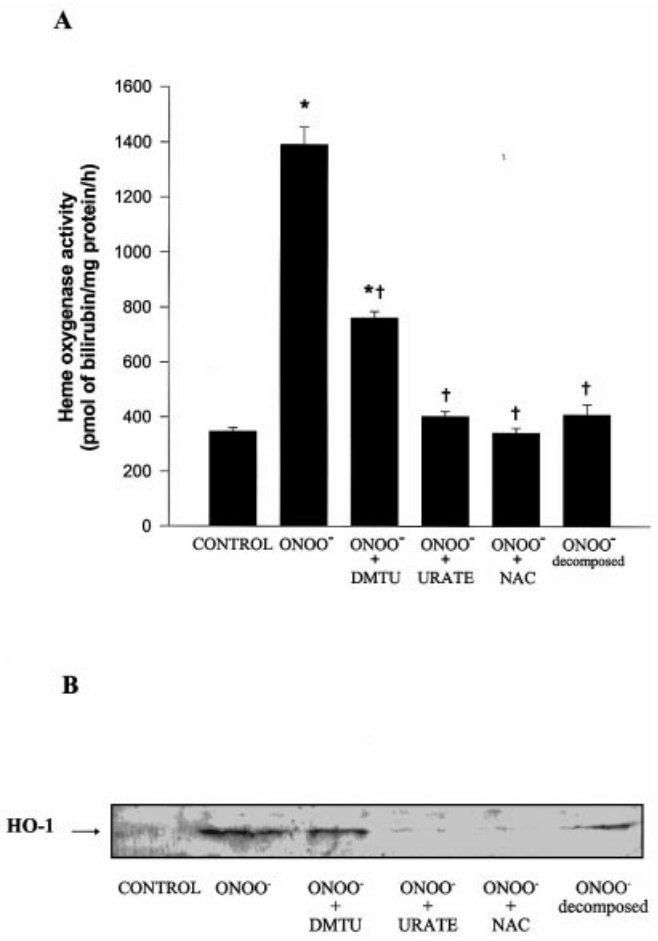

Figure 2 Effect of uric acid and *N*-acetylcysteine on ONOO⁻-mediated increase in haem oxygenase activity and HO-1 expression in endothelial cells

(**A**) Haem oxygenase activity was measured 6 h after exposure of endothelial cells to ONOO⁻ (750 μM) alone or to ONOO⁻ in the presence of 1 mM 1,3-dimethyl-2-thiourea (DMTU), 1 mM uric acid (urate) or 1 mM *N*-acetylcysteine (NAC). Decomposed ONOO⁻ was also tested at 750 μM. Control experiments were performed by exposing cells to complete medium alone. Each bar represents the mean $\pm$ S.E.M. for five or six independent experiments. $^*P < 0.05$ compared with control; $\dagger P < 0.05$ compared with ONOO⁻ alone. (**B**) HO-1 protein expression was analysed by the Western immunoblot technique (as described in the Materials and methods section) in samples of cells treated as above.

1). Exposure of cells to ONOO⁻ (750 μM) in phosphate buffer for 2, 5 or 10 min followed by incubation with fresh medium also resulted in a significant increase in haem oxygenase activity at 6 h (Table 1). Control experiments performed by exposing cells to phosphate buffer alone for 10 min (but not 2 and 5 min) showed only a slight increase in haem oxygenase activity (results not shown). Incubation of cells with ONOO⁻-treated medium also produced a minor change in enzyme activity (Table 1). In an additional set of experiments, the stock solution of ONOO⁻ was treated with MnO_2 to remove the possible residual H_2O_2. At 500 μM ONOO⁻, endothelial haem oxygenase activity was 785 ± 25 pmol bilirubin/h per mg of protein with ONOO⁻ pretreated with MnO_2, compared with 805 ± 40 pmol bilirubin/h per mg of protein with ONOO⁻ (not significant).

Uric acid and *N*-acetylcysteine, a glutathione precursor with antioxidant properties, are commonly used as ONOO⁻ scavengers. In our experiments we observed a direct effect of both scavengers in preventing the increase in haem oxygenase activity and HO-1 expression by ONOO⁻ (Figure 2). In contrast, when the hydroxyl radical scavenger 1,3-dimethyl-2-thiourea was used, only a partial attenuation of the enzyme activity and HO-1 expression was seen. Taken together, these results suggest a primary role for ONOO⁻ itself and a contribution of hydroxyl radicals to up-regulating HO-1 in endothelial cells. This was further sustained by the finding that decomposed ONOO⁻ did not produce any significant change in the levels of haem oxygenase (Figure 2).

Effect of ONOO⁻ on apoptosis and cell viability in endothelial cells

As shown in Figure 3(A), treatment of endothelial cells with ONOO⁻ resulted in apoptotic cell death. Cells showed morphological characteristics typical of the apoptotic event, such as chromatin condensation and segregation, and condensation of the cytoplasm with preservation of the integrity of the organelles; in some experiments, a careful examination of the fixed tissues also revealed the presence of some cell death by necrosis at the highest concentrations of ONOO⁻ used. ONOO⁻-mediated apoptosis was concentration-dependent, with a considerable number of dead cells at the concentration of 1000 μM (Figure 3B). The possibility that in our experiments apoptosis was induced by NaOH, the vehicle in which ONOO⁻ is solubilized, can be excluded. In fact, endothelial cells appeared perfectly viable when, instead of ONOO⁻, the same amount of 0.3 M NaOH was added to the culture medium (results not shown). The apoptotic response to ONOO⁻ was, however, markedly attenuated by exposing cells to ONOO⁻ in the presence of uric acid or *N*-acetylcysteine, as shown in Table 2. Uric acid or *N*-acetylcysteine at 1 mM were capable of decreasing ONOO⁻-mediated apoptosis by approx. 80% when compared with ONOO⁻ alone.

As shown in Figure 4(A), exposure of endothelial cells to ONOO⁻ resulted in a concentration-dependent decrease in cell viability. Pronounced damage was observed at 1000 μM ONOO⁻, with approx. 55% of cells being metabolically inactive. Similarly to our observations with apoptosis, we found that uric acid and *N*-acetylcysteine prevented the ONOO⁻-mediated cytotoxic effect, restoring cell viability to 92% and 82% of control respectively (Figure 4B).

Effect of a haem oxygenase inhibitor (SnPPIX) on ONOO⁻-mediated apoptosis and cell viability

Because ONOO⁻ induced endothelial HO-1 expression and caused apoptosis in a concentration-dependent manner, we wished to test whether haem oxygenase had a role in the process of apoptotic cell death produced by the powerful oxidant. When SnPPIX (10 or 25 μM), an inhibitor of haem oxygenase activity, was added to the culture medium just before ONOO⁻ (1000 μM), cells showed a significant decrease in programmed cell death (Table 2). Similarly, 10 μM SnPPIX prevented ONOO⁻-mediated cell injury, recovering cell viability to 96% of that of the control (Figure 4B). We also found that addition of SnPPIX (10 or 25 μM) 5 min after ONOO⁻ (1000 μM) decreased apoptosis by only 40%, as opposed to a decrease by 70% and 55% when 10 and 25 μM SnPPIX respectively were added before ONOO⁻ (Table 2). At higher concentrations (50 μM), SnPPIX added before ONOO⁻ did not provide any significant protection against cell damage mediated by the oxidant (results not shown) and caused a 50% increase in apoptosis compared with ONOO⁻ alone. At this concentration, SnPPIX has been reported to partly inhibit the activity of constitutive NO synthase and guanylate

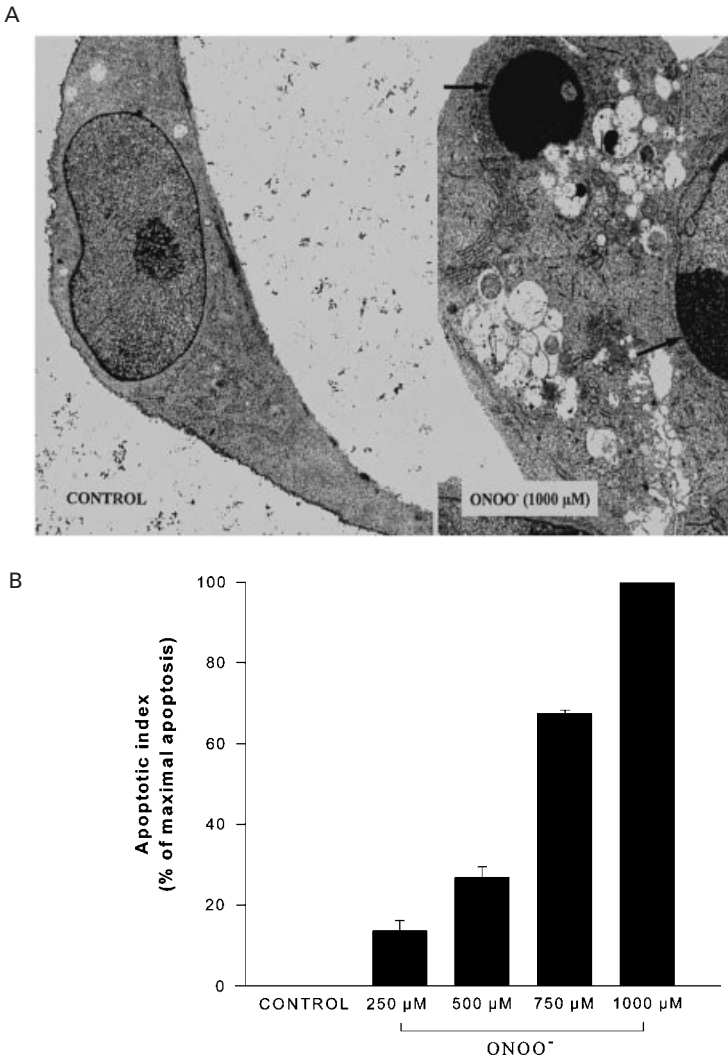

Figure 3 Effect of $ONOO^-$ on apoptosis in endothelial cells

(**A**) Representative pictures of endothelial cells exposed to complete medium alone (control) or 1000 μM $ONOO^-$ added as a bolus to the culture medium. After 6 h incubation, cells were fixed and stained, and apoptosis was assessed by electron microscopy as described in the Materials and Methods section. Morphological changes typical of apoptosis, such as chromatin condensation and endoplasmic reticulum alignment (arrowed), are visible in cells treated with $ONOO^-$. Magnification $\times$ 3696 for the control and $\times$ 5676 for $ONOO^-$. (**B**) Apoptosis was assessed 6 h after treatment with $ONOO^-$ as described in the the Materials and methods section. Apoptotic index was calculated by considering the maximal apoptosis caused by 1000 μM $ONOO^-$ as 100% and expressing all results from the various protocols as a percentage of this value. Each bar represents the mean $\pm$ S.E.M. for three independent experiments.

Table 2 Effect of various compounds on $ONOO^-$-mediated apoptosis

$ONOO^-$ (1000 μM) was added as a single bolus to endothelial cells in complete medium; apoptosis was assessed after 6 h as described in the Materials and methods section. SnPPIX, an inhibitor of haem oxygenase activity, was added to the cell culture medium either before or 5 min after $ONOO^-$. *N*-Acetylcysteine and uric acid were added before $ONOO^-$. Haemin pretreatment was performed by exposing cells to culture medium containing 100 μM haemin for 2 h followed by an additional 16 h of incubation in medium alone. Apoptotic index was calculated by considering the maximal apoptosis caused by $ONOO^-$ as 100% and expressing all results as a percentage of this value. The results are expressed as means $\pm$ S.E.M. for three to five independent experiments. $^*P <$ 0.05 compared with $ONOO^-$.

Compound added *before* $ONOO^-$	Apoptotic index (%)	Compound added 5 min *after* $ONOO^-$	Apoptotic index (%)
$ONOO^-$	100	$ONOO^-$	100
SnPPIX (10 μM) + $ONOO^-$	30 $\pm$ 7*	$ONOO^-$ + SnPPIX (10 μM)	60 $\pm$ 4*
SnPPIX (25 μM) + $ONOO^-$	46 $\pm$ 1*	$ONOO^-$ + SnPPIX (25 μM)	60 $\pm$ 12*
N-Acetylcysteine (1 mM) + $ONOO^-$	20 $\pm$ 8*		
Uric acid (1 mM) + $ONOO^-$	21 $\pm$ 9*		
Haemin pretreatment + $ONOO^-$	65 $\pm$ 4*		

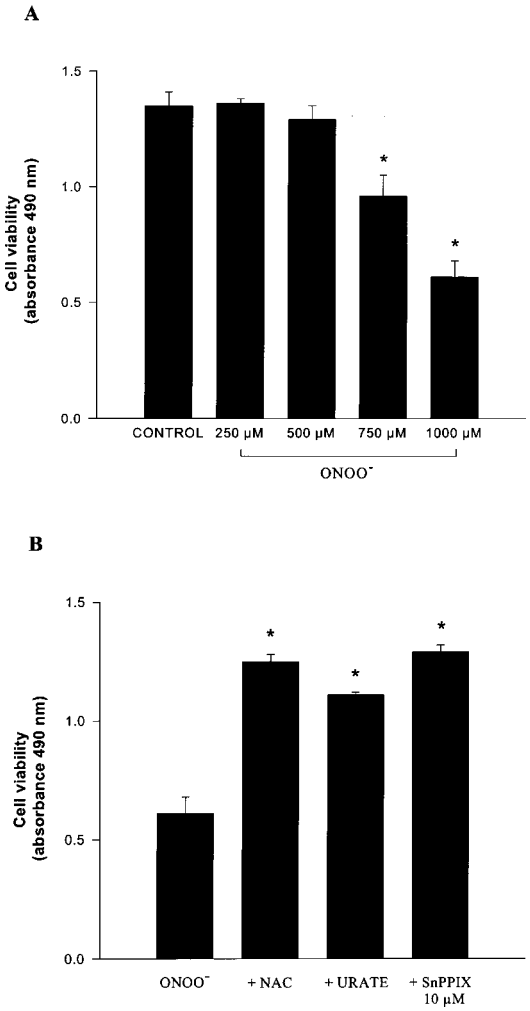

Figure 4 Effect of $ONOO^-$ on cell viability and effect of antioxidants and SnPPIX on $ONOO^-$-mediated cell injury

(**A**) Viability was assessed in endothelial cells 6 h after exposure to various concentrations of $ONOO^-$ (250–1000 μM) added as a bolus to the culture medium. Each bar represents the mean ± S.E.M. for four independent experiments. $^*P < 0.05$ compared with control. (**B**) Cell viability after exposure to $ONOO^-$ alone (1000 μM) or $ONOO^-$ in the presence of 1 mM *N*-acetylcysteine (NAC), 1 mM uric acid (urate) or 10 μM SnPPIX. The absorbance at 490 nm is directly proportional to the number of living cells. Each bar represents the mean ± S.E.M. for four independent experiments. $^*P < 0.05$ compared with $ONOO^-$ alone.

cyclase *in vitro* [29,30]. Therefore the experiments using 50 μM SnPPIX cannot be interpreted solely on the basis of haem oxygenase activity blockade.

Effect of haemin pretreatment on endothelial haem oxygenase activity, bilirubin production and $ONOO^-$-mediated apoptosis

To define whether increased haem oxygenase activity is protective against apoptosis caused by $ONOO^-$, endothelial cells were pretreated for 2 h with 100 μM haemin, a well-known HO-1 inducer [26], followed by an additional 16 h incubation in complete medium. Endothelial haem oxygenase activity after this treatment was significantly higher than in the control (Table 1). We also observed that the rate of bilirubin released in the culture medium of haemin-pretreated cells was considerably enhanced (see Table 1). Interestingly, compared with untreated cells, exposure of haemin-preconditioned cells to 1000 μM $ONOO^-$ added as a bolus to the cell culture resulted in a decrease in apoptosis by 35% (Table 2). These results suggest that stimulation of the haem oxygenase/bilirubin pathway protects against apoptosis mediated by $ONOO^-$. No apoptosis was observed in cells pretreated with haemin alone (results not shown).

DISCUSSION

Strong evidence supports a major role for $ONOO^-$ in the cellular injury and death that was once attributed entirely to nitric oxide (NO) [21,31]. In fact, $ONOO^-$, formed from the reaction of NO and $O_2^{-\bullet}$ [17], is a powerful oxidant that has been implicated in the pathophysiology of conditions such as ischaemia/reperfusion, atherosclerosis and shock [18]. $ONOO^-$ decomposes at pH 7.4 to generate a hydroxyl radical and nitrogen dioxide [16], two highly reactive species that cause oxidative stress. $ONOO^-$ itself also reacts directly with biological targets including thiols, iron–sulphur centres and zinc fingers and is responsible for the production of nitrotyrosine [19]. Indeed, nitrotyrosine has been detected in human atherosclerotic lesions and as a free amino acid in plasma from patients with rheumatoid arthritis [32,33]. Another important characteristic of $ONOO^-$ is its ability to promote nitrosative stress [34]. The hypothesis that $ONOO^-$ might also be involved in the induction of the tissue stress response has not, however, been explored directly. In previous studies we have shown that NO donors are able to modulate the activity [25] and expression of the stress protein HO-1 in vascular endothelial cells [26] and other cell types [35]. Furthermore, we have demonstrated that thiols interact with NO to regulate HO-1 expression, and suggested that $O_2^{-\bullet}$ and $ONOO^-$ might have a role in the stimulation of this protein [26].

In the present study we report that $ONOO^-$ is capable of inducing HO-1 protein expression and increasing haem oxygenase activity in a concentration-dependent manner in cultured endothelial cells. This effect is attributable to $ONOO^-$ itself rather than to an oxidized or nitrated component of the medium, because a brief exposure of cells (2–10 min) to $ONOO^-$ in phosphate buffer also resulted in a marked increase in activity. Furthermore, $ONOO^-$-treated medium produced only a minor effect on haem oxygenase activity. Uric acid and *N*-acetylcysteine, two antioxidants known to scavenge $ONOO^-$, completely abolished the increase in haem oxygenase activity and HO-1 protein. Although the concentrations of $ONOO^-$ used in our experiments might seem high, the amount of $ONOO^-$ reacting with cells is considerably lower than the actual concentration used because of the interaction of $ONOO^-$ with components of the culture medium [20]. In addition, the net exposure of cells to $ONOO^-$ is brief because $ONOO^-$ decomposes with a half life of less than 1 s at pH 7.4 [36]. Ischiropoulos et al. [37] have also shown that in activated murine macrophages the formation of $ONOO^-$ might be as high as 0.11 nmol/min per 10^6 cells, which might result in local maximal rates of as much as 1 mM/min.

$ONOO^-$-mediated HO-1 induction might occur via at least two possible pathways. First, $ONOO^-$ itself could, by virtue of its powerful oxidant and/or nitrosative characteristics, participate directly in triggering cellular signals responsible for the induction of the protein. Alternatively, hydroxyl radicals and nitrogen dioxide, the decomposition products of $ONOO^-$, could be the mediators of the process. Moreover, the possibility that both mechanisms might take place simultaneously cannot be excluded. Our results showing that 1,3-dimethyl-2-thiourea partly attenuated HO-1 induction by $ONOO^-$ indicate a contribution of hydroxyl radicals to the observed effect. Evidence for the involvement of hydroxyl radicals in the induction of the haem oxygenase gene has already been reported in human skin

fibroblasts by UV-A radiation [38] and in the rat retina by intense visible light [39]. It has to be noted that previous reports suggested the possibility that DMTU could also have a direct scavenging effect towards $ONOO^-$ [40,41]. Because scavengers that effectively intercept nitrogen dioxide, such as β-carotene, also react with $ONOO^-$ [42], it was more difficult in the present study to determine a potential contribution of nitrogen dioxide to the modulation of the expression of this stress protein.

We also observed a concentration-dependent increase in apoptosis in endothelial cells by $ONOO^-$. $ONOO^-$-mediated apoptosis has been described in cultures *in vitro* of PC12 cells [43], HL-60 cells [20] and human endothelial cells [21]. In the last study mentioned, it was shown that extracellular glutathione protected human endothelial cells against $ONOO^-$-induced cytotoxicity. Recently, uric acid has also been reported to prevent apoptosis produced by $ONOO^-$ in neural cell lines [44]. In our experiments, *N*-acetylcysteine, a precursor of glutathione biosynthesis, and uric acid significantly diminished the number of apoptotic cells observed with $ONOO^-$ alone. In line with our findings on $ONOO^-$-mediated apoptosis, we observed a loss of cell viability after exposure of endothelial cells to increasing concentrations of $ONOO^-$. Once again, uric acid and *N*-acetylcysteine significantly prevented the cytotoxic action of $ONOO^-$.

Of major interest in this study is the effect of SnPPIX, an inhibitor of haem oxygenase activity, on $ONOO^-$-mediated apoptotic cell death and cytotoxicity. Our results show that 10 μM SnPPIX, a concentration of inhibitor that has been shown *in vitro* to block haem oxygenase activity specifically [29], effectively protected against cell injury and death when added before $ONOO^-$. This would suggest that haem oxygenase, and in particular HO-1 induction, is associated with increased apoptosis. This is rather surprising, considering the substantial body of evidence pointing to HO-1 up-regulation as an important intracellular protective system against stress stimuli [3,6,15]. Moreover, recent findings have demonstrated that endothelial cells transfected with HO-1 are resistant to tumour-necrosis-factor-α-mediated apoptosis [45]. This apparent contradiction could be partly explained if SnPPIX were to react directly with $ONOO^-$ in our experimental system. Interestingly, a group of metalloporphyrins capable of catalysing $ONOO^-$ decomposition to nitrate has recently been identified [31,46,47]. It was therefore plausible to postulate that SnPPIX, a protoporphyrin that contains tin as a metal centre, could also act as a catalyst for $ONOO^-$ decomposition. Our hypothesis is corroborated by the results of this study showing that the addition of SnPPIX (10 or 25 μM) 5 min after $ONOO^-$ decreased apoptosis by only 40%, as opposed to decreases by 70% and 55% when 10 and 25 μM SnPPIX respectively were added before $ONOO^-$. These results are best explained by a direct interaction of SnPPIX with $ONOO^-$ when both compounds are present simultaneously in the culture medium. We still cannot explain why 10 μM SnPPIX added 5 min after $ONOO^-$ resulted in decreased apoptosis compared with $ONOO^-$ alone. It is possible that SnPPIX might have effects other than haem oxygenase inhibition and interaction with $ONOO^-$, potentially related to the cascade of events leading to apoptosis.

The contribution of HO-1 induction against $ONOO^-$-mediated damage was sustained by our findings showing that cells preconditioned with haemin, a potent inducer of HO-1, were less susceptible to apoptosis caused by $ONOO^-$. We also observed that the rate of release of the antioxidant bilirubin, the end product of haem degradation by haem oxygenase, was significantly higher in the culture medium of haemin-treated cells, suggesting that bilirubin could participate in the mechanism of protection. In agreement with this concept, it has been recently demonstrated that bilirubin is effective in preventing $ONOO^-$-mediated protein oxidation in human blood plasma [48]. However, we cannot exclude the possibility that in our experiments other important products of haem catabolism, such as biliverdin and CO, might have a defensive role against $ONOO^-$.

In conclusion, our results demonstrate that $ONOO^-$ can stimulate the tissue stress response by modulating HO-1 protein expression and that exogenously applied antioxidants prevent this effect. Our findings also provide evidence that induction of the HO-1 pathway is cytoprotective against cell death caused by $ONOO^-$.

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