Haem oxygenase shows pro-oxidant activity in microsomal and cellular systems: implications for the release of low-molecular-mass iron

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Haem oxygenase-1 (HO-1) is a highly inducible stress protein that removes haem from cells with the release of biliverdin, carbon monoxide and low-molecular-mass iron (LMrFe). Several antioxidant functions have been ascribed to HO; its induction is considered to be a protective event. However, LMrFe produced during haem catabolism might elicit a pro-oxidant response, with deleterious consequences. We therefore investigated the delicate balance between pro-oxidant and antioxidant events with the use of a microsomal lipid peroxidation (LPO) system. By using microsomal-bound HO in an NADPH-dependent LPO system, we assessed the pro-oxidant nature of the released LMrFe and the antioxidant effect of the released bilirubin. Hb, a biologically relevant substrate for HO, was included with the microsomes to supplement the source of haem iron and to promote LPO. We found significant increases in microsomal LPO, by using the thiobarbituric acid (TBA) test, after incubation with Hb. This Hb-stimulated peroxidation was inhibited by HO inhibitors and by iron chelators, suggesting a HO-driven, iron-dependent mechanism. GLC–MS was employed to measure the specific LPO product 4-hydroxy-2-nonenal and to confirm our TBA test results. A HO inhibitor attenuated an increase in intracellular LMrFe that occurred after treatment of rat pulmonary artery smooth-muscle cells with Hb. Additionally, exogenously added bilirubin at an equimolar concentration to the LMrFe present in both microsomal and liposomal systems was unable to prevent the pro-oxidant effect of the iron. Under certain circumstances HO can act as a pro-oxidant and seems to have a role in stimulating microsomal LPO.

Key words: antioxidants, bilirubin, heat shock protein 32, lipid peroxidation.

INTRODUCTION

Haem oxygenase (HO) catalyses the oxidative degradation of the haem moiety of Hb to biliverdin, carbon monoxide and free iron. Three isoforms have been isolated, namely the inducible haem oxygenase-1 (HO-1), the constitutive haem oxygenase 2 (HO-2) (reviewed in [1,2]) and the recently discovered haem oxygenase 3 (HO-3) [3], which unlike the other two breaks haem down only poorly [3]. HO-1 is a stress protein known as heat shock protein 32. Its transcription can be induced by a whole array of stresses, including endotoxin [4–7], transition-metal ions [8], haem, haemin, Hb and other haem proteins [9–12], and oxidative stress [13–15]. Indeed, it has been suggested that HO-1 induction might represent a generalized response to oxidative stress [16–18]. Several authors have suggested that HO-1 confers cellular protection against a wide range of oxidant stresses both *in vitro* and *in vivo* [16–20]. The mechanism of protection is unclear, although the removal of pro-oxidant haem, the antioxidant properties of bilirubin *in vitro* [21,22] and the induction of ferritin synthesis, which sequesters redox-active iron [23], are thought to be involved. However, recent evidence suggests that HO-1 induction might not always be beneficial and that the release of redox-active, low-molecular-mass iron (LMrFe) from haem might be responsible for cellular damage [24–26].

The present study had two aims: (1) to assess the effect of the equimolar release of LMrFe (a pro-oxidant) and bilirubin (an antioxidant) on peroxidizing microsomes brought about by HO's acting on a haem substrate, and (2) to resolve an outstanding question about the source of relatively large amounts of LMrFe associated with microsomal preparations. Microsomes have been used to study biological lipid peroxidation (LPO) for several decades. However, the initiation process, and the role of iron complexes in this, have remained topics of debate because both enzymic and non-enzymic components are involved [27–29]. The participation of HO in microsomal LPO seems likely from the results that we obtained.

EXPERIMENTAL

Reagents

4-Hydroxy-2-nonenal (HNE) was obtained from Calbiochem–Novabiochem (Nottingham, Notts., U.K.). Tin protoporphyrin (SnPP) was provided by Porphyrin Products (Logan, UT, U.S.A.) and zinc protoporphyrin (ZnPP) by Aldrich (Gillingham, Dorset, U.K.); they were each made up in 1 mM NaOH and diluted in PBS [0.1 M NaH_2PO_4/0.15 M NaCl (pH 7.4)]. Methanol, ethanol and acetone were obtained from Fisher Scientific (Loughborough, Leics., U.K.). HO-1 antibody was supplied by Bioquote Ltd. (York, Yorks., U.K.). Desferrioxamine (Desferal) was from Ciba–Geigy (Basle, Switzerland). All other chemicals were supplied by Sigma–Aldrich (Poole, Dorset, U.K.). All porphyrin-based compounds were stored in the dark before use.

Isolation of microsomes

Normal male Wistar rats (275–300 g) were anaesthetized with pentobarbitone (60 mg/kg, intraperitoneally) and asphyxiated; the liver tissue was removed and rinsed with ice-cold Tris buffer [0.25 M sucrose/0.01 M Tris/HCl (pH 7.4)]. Microsomes were prepared essentially as described by Trakshel et al. [30]. In brief,

Abbreviations used: BHT, butylated hydroxytoluene; HNE, 4-hydroxy-2-nonenal; HO, haem oxygenase; LMrFe, low-molecular-mass iron; LPO, lipid peroxidation; LPS, lipopolysaccharide; MDA, malondialdehyde; SnPP, tin protoporphyrin; TBA, thiobarbituric acid; TMS, trimethylsilyl; ZnPP, zinc protoporphyrin.

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liver tissue was cut into small pieces and homogenized with Tris buffer. The homogenate was spun at 10000 ***g*** for 20 min at 4 °C; the supernatant was retained and the pellet was rehomogenized with 2 vol. of sucrose buffer. After centrifugation at 10000 ***g*** for 20 min at 4 °C, the two supernatant fractions were combined and centrifuged at 150000 ***g*** for 1 h at 4 °C. After resuspension in 125 mM KCl, these final pellets were used as the microsomal fraction. The protein concentration was determined by the Lowry method with a kit obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.); the microsomes were then stored at −80 °C until use.

Conditions of incubation

To iron-free polypropylene tubes were added reagents in the following order: 780 μl of PBS, 20 μl of rat liver microsomes (0.5 mg/ml protein), 100 μl of phospholipid liposomes (5 mg/ml in PBS) and 100 μl NADPH (2 mM). This reaction mixture served as a control system. Test incubations contained 100 μl of Hb (0.1 mg/ml, final concentration) in place of buffer. Hb has been used previously in studies with HO [12,20]. It might not be the ultimate form of haem utilized by the enzyme *in vivo* but it is definable, readily soluble and biologically relevant. In the first set of experiments, microsomes were incubated for up to 180 min to establish the time course of the reaction; a set of matched controls lacking various reaction components was also incubated for comparison. In the second set of experiments the role of iron in HO-catalysed LPO was investigated by the use of chain-breaking antioxidants, iron chelators and HO inhibitors: 100 μl of an iron chelator (EDTA, desferrioxamine or ferrozine) was added to the test reaction mixture as appropriate controls (1 mg/ml in PBS); 100 μl of either of the lipid-soluble chain-breaking antioxidants butylated hydroxytoluene (BHT) or α-tocopherol (1 mg/ml) served as additional controls. The HO inhibitors SnPP and ZnPP were also added as controls at 20 μM in 100 μl volumes. Volume discrepancies were corrected by the addition of an equivalent volume of PBS to the test mixture. The LMrFe content of the buffer was 0.5 μM, which was insufficient to effect the peroxidative process. Microsomes heated for 5 min at 100 °C served as a control for denatured HO enzyme activity. Finally, a Hb solution (1 mg/ml) was dialysed against conalbumin for 48 h to remove contaminating chelatable iron, to establish the extent to which such adventitious non-haem iron contributed to the LPO seen. All reactions were set up in triplicate and were incubated at 37 °C in a circulating-water bath for the immediate analysis of markers of LPO.

Thiobarbituric acid (TBA) assay

The TBA assay [31] was employed as an index of LPO in the microsomal system. After incubation of the microsomal reaction sample, 0.5 ml of TBA [1 % (w/v) in 50 mM NaOH] was added, followed by 0.5 ml of HCl (25 %, v/v). The tubes were capped and heated for 15 min at 100 °C. When cool, 1.5 ml of butan-1-ol was added; the tube contents were then vortex-mixed vigorously for 2 min before centrifugation at 3000 ***g*** for 10 min. The upper organic layer was carefully removed; the fluorescence was measured at 553 nm with excitation at 532 nm. Relative fluorescence intensity units were ascribed to the samples on the basis of a standard of rhodamine B (3 μM) set to 100 units at the same wavelength settings. TBA-reactive substances were measured against a pure standard of malondialdehyde (MDA); results are expressed as μmol of MDA.

Extraction and derivatization of HNE, and measurement by GLC–MS

HNE was extracted and derivatized as described previously [32]. In brief, 1 ml of sample and 5 μl of BHT (2 mM in methanol) were mixed well and divided into two portions. Nonanoic acid served as an internal standard (6 μl, diluted 1:10000 in methanol). To each was added 4 ml of chloroform/methanol (2:1, v/v) and 0.4 ml of saline solution (0.15 M). Tubes were vortex-mixed and centrifuged at 3400 ***g*** (4500 rev./min) for 6 min. Lower layers were collected and stored. The residue was extracted with 2 ml of chloroform/methanol (2:1, v/v). After vortex-mixing and centrifugation, all lower layers were collected and combined, then evaporated to dryness under a stream of oxygen-free nitrogen at room temperature. Extracted HNE was derivatized with 200 μl of dry acetone and 200 μl of bis(trimethylsilyl)-trifluoroacetamide containing 1 % (v/v) trimethylchlorosilane. Tubes were capped and left at room temperature for 1 h to form trimethylsilyl (TMS) esters. Insoluble residues were removed by centrifugation; samples were placed in clean vials, capped and stored at −20 °C ready for GLC–MS.

HNE-TMS (2 μl) in dry acetone was injected with a splitless injection technique. HNE-TMS standards and nonanoic acid-TMS standards were detected as total ion chromatograms to gain information about mass spectra and retention times. HNE, although present in microsomal samples in small amounts, could nevertheless be detected with the sensitive technique of selective ion monitoring. Three characteristic masses were selected from the HNE standard total ion chromatogram (m/z 81, m/z 85 and m/z 138) and monitored at the expected retention times. Identification was aided by the relative intensities of these ions. Peak areas were corrected to an internal standard of nonanoic acid methyl ester. The selected ions used from the internal standard were m/z 74, m/z 87 and m/z 129. Corrected areas were related to a standard curve of HNE produced from the original standard (range 0.1–20 nmol/ml). Results were expressed as nmol of HNE/ml of reaction mixture.

HO activity

HO activity in rat liver microsomes was measured with a chromatographic method adapted from that of Ryter et al. [33]. The reaction mixture consisted of 100 μl of microsomes (0.5 mg/ml protein), 100 μl of NADPH (1 mM), 60 μl of glucose 6-phosphate (5 mM) and 1 unit of glucose-6-phosphate dehydrogenase. The mixture was buffered with 250 mM sucrose/20 mM Tris/HCl (pH 7.4) to make a final volume of 300 μl. Reactions were started by the addition of 2 μl of haemin [2.5 mM, in 25 % (v/v) DMSO], incubated for 1 h and then stopped on ice by the addition of 300 μl of ethanol/DMSO (95:5, v/v) containing 0.8 M mesoporphyrin as an internal standard. Samples were analysed by HPLC with a linear gradient of 100 % solvent A [100 mM ammonium acetate (pH 5.1)/60 % (v/v) methanol] to 100 % solvent B (methanol) over 23 min. Pure solutions of bilirubin were used to identify peaks and to assess recovery within the system. Results are expressed as nmol of bilirubin/h per mg of protein.

SDS/PAGE and Western blot analysis of HO-1

The presence of HO-1 in rat liver microsomes was confirmed by immunoblotting. In brief, microsomes were subjected to electrophoresis in SDS/14 % (w/v) PAGE running gels, with 10 % (w/v) stacking gels. The gels were transferred to Hybond-C nitrocellulose membranes (Amersham International, Little Chal-

font, Bucks., U.K.). After blocking with PBS/Tween/5 % (w/v) dried skimmed milk, the blots were probed by a polyclonal antibody against HO-1, then incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Sigma–Aldrich). After being washed, the membranes were treated with enhanced chemiluminescence reagent (ECL®; Amersham) to reveal immunoreactive proteins. Blots of microsomes from normal and lipopolysaccharide (LPS)-treated rats were compared to confirm the presence of HO-1.

LMrFe measurement

The bleomycin assay was used as described previously [34] for the measurement of chelatable LMrFe. In brief, the reaction mixture contained calf thymus DNA (1 mg/ml), bleomycin sulphate (1.5 i.u./ml) and the microsomal sample buffered to pH 7.4 with a Tris salt (1 M). In the presence of added ascorbate (7.5 mM, freshly made), iron that is able to be chelated from the sample by bleomycin can degrade DNA *in vitro* with the release of MDA from its deoxyribose moiety. The MDA released is measured by its reaction with 2-thiobarbituric acid and is proportional to the amount of chelatable iron present.

Application of the LMrFe assay to microsomes showed that they had a baseline level of 0.7 ± 0.06 μmol (mean $\pm$ S.E.M.) of LMrFe. The production of LMrFe by rat pulmonary artery smooth-muscle cells after the induction of HO-1 was also determined. Rat pulmonary artery smooth-muscle cells were cultured by explant from rat pulmonary arteries and were confirmed by immunostaining with α-actin. Cells were incubated for 24 h with Hb (50 μl of 1 mg/ml), then washed with Hanks medium and lysed with a 1 % (v/v) Triton X-100 extraction buffer containing protease inhibitors. The intracellular contents were analysed by using the bleomycin assay and were found to contain 0.39 ± 0.08 μmol of LMrFe. The protein was blotted for HO-1 immunodetection as described above.

Deoxyribose assay

The deoxyribose assay for iron-catalysed hydroxyl radical formation [35] was used to establish whether the HO inhibitors had any iron-chelating properties that could conceivably influence the interpretation of results. In brief, we incubated SnPP or ZnPP with Fe^{2+} (1 mM) and deoxyribose (10 mM) in 0.1 M phosphate buffer, pH 7.4, for 30 min, against appropriate controls; 0.5 ml of 2.8 % (w/v) trichloroacetic acid and 0.5 ml of TBA reagent were added and heated for 15 min at 100 °C. After extraction of the pink chromagen into butan-1-ol, iron-catalysed damage to deoxyribose was measured as the release of MDA with a spectrophotometer set to 532 nm.

Exogenously added iron and bilirubin

To determine the relative pro-oxidant and antioxidant effects of bilirubin and LMrFe during LPO under our conditions, we added a range of equimolar concentrations to liposomes and microsomes.

Statistical treatments

Unless stated otherwise, all experiments were performed in triplicate and results are expressed as means $\pm$ S.E.M. Results were compared by analysis of variance followed by Dunnett's *post hoc* test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The incubation of microsomes with Hb in the presence of NADPH revealed an initial formation of TBA-reactive substances (measured as μmol of MDA), the levels of which increased rapidly for 1 h and from then on more slowly. Matched controls lacking added Hb but including all the other reaction constituents showed an increase in MDA levels. This increase was apparent after 30 min of incubation, but only at levels significantly below those seen in the test incubations. Controls lacking NADPH showed a similar response to those lacking Hb but with correspondingly lower levels of MDA formation. A blank lacking both Hb and NADPH showed the lowest level of MDA formation over time. Results are shown in Figure 1 and demonstrate a substrate effect for Hb during NADPH-dependent microsomal LPO. The cause of some degree of MDA formation in the various controls, and to a smaller extent the blank, was probably the availability of endogenous substrates such as haem-containing molecules and NADPH in this acellular system. We subsequently decided to perform all experiments involving microsomes with an incubation duration of 30 min. In Figure 1 it can be seen that after this time there is the greatest difference between test and controls, representing the midpoint for maximum LPO under the conditions described. However, other time points could have be used because differences between test and controls remained significant throughout.

MDA formation in test incubations compared with controls containing either BHT or α-tocopherol (both lipid-phase, chain-breaking antioxidants) revealed significant levels of inhibition (73 %) over the baseline control levels (Figure 2). The TBA-reactive substances detected clearly arose from the peroxidation of polyunsaturated fatty acids. MDA levels from test incubations compared with controls containing the iron chelators desferrioxamine, EDTA or ferrozine also showed them to inhibit significantly the LPO observed (Figure 2). This finding indicated strongly that the iron was both chelatable and pro-oxidant under the conditions described. Test incubations with added Hb from which adventitious LMrFe had been removed by dialysis [36] were no different from those with untreated Hb (Figure 2) in stimulating LPO. We can therefore assume that the iron responsible for driving LPO arises from the haem moiety of Hb and not from contaminating LMrFe.

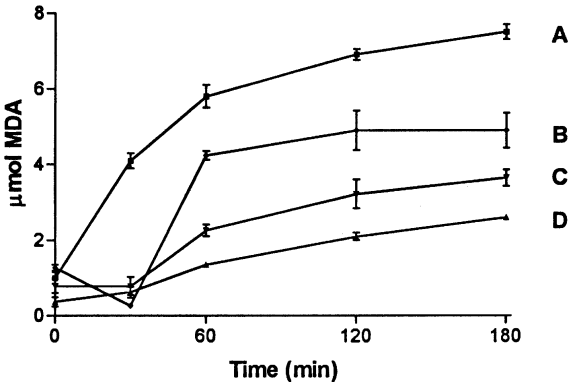

Figure 1 Time-dependent increases in microsomal LPO

Rat liver microsomes (a source of HO) were incubated with Hb (1 mg/ml), NADPH (2 mM) and phospholipid liposomes (5 mg/ml in PBS) at 37 °C. MDA formation was monitored as an index of LPO at the time points shown (line A). Incubations without NADPH (line C), without Hb (line B) or without both (line D) served as controls.

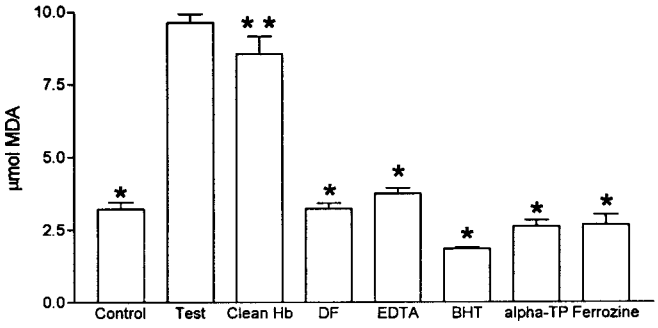

Figure 2 Iron-mediated microsomal LPO

Rat liver microsomes were incubated with NADPH, Hb and liposomes at 37 °C; MDA formation was measured after 30 min (test). Tubes without Hb served as a control. Hb was also dialysed with conalbumin to remove adventitious iron (clean Hb). The addition of the iron chelators EDTA, desferrioxamine (DF) and ferrozine, and the chain-breaking antioxidants BHT and α-tocopherol (alpha-TP), served as controls. *LPO significantly lower than that of test ($P < 0.05$); **LPO not significantly different from that of test.

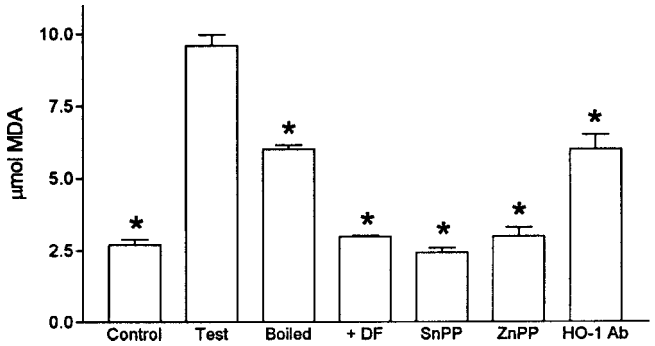

Figure 3 HO-mediated microsomal LPO

Rat liver microsomes were incubated with NADPH, Hb and liposomes at 37 °C; MDA formation was measured after 30 min (test). Tubes without Hb served as a control. Microsomes were boiled for 5 min to denature HO. SnPP and ZnPP, both selective inhibitors of HO, were added as controls. Abbreviation: + DF, microsomes were boiled and then desferrioxamine was added; HO-1 Ab, polyclonal antibody against HO-1.

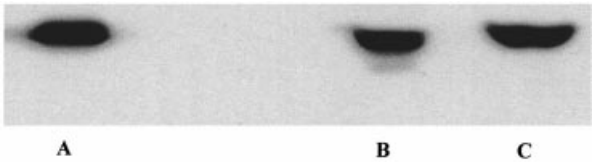

Figure 4 Western blotting of rat liver microsomes

Confirmation of the presence of HO-1 in the rat liver microsome preparation. Normal (lane B) and LPS-treated (lane C) rats are compared, with increased expression in the LPS-treated animals. Pure HO-1 is shown in lane A. The autoradiogram was analysed with an Agfa Studio Scan II and NIH densitometric software.

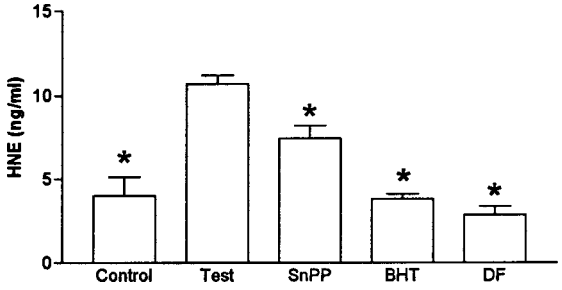

Figure 5 HNE formation

We measured the specific LPO product HNE in our microsomal system by GLC–MS. Rat liver microsomes were incubated with NADPH, Hb and liposomes at 37 °C; HNE formation was measured after 30 min (test). Tubes without Hb served as a control. SnPP was used to inhibit HO; the chelators BHT and desferrioxamine (DF) were used to demonstrate iron-dependent HNE formation.

To establish the role of HO in this iron-catalysed LPO, we compared the levels of MDA formed in test incubations with those of controls containing selective HO inhibitors. Both SnPP and ZnPP significantly inhibited MDA formation (Figure 3). These results indicate that HOs (probably both HO-1 and HO-2) are involved in our LPO reaction and are a likely source of the chelatable iron that significantly increases the pro-oxidant activity of the whole system. It was considered possible that the HO inhibitors were acting as iron chelators and were therefore influencing the results observed. To investigate this we used the deoxyribose assay, which is made dependent on iron-catalysed hydroxyl radical formation [35]. In the presence of iron(II) salts, H_2O_2 is decomposed to form hydroxyl radicals by a Fenton reaction, which degrade 2-deoxyribose and release TBA-reactive material. When iron is rendered redox-inactive by certain chelators, hydroxyl radical damage to deoxyribose is inhibited. It should be noted that many iron chelators can stimulate damage in such a system [37]; however, we were looking only for evidence of inhibition. Because there was no decrease in the amount of deoxyribose damaged when either of the HO inhibitors (results not shown) was added, it is most likely that SnPP and ZnPP act solely by inhibiting HO activities.

Western blots performed on microsomal preparations from normal and LPS-treated rats (Figure 4) confirmed the presence of HO-1. Densitometry of the blots revealed a 21% increase in HO-1 immunoreactivity in microsomes isolated from LPS-treated rats (1049324 attenuance units) compared with normal ones (827130 attenuance units). A 1 ng sample of pure HO-1 served as a positive control (1083214 attenuance units). When a polyclonal antibody to HO-1 was incubated with the microsomes there was a significant decrease in observed LPO, indicating a role for inducible HO in this process (Figure 3). However, inhibition was not brought down to the levels in the control samples without the HO-1 antibody. This finding could be explained by contributions to LPO made by HO-2 and the possibility that antibody binding does not completely block the active site of HO-1.

Boiling microsomes to heat-denature proteins caused a significant decrease in the quantities of MDA formed during LPO, indicating an enzyme-catalysed process rather than a non-specific protein effect. However, levels of MDA formation were still above those seen in the control and seem to be related to the release of catalytic iron by the heat-denaturing process because the iron chelator desferrioxamine completely inhibited LPO (Figure 3).

The use of TBA-reactive substances as a measure of LPO has limitations in biological systems [38]. To corroborate our findings we used a GLC–MS technique to measure HNE (a specific

aldehydic $n-6$ fatty acid oxidation product formed when catalytic iron is present [39]) in a selected set of repeated experiments (Figure 5). HNE levels in test incubations compared with controls, in which the chain-breaking antioxidant BHT, the iron chelator desferrioxamine or a HO inhibitor was included, all showed significant levels of inhibition, supporting our findings with the TBA assay.

The role of redox-active LMrFe iron as a catalyst for peroxidation in this system was demonstrated by measuring bleomycin-detectable iron [34]. A significant increase in bleomycin-detectable iron (a measure of LMrFe) was seen after the incubation of microsomes with Hb and NADPH (1.28 ± 0.14 μmol of bleomycin-detectable iron). The HO inhibitor SnPP significantly decreased this value to 0.42 ± 0.04 μmol bleomycin-detectable iron, whereas boiling microsomes to inactivate HO also caused a significant decrease in the release of LMrFe (0.61 ± 0.09 μmol) after incubation.

HO activity was demonstrated in our microsomes by measuring the formation of bilirubin with an HPLC technique [33]. Bilirubin is formed from biliverdin by the enzyme biliverdin reductase; however, biliverdin peaks were not detected by HPLC, probably reflecting this enzymic process [33]. Bilirubin formation within the microsomal preparations was inhibitable with SnPP when being formed at a rate of 1.03 ± 0.22 μmol/h per mg of protein. No HO activity could be measured in controls (no NADPH) or when incubated with the HO inhibitor SnPP. In our microsomes we detected a formation equilibrium of 1.28 μmol of LMrFe and 1.03 μmol of bilirubin. From model studies described below, such levels of bilirubin are not sufficient to prevent the pro-oxidant effect of LMrFe.

Equimolar concentrations of bilirubin and an iron salt were added to peroxidizing liposomes to assess the pro-oxidant effects of iron and the antioxidant effects of bilirubin. With this system, 10 μmol of bilirubin with equimolar concentrations of iron salt present showed a 147.5% increase in overall LPO from a baseline control. This compared with a 335% increase in LPO when 10 μmol of iron salt was added alone. When pure bilirubin was incubated in the microsomal system, at a concentration of 2.5 μmol/l or higher, bilirubin was seen to act as a successful antioxidant (levels well in excess of measured iron concentrations). However, at 1.25 and 0.63 μmol (to match the levels of LMrFe present) bilirubin did not inhibit HO-dependent LPO. One reason why LMrFe, as a pro-oxidant, could outcompete bilirubin as an antioxidant in our acellular systems might relate to its catalytic recycling properties, whereas bilirubin would be consumed stoichiometrically, thereby limiting its antioxidant capacity.

Our results have so far described the use of an acellular system to explore the pro-oxidant potential of HO. This obviously does not represent the case *in vivo*, in which HO is spatially separated from Hb by a cell membrane. However, recent evidence suggests that Hb can enter aortic endothelial cells [40]; this might be a generalized cellular response [41]. To examine possible pro-oxidant effects of HO under circumstances in which a cell membrane is present, we incubated rat pulmonary artery smooth-muscle cells with Hb and measured LMrFe in the cellular contents. After 24 h of incubation we found a significant increase in LMrFe in cells treated with Hb (0.90 ± 0.22 μmol/l) compared with controls not treated with Hb. A similar increase was found when dialysed Hb was substituted (0.72 ± 0.03 μmol/l). Addition of the HO inhibitor SnPP decreased cellular levels of LMrFe to 0.49 ± 0.13 μmol/l, supporting the pro-oxidant effect of HO through LMrFe in cellular systems. There is, however, a possibility that the release of proteases from dead cells in the medium might contribute to an increased release of LMrFe from Hb. To minimize this, cells were washed thoroughly with Hanks buffer before being lysed. Additionally, our results showed that the HO inhibitor SnPP decreased cellular LMrFe.

LPO is considered a pathological consequence of oxidative stress and cellular injury in many diseases, if not an inevitable result of cell death [42], and liver microsomes have been used as a biological model with which to study LPO for over 35 years. Two types of microsomal LPO have been identified: a non-enzymic process, induced by ascorbate and EDTA-Fe(III), and an enzymic process, which is NADPH-dependent [27–29,43]. NADPH-dependent LPO is catalysed by NADPH:cytochrome P450 reductase. The mechanisms are complex, with some groups evoking the generation of superoxide radicals or singlet oxygen, as key events that ultimately lead to the initiation of LPO through iron-dependent mechanisms [43,44]. Microsomal LPO is known to be greatly enhanced by the addition of chelated iron; Tampo and Yonaha [29] identified a microsomal membrane component involved in iron reduction by co-operation with NADPH:cytochrome P450 reductase. On the basis of our findings it seems likely that both HO-1 and HO-2 are components of the liver microsomal LPO system, being a source of the LMrFe associated with microsomal peroxidation systems. Hb is not normally found in microsomes but other microsome-bound haem proteins, such as cytochrome P450, NADPH:cytochrome *c* reductase and cytochrome b_5, could serve equally well as substrates for the enzyme, particularly because HO is known to use various sources of haem for its enzymic activity.

There is much debate at present concerning the pro-oxidant or antioxidant nature of HO in various settings [2,24–26]. Haem has pro-oxidant properties, so its removal by HO can be considered an important antioxidant effect [45]. However, haem breakdown by HO leads to the formation of low-molecular-mass redox-active iron, which is a more versatile catalyst of oxidative damage than haem [46]. If iron transport and storage proteins do not rapidly and effectively remove this LMrFe, the argument for HO as an antioxidant becomes flawed. Experiments with HO-2 knock-out mice reveal the accumulation of iron in the lung after hyperoxic exposure; this occurs without the induction of ferritin [24]. Indeed, the protective effects attributed to HO might actually reside in the induction of ferritin, rather than in HO-1 itself, as was seen in a rodent rhabdomyolysis model [12]. Similar findings have been confirmed in endothelial cells, where it was the induction of ferritin and not HO up-regulation that protected cells against the toxicity of H_2O_2 [23]. More recently, intratracheal LPS was found to induce ferritin protein rapidly in the rat lung independently of its mRNA synthesis or HO enzyme activity [7]. Additionally, the intratracheal administration of Hb was found to induce lung HO-1 in the rat and to protect against hyperoxia, although inhibitor studies showed that the protection was not mediated by increased HO enzyme activity [47]. Furthermore, HO does not protect MCF-7 cells against menadione-induced oxidant stress [48]. Finally, the antioxidant enzyme peroxiredoxin-1 (HBP-32), a haem-binding protein with thiol peroxidase activity, has been found to be co-expressed with HO-1 [49]. Similarly, metallothionein shows a parallel induction with HO-1 [50], so it is not inconceivable that these inducible proteins contribute to the so-called 'antioxidant' effects of HO-1 in animal experiments.

The findings of this study show that HO contributes to NADPH-dependent LPO in microsomes and that iron generated from Hb, and other haem sources, acts as a catalyst in this process. Further, we have shown that in the microsomal system equimolar levels of bilirubin are not sufficient to outcompete the pro-oxidant effect of iron, for reasons stated above; these findings were corroborated by studies in a pure liposomal system. A pro-

oxidant response has been demonstrated by two different assays of LPO, by HO inhibition studies and by studies involving intact cells. Inducible HO contributes, at least in part, to this oxidative process as demonstrated by HO-1 antibody inhibition studies. The significance of this apparent pro-oxidant pathway has yet to be established, and indeed it could have beneficial functions to the cell through an as yet unidentified iron-signalling mechanism.

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