Iron overload upregulates haem oxygenase 1 in the lung more rapidly than in other tissues

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Abstract Haem oxygenase-1 is upregulated by numerous insults, including oxidative stress, and under such circumstances it is considered to be a protective stratagem. We have measured the haem oxygenase-1 expression in heart, lung and liver tissues of control and iron-overloaded rats. Lung tissue from iron-overloaded rats displayed a significant increase in the haem oxygenase-1 protein but no changes in haem oxygenase-1 mRNA. Conversely, heart tissue showed a significant increase in haem oxygenase-1 mRNA but no changes in haem oxygenase-1 protein. We conclude that during oxidative stress caused by iron overload, lung tissue responds with a rapid upregulation of haem oxygenase-1 levels.

Key words: Haem oxygenase; Oxidant damage; Iron

1. Introduction

The lungs are particularly sensitive to oxidant damage caused by inhaled toxins and by activated phagocytic and inflammatory cells within the lungs [1]. The respiratory tract lining fluid contains several scavenging antioxidants, such as mucin, uric acid, glutathione and ascorbate, as well as the enzyme superoxide dismutase [2]. The protective function of iron-binding and iron-oxidising proteins, however, in preventing lung damage is less clear, since their concentration only greatly increases when there is lung damage and leak of plasma into the lung lining fluid [3]. In order to better understand relationships between constitutive antioxidants described above, and inducible antioxidants produced in response to oxidative stress, we have followed the upregulation of haem oxygenase-1 (HO-1) in rat lung tissue after an insult with an iron salt sufficient to cause iron overload.

2. Materials and methods

2.1. Animals and anaesthesia

All procedures and protocols were performed in accordance with the Animal (Scientific Procedures) Act, 1986 and approved by the Home Office (UK) Inspectorate. Male Wistar rats (275–300 g) were anaesthetised with pentobarbitone (60 mg/kg i.p.) and placed supine on an electric heating pad, maintaining the body temperature at 37°C. A tracheotomy was performed and the animals were ventilated using a mixture of 21% O_2, 5% CO_2, balanced with N_2 using a volume-cycled, small animal ventilator (Harvard Apparatus, Chatham, Kent, UK). A cannula was inserted into the left femoral artery and attached to a pressure transducer (Hewlett-Packard, Bracknell, UK) for continuous monitoring the mean arterial blood pressure and the withdrawal of blood samples. A 1Fr-thermistor microprobe (Columbus Instruments, OH, USA) was inserted into the aortic arch via the right carotid artery. A cannula was inserted into the right atrium via the right jugular vein and attached to a microinjector (Columbus Instruments) allowing 150 μl injection of room temperature saline. The thermistor cardiac output was measured (Cardiotherm 500 AC-R cardiac output computer, Columbus Instruments, OH, USA) and the mean of two consecutive measurements within 10% of each other were recorded. Intermittent boluses of i.p. pentobarbitone were given to supplement anaesthesia as required.

After cannulation was completed, the continuous measurement of mean arterial blood pressure was performed. Cardiac output measurements were measured at t = 120, 150, 180, 210 and 240 min after administration of ferric chloride or saline. At t = 120 and t = 240 min, a 0.75 ml blood sample was taken into a lithium heparin tube. At t = 240 min, the animal was killed and the heart, lungs and liver were harvested for RT-PCR and Western blotting.

Animals were divided at random into the following groups: Sham-treated animals (group 1, $n = 4$) given 0.75 ml saline and iron-treated animals (group 2, $n = 4$), given ferric chloride (0.75 ml i.v. bolus of 1 mM $FeCl_3$, pH 3.0) applied at t = 120 min.

Initially we attempted to prepare the ferric chloride solution in pH 7.4 buffered saline but this resulted in precipitation of the iron. We therefore decided to use a non buffered $FeCl_3$ solution in our experiments. Analysis of blood with a blood gas analyser (Ciba-Corning) prior to $FeCl_3$ treatment and at 5 min, 30 min and 1 h after $FeCl_3$ administration showed no differences in the blood pH, values which remained unaltered at pH 7.3.

2.2. Bleomycin detectable iron

Iron chelatable to bleomycin was determined as previously described [4]. Briefly, the reaction mixture contained DNA, bleomycin and the plasma sample buffered to pH 7.4 with a Tris salt. In the presence of added ascorbate, iron chelated from the plasma sample by bleomycin degraded DNA with the release of malondialdehyde from its deoxyribose moiety. Iron responsible for DNA degradation was quantitated with a reference to ferric chloride standards.

2.3. Total plasma iron and iron-binding capacity

Total plasma iron and iron-binding capacity were determined using a Sigma kit assay based on the ferrozine spectrophotometric technique. The percentage saturation of transferrin was derived from the measured total iron-binding capacity.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

Tissues were homogenised and RNA extracted using a Tri reagent kit (Sigma Chemical Co.). Single stranded cDNA was synthesised from 600 ng of total RNA with the aid of Moloney Murine Leukemia Virus Reverse Transcriptase (Life Technologies) and Oligo $(dT)_{12-18}$ primers (Life Technologies).

Oligo primers for PCR amplification were chosen from homologous parts of the encoding region of the rat gene haem oxygenase-1 [5]. Double stranded cDNAs were synthesised and amplified by use of Taq DNA polymerase (Life Technologies). Amplification was performed in a DNA thermal cycler at 94, 60 and 72°C for 30 s, 1 min and 1 min respectively for a total of 26 cycles. Semi-quantitative analyses were performed with reference to β-actin, which was selected for use as a housekeeping gene. PCR fragments were analysed by gel electrophoresis followed by ethidium bromide staining. Gels were photographed and analysed by densitometry.

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2.5. SDS-PAGE and Western blotting analysis

Liver, lung and heart tissue samples (all $n=4$) from the control (saline-treated) and iron-treated groups were homogenised with 0.25 M sucrose/0.01 M Tris buffer pH 7.4 and centrifuged at $10\,000\times g$ for 20 min. Supernatants of equal protein content were subjected to electrophoresis in 14% w/v SDS-PAGE gels, with 10% w/v stacking gels. The gels were transferred onto Hybond-C nitrocellulose membranes (Amersham, UK). After blocking with PBS-Tween containing 5% w/v non-fat milk, the blots were probed by a polyclonal antibody against HO-1 and then incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich, UK). After washing, the membranes were treated with enhanced chemiluminescence reagent (ECL, Amersham, UK) to reveal immunoreactive proteins. Mean results are expressed as densitometric readings, normalised to a pure HO-1 standard run on each gel.

2.6. HO activity

To assess whether the increased formation of HO-1 protein in lung tissue correlated with the increased enzyme activity, we measured the total HO enzyme activity using the following technique: the HO activity was measured in lung tissue from rats treated with the iron salt and compared with control rats. The method of Ryter and co-workers [6] was used, with the following modifications: reactions were incubated for 30 min instead of 1 h and solvent A was changed to pH 4.5 to improve the peak separation. Bilirubin peaks were found to be an inconsistent measure of HO activity and so we measured biliverdin, the primary product of HO activity. The HO activity was expressed as nmol biliverdin/mg protein/h, corrected to an internal standard of mesoporphyrin.

2.7. Data analysis

All results are expressed as mean ± S.E.M. Differences between individual group means were tested using the one way, non-parametric Mann Whitney test. P values less than 0.05 were considered significant.

3. Results

3.1. Cardiovascular changes

Infusion of iron into control animals induced a transient increase in the mean arterial blood pressure (peak increase 147 ± 5.2 versus 115 ± 6.9 mm Hg at 30 min, $P<0.05$) which returned to baseline by the end of the experiment. The cardiac output was unchanged throughout (data not shown).

3.2. Iron-overload status of rats

Rats were shown to be in a state of iron overload after the administration of ferric chloride, by measuring bleomycin detectable iron in the plasma. No bleomycin detectable iron was present in rat plasma before iron administration, however, bleomycin detectable iron was present after administration (0 μM versus 19.5 ± 1.73 μM, $P<0.001$). Further, the iron saturation of transferrin was found to be 100% in iron-overloaded rats, but only 55.0 ± 5.48% pre iron administration.

3.3. Message upregulation

HO-1 mRNA was detectable in all samples studied and there was a significant upregulation in the hearts of rats after iron treatment. In lung samples, however, there was no significant increase in HO-1 mRNA during iron overload (see Fig. 1). Levels of HO-1 mRNA in the liver were also unchanged (see Fig. 1)

HO-1 protein was detectable in all tissues examined, both before and after iron administration. In the heart, basal levels of HO-1 protein were elevated compared with levels in the liver. Treatment with iron did not significantly increase HO-1 protein levels in either tissue. However, in the lung there was a significant ($P<0.05$) increase in HO-1 protein during iron overload (see Fig. 2).

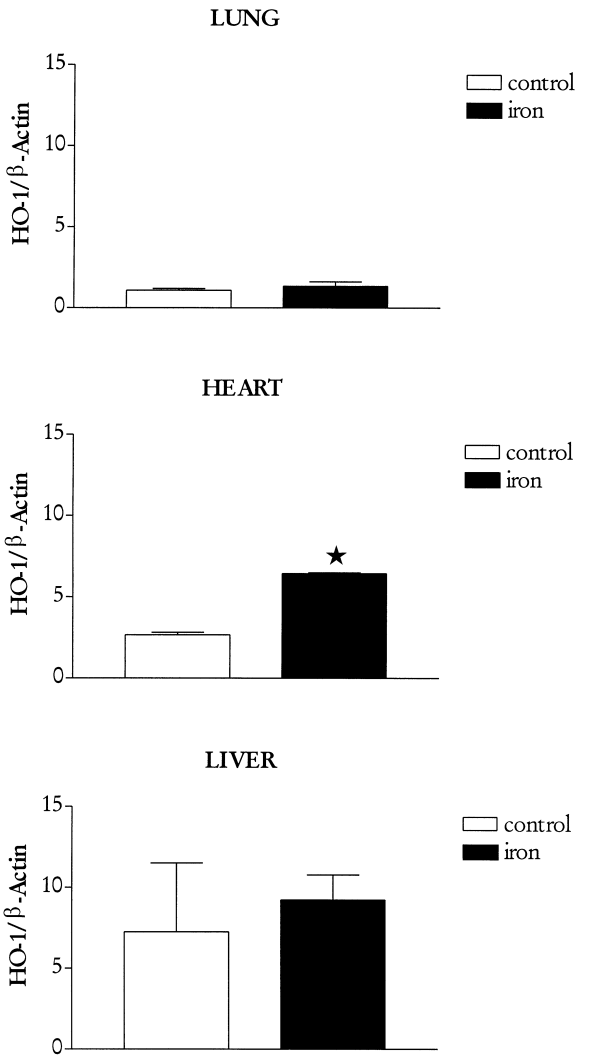

Fig. 1. Graphs showing haem oxygenase-1 mRNA, expressed as densitometric ratios of HO-1 to β actin in control and iron-overloaded rats from lung, heart and liver, $^*P<0.05$.

3.4. HO enzyme activity

An increased HO activity was observed in the lungs of iron-overloaded rats compared to controls although this was not quite significant (292.7 ± 101.9 versus 78.2 ± 62.4 nmol biliverdin/mg protein/h respectively, both $n=4$, $P=0.056$).

4. Discussion

The HO family of enzymes is responsible for the catabolic decomposition of haemoglobin (haem) to biliverdin, carbon monoxide and free iron. Three isomers of the enzyme have been isolated, namely the inducible isoform HO-1, the constitutive isoform HO-2 [7] and the recently discovered isoform HO-3 [8]. HO-1 is also a stress protein, often referred to as heat shock protein 32. Its transcription can be induced by an array of stresses including endotoxin [9], heavy metals [10,11], transition metal ions [12], haemoglobin and other haem proteins [13,14]. Oxidative stress leads to HO-1 induction [15] and

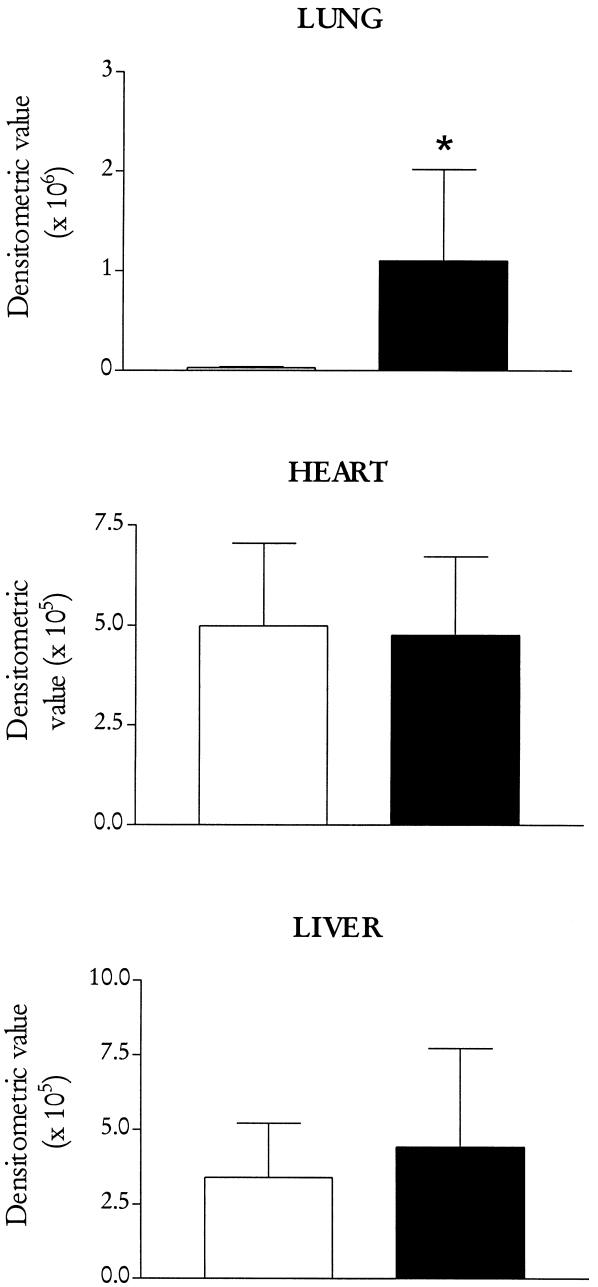

Fig. 2. Graphs showing haem oxygenase-1 protein, expressed as densitometric values in control and iron-overloaded rats from lung, heart and liver. Results were normalised to an HO-1 positive control run on each gel, $*P < 0.05$.

under such circumstances it is considered to be protective against oxidative damage [16]. It has been suggested that this is due to the antioxidant properties of bilirubin [17] and an increased ferritin synthesis [18]. There is, however, some debate over whether HO-1 induction can solely be considered protective, since it has recently been found that ferritin synthesis occurs before HO-1 induction in the lungs of rats exposed to hyperoxia or haemoglobin [19]. In addition, lungs from HO-2 knockout mice were found to accumulate redox active iron and to be susceptible to oxidative damage during hyperoxia despite HO-1 induction [20]. It is more likely that the levels of HO-1 produced and the circumstances in which it is induced may ultimately determine whether the effects of HO-1 induction are beneficial or adverse. Mechanisms of cellular upregulation of HO-1 by extracellularly administered haemoglobin remains unclear since it cannot directly enter cells. It is therefore possible that low molecular mass iron derived from haemoglobin may provide a key signal for HO-1 induction under such circumstances through the constitutive form. Indeed, iron has been shown to induce HO-1 in cells in culture [21,22] and in other animal models of iron overload [23]. Furthermore, it has been shown that methaemoglobin, but not oxyhaemoglobin, can release the hydrophobic iron-containing haem moiety, which is taken up by endothelial cells in vitro, perhaps providing a mechanism by which haemoglobin-derived iron can enter cells [24].

To support this proposal, we observed a significant upregulation of HO-1 message in heart tissue of iron-overloaded rats. In contrast, however, we found no change in HO-1 message in the lungs. Paradoxically, a significant increase in HO-1 protein levels was observed in lung tissue of iron-overloaded rats whereas no changes in HO-1 protein were observed in heart tissue. Moreover, the increase in HO enzyme activity further supported our observations that active HO-1 protein is upregulated in the lung. Failure to increase HO-1 protein levels in heart tissue, even though mRNA levels were significantly increased, most probably reflects the delay between message formation and protein synthesis. There were also no significant differences in HO-1 mRNA or HO-1 protein levels in livers of control or iron-overloaded rats, probably reflecting the high basal levels of HO-1 normally found in this organ. Our results indicate that lung tissue of rats responds to oxidative stress caused by iron overload with a rapid upregulation of HO-1 levels. This early increase in HO-1 levels may be of considerable importance to the lung, either as a protective antioxidant stratagem or as a deleterious event producing high levels of low molecular mass iron.

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