

RESEARCH PAPER

Haem oxygenase-1 induction protects against tumour necrosis factor α impairment of endothelial-dependent relaxation in rat isolated pulmonary arteryHany M. El-Bassossy^{1,2}, Nabila N. El-Maraghy², Hassan M. El-Fayoumi² and Malcolm L. Watson¹¹Department of Pharmacy and Pharmacology, University of Bath, UK, and ²Department of Pharmacology, Faculty of Pharmacy, University of Zagazig, Egypt

Background and purpose: Disturbances in pulmonary vascular reactivity are important components of inflammatory lung disease. Haem oxygenase-1 (HO-1) is an important homeostatic enzyme upregulated in inflammation. Here we have investigated the potentially protective effect of HO-1 against cytokine-induced impairment in pulmonary artery relaxation.

Experimental approach: Haem oxygenase-1 protein levels were assessed by immunofluorescence. HO activity was assessed by conversion of haemin to bilirubin. Rings of rat isolated pulmonary artery in organ baths were used to measure relaxant responses to the endothelium-dependent agent ACh and the endothelium-independent agent sodium nitroprusside (SNP). Production of nitric oxide (NO) and reactive oxygen species (ROS) was assessed by confocal fluorescence microscopy and fluorescent probes.

Key results: Haem oxygenase-1 protein expression was strongly induced in pulmonary artery after 24-h incubation with either haemin (5 μ M) or curcumin (2 μ M), accompanied by a significant increase in HO activity. Incubation with tumour necrosis factor α (TNF α , 1 ng·mL⁻¹, 2 h) significantly decreased relaxation of arterial rings to ACh, without affecting responses to SNP. Induction of HO-1 by curcumin or haemin protected against TNF α -induced hyporesponsiveness to ACh. The competitive HO inhibitor, tin protoporphyrin (20 μ M), abolished the protective effect of haemin. HO-1 induction prevented a TNF α -induced increase in NO generation without affecting the TNF α -induced increase in ROS generation. HO-1 induction prevented the TNF α -induced decrease in ACh-stimulated NO generation.

Conclusions and implications: Induction of HO-1 protected against TNF α impairment of endothelium-dependent relaxation in pulmonary artery, by a mechanism involving a reduction in inducible NO synthase-derived NO production.

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Keywords: pulmonary artery; endothelial-dependent relaxation; haem oxygenase; cytokines; tumour necrosis factor; immunofluorescence; immunoblotting; nitric oxide

Abbreviations: 1400 W, N[[3(aminomethyl)phenyl]methyl]ethanimidamide dihydrochloride; BH₄, tetrahydrobiopterin; DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; DHE, dihydroethidium; eNOS, endothelial nitric oxide synthase; HO-1, haem oxygenase-1; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; SHR, spontaneously hypertensive rat; SNP, sodium nitroprusside; SnPP, tin protoporphyrin; SOD, superoxide dismutase; TNF α , tumour necrosis factor α

Introduction

Vascular endothelial cells have an essential role in the control of the human pulmonary circulation. Endothelial cells release

potent vasodilators that regulate the vascular reactivity to various stimuli (Hida *et al.*, 2002). Many studies have implicated endothelial dysfunction in the development of chronic obstructive pulmonary disease (Peinado *et al.*, 1998), a disease associated with local and systemic inflammation (Wouters, 2005). Lung inflammation was found to induce pulmonary vascular hyporesponsiveness to vasodilators (Sheridan *et al.*, 1996). Tumour necrosis factor (TNF α) is a major cytokine that is elevated in many diseases and seems to be involved in the

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associated disturbances in vascular reactivity (Vila and Salaices, 2005).

Some of the proinflammatory actions of cytokines are mediated by inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO) production. Three isoforms of NOS have been described: constitutive-type isoforms like neuronal NOS and endothelial NOS (eNOS), and the inducible type of the enzyme (see Ricciardolo *et al.*, 2004). Also, overproduction of reactive oxygen species (ROS) under pathophysiological conditions is believed to be integral to the development of cardiovascular diseases (see Madamanchi *et al.*, 2005). One of the most important ROS in the vasculature is superoxide, which is formed by the univalent reduction of oxygen (Droge, 2002). Although superoxide can itself exert effects on vascular function, it is also pivotal in generating other reactive species. Reaction of superoxide with NO generates peroxynitrite, a potentially deleterious ROS. Dismutation of superoxide by superoxide dismutase (SOD) produces the more stable ROS, hydrogen peroxide. H₂O₂ can also react with reduced transition metals to be converted to the highly reactive hydroxyl radical, or it can be metabolized by myeloperoxidase to form hypochlorous acid. Virtually all types of vascular cells produce superoxide and H₂O₂ (Taniyama and Griendling, 2003).

Of the three known isoforms of the enzyme haem oxygenase (HO), HO-1 is inducible. HO-1 is highly expressed in erythropoietic tissues, where its function is haem degradation, but it is also expressed in vascular smooth muscle cells, where its role may include regulation of vascular tone (Soares and Bach, 2009). Strong expression of HO-1 has been associated with a major anti-inflammatory response and, as it is induced in most organs in the body, it has been implicated in protection against several disease states (reviewed by Ryter *et al.*, 2006). HO is the rate-limiting enzyme in the degradation of haem. In breaking down haem, HO releases iron, biliverdin (which is subsequently converted to bilirubin) and carbon monoxide (CO). Each of these products of this enzymic activity plays unique and often protective roles in the human body (Morse and Choi, 2005).

The present work aimed to investigate the potentially protective effect of HO-1 against TNF α -induced impairment of relaxation in isolated pulmonary arteries.

Methods

Animals and tissues

All animals were housed and maintained in compliance with the University of Bath and the UK Home Office Guidelines and Standards of Care. Male Wistar rats (270–290 g, University of Bath) were killed by cervical dislocation and the lungs and the heart were rapidly isolated. The pulmonary artery branches were dissected and 2–3 mm rings from each animal were divided between the different treatment groups. The isolated tissue was then incubated for 24 h with or without haemin or curcumin in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum, penicillin/streptomycin (100 iu·mL⁻¹/100 μ g·mL⁻¹) and HEPES buffer (25 mM) at 37°C in a humidified 5% CO₂ atmosphere. For assessment of HO-1 induction,

haemin or curcumin was added at different times before the end of the 24-h incubation period.

HO-1 immunofluorescence

Immunofluorescence was assessed as previously described (Hemmings *et al.*, 2004) after optimization for HO-1 detection. Briefly, at the end of 24-h incubation, pulmonary artery rings were embedded in OCT compound (Raymond A Lamb, East Sussex, UK) and snap-frozen in liquid nitrogen. The frozen sections were cut on a cryostat and consecutive 7 μ m sections adhered to Superfrost Plus glass slides (VWR, Leics., UK) and then fixed with 4% paraformaldehyde for 40 min. Perforation was carried out by incubation with cold methanol for 5 min at –20°C. Non-specific binding was blocked by incubating the sections with 70 μ L of fluorescence-enhanced blocking buffer (Molecular Probes, Paisley, UK) for 30 min. The sections were then incubated with 70 μ L of the rabbit anti-HO-1 primary antibody (dilution 1:200 in 0.2% BSA; StressGen, Ann Arbor, MI, USA) for 1 h at room temperature followed by incubation with 70 μ L of the Alexa fluor conjugated goat anti-rabbit secondary antibody (dilution 1:1000 in 0.2% BSA; Molecular Probes, Paisley, UK) for 1 h in the dark. A coverslip was mounted with 20 μ L of fluorescence mounting media (Molecular Probes, Paisley, UK) and slides were left in dark for 20 h before examination by Zeiss LSM510 confocal microscope (Carl Zeiss, Göttingen, Germany) with argon laser excitation at 488 nm and a 505 nm longpass emission filter. Sections treated with the secondary antibody alone did not show specific staining.

HO activity assay

Activity of HO was assessed by conversion of haemin to bilirubin as previously described (McNally *et al.*, 2004) with slight modifications. After 24-h incubation, chopped lung tissues were homogenized on ice with 0.1 M phosphate buffer supplemented with 2 mM MgCl₂ and the homogenate centrifuged at 1000 \times g for 10 min at 4°C. The supernatant was added to 400 μ L reaction mixtures containing 1 mg of rat liver cytosol, 50 mM haemin, 2 mM glucose 6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase and 0.8 mM NADPH and incubated for 1 h at 37°C in the dark. Liver cytosol was prepared by homogenizing a rat liver in one volume of 20 mM Tris HCl buffer plus 154 mM KCl, pH 7.4. The homogenate was centrifuged at 10 000 \times g for 20 min 4°C, and the supernatant was further centrifuged at 100 000 \times g for 1 h at 4°C. The formed bilirubin was extracted with chloroform and the absorption measured as the difference between 464 and 530 nm (extinction coefficient, 40 mM⁻¹·cm⁻¹ for bilirubin). HO activity is expressed as pmol of bilirubin formed per mg protein per h.

Pulmonary artery relaxation

At the end of 24-h incubation, pulmonary artery rings were suspended under 8 mN resting tension in individual 21 mL organ chambers containing Krebs–Henseleit buffer (containing in mM: NaCl 118.1, KCl 4.69, KH₂PO₄ 1.2, NaHCO₃ 25.0, glucose 11.7, MgSO₄ 0.5, CaCl₂ 2.5) at 37°C and aerated with

95% oxygen, 5% carbon dioxide. Ring tension was determined by use of an isometric force transducer (K30, Hugobachs Elektronik, March, Germany). Force displacement was recorded with a PowerLab Data Interface Module connected to a PC running Chart software (v4.2, ADInstruments, Oxon, UK). Rings were equilibrated for 60 min, during which time the bath solution was changed every 15 min. Before beginning the studies, vessel viability was assessed by exposing arteries to KCl (80 mM). This was repeated until stable responses were achieved (usually two exposures). Rings were then precontracted with a submaximal phenylephrine concentration (3×10^{-7} M, as determined in preliminary experiments). The contraction was allowed to reach plateau and then cumulative concentrations of ACh or sodium nitroprusside (SNP) (10^{-8} M to 10^{-5} M) were tested. After washing, rings were then incubated with or without rat TNF α ($1 \text{ ng}\cdot\text{mL}^{-1}$) in the organ bath for 2 h before repeating the cumulative response curve to ACh. In some experiments, tetrahydrobiopterin (BH $_4$) ($3 \mu\text{M}$), N[[3(aminomethyl)phenyl]methyl]ethanimidamide dihydrochloride (1400 W) ($1 \mu\text{M}$) or tempol (1 mM) were added for the final 30 min of the TNF α treatment period. The change in E_{max} and EC_{50} in each tissue because of the 2-h incubation was used to compare the effect of treatments.

Determination of intracellular NO and ROS levels

The intracellular levels of NO and ROS were determined by the fluorescent probe 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) and dihydroethidium (DHE) (Molecular Probes, Paisley, UK) according to the method described by Somers *et al.* (2000). DAF-FM is virtually nonfluorescent until it reacts with NO to form a fluorescent benzotriazole, while ROS oxidize DHE forming ethidium which forms a highly fluorescent complex with DNA. After 24-h incubation, artery rings were transferred into fresh phenol red-free DMEM media and incubated with or without rat recombinant TNF α ($1 \text{ ng}\cdot\text{mL}^{-1}$). Rings were then embedded in OCT Compound and snap-frozen in liquid nitrogen. The frozen sections were cut on a cryostat in consecutive $30 \mu\text{m}$ sections and adhered to a Superfrost Plus glass slide (VWR, Leics., UK). The cryostat sections were then allowed to thaw at room temperature and double loaded for 40 min at room temperature with $5 \mu\text{M}$ of both DAF-FM and DHE in Krebs–Henseleit buffer containing 25 mM HEPES and 0.1% Pluronic F-127 (Molecular Probes, Paisley, UK). Segments were then washed twice with Krebs–Henseleit buffer, covered with a coverslip and immediately examined by Zeiss LSM510 confocal microscope (Carl Zeiss, Göttingen, Germany) with excitation at 488 nm and a 505–530 nm or 590–615 nm bandpass emission filter systems.

Fluorescence measurements from confocal images

Images were acquired with identical acquisition parameters, with minimum gain to avoid interference by tissue autofluorescence. Quantitative comparisons of fluorescence were made with Image J software (National Institutes of Health, Bethesda, MD, USA). For printing purposes, the contrast and the brightness of the confocal pictures were adjusted equally

after the fluorescence measurements were carried out on unmanipulated pictures.

Real-time measurement of intracellular NO and ROS generation

The real time generation of intracellular NO and ROS were investigated with the fluorescence probes DAF-FM diacetate (Itoh *et al.*, 2000) and 2',7'-dichlorodihydrofluorescein diacetate (H $_2$ DCFDA, Szabados *et al.*, 1999) respectively. Briefly, at the end of 24-h incubation, artery segments were fixed with stainless steel pins in a 96 black multiwell plate coated with 2 mm thickness layer of Sylgard 184 (Dow Corning, MI, USA). The segments were then loaded for 40 min at room temperature with $5 \mu\text{M}$ of either DAF-FM diacetate or H $_2$ DCFDA plus 0.1% Pluronic F-127 in Krebs–Henseleit buffer containing 25 mM HEPES. Segments were then washed twice and incubated with $200 \mu\text{L}$ of Krebs–Henseleit buffer containing HEPES for 30 min at 37°C before addition of TNF α ($1 \text{ ng}\cdot\text{mL}^{-1}$). Readings ($\lambda_{\text{ex}} = 490 \text{ nm}$ and $\lambda_{\text{em}} = 510 - 570 \text{ nm}$) were taken before and every 15 min after cytokine addition in a Fluoroskan II plate reader (ICN-Flow Laboratories, High Wycombe, Oxon, UK). For studying the formation of intracellular nitric oxide because of ACh stimulation, cumulative ACh concentrations (10^{-6} to 10^{-4} M) were added after 2-h incubation with TNF α . Readings were taken before and 3 min after each addition of ACh. The NO donor diethylamine NONOate (100 nM) and the ROS stimulus ATP (3 mM) were used as a positive control for DAF and DCF respectively.

Statistical analysis

All data are expressed as mean \pm SEM. Statistical analysis was performed by analysis of variance followed by Newman–Keuls' *post hoc* test. Sigmoidal curve fitting was applied using 3-parameter non-linear regression using Prism 4 software (Graphpad, La Jolla, CA, USA) on a PC.

Materials

The following chemicals were used: rat recombinant TNF α (Peprotec, London, UK), haemin and tin protoporphyrin (SnPP, Frontier, Aberdeen, UK), PE, ACh, BH $_4$, tempol and curcumin (Sigma-Aldrich, Poole, Dorset, UK), 1400 W (Tocris, Bristol, UK). 1400 W, BH $_4$, tempol PE, ACh, ATP and diethylamine NONOate were dissolved in distilled water. Haemin, curcumin and SnPP were freshly dissolved in 0.5 N NaOH in dark and quickly diluted 200-fold with phosphate buffer saline before adding to the media to reach the required concentration.

Results

Assessment of HO-1 induction and inhibition

Haemin ($5 \mu\text{M}$) and curcumin ($2 \mu\text{M}$) were able to induce HO-1 protein in pulmonary artery segments (Figure 1) with a significant increases from control values of 3.03 ± 0.03 to 12.38 ± 2.04 ($P < 0.01$, $n = 3$) and 7.08 ± 0.55 fluorescence units per pixel at 24 h ($P < 0.05$, $n = 3$) for haemin and

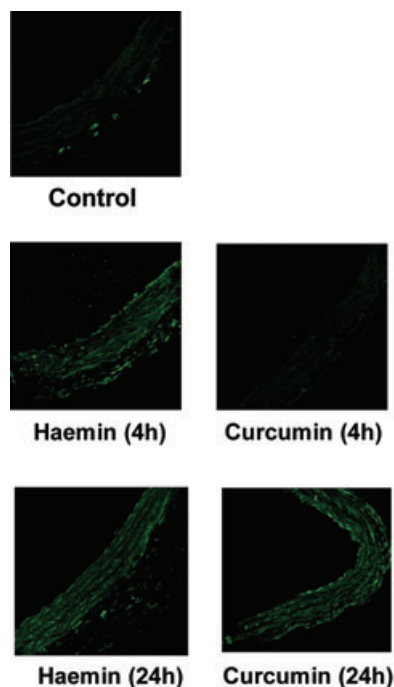


Figure 1 Effect of *in vitro* incubation with haemin (5 μ M) or curcumin (2 μ M) on haem oxygenase-1 protein expression after 4- or 24-h incubation, as indicated on the figure, in isolated rat pulmonary artery cryostat sections. Micrographs (325 \times 325 μ m frame) are representative of data from three independent experiments each using tissue from different animals.

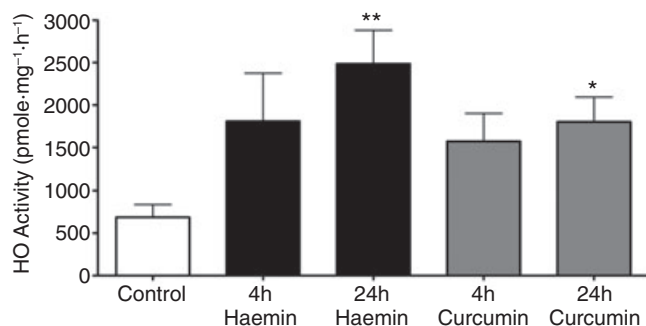


Figure 2 Induction of haem oxygenase activity in rat lung tissue incubated with haemin (5 μ M) or curcumin (2 μ M). Bars indicate mean \pm SEM for $n = 3$ –4 independent determinations. * $P < 0.05$ and ** $P < 0.01$; significantly different from control. HO, haem oxygenase.

curcumin-treated tissues respectively. The induction of protein expression was accompanied by an increase in HO enzyme activity, which was apparently increased at 4 h and significantly increased after 24-h incubation with haemin (5 μ M, $P < 0.01$) or curcumin (2 μ M, $P < 0.05$; Figure 2). The induction of HO-1 by haemin and curcumin over this time-course was confirmed by Western blotting (data not shown). The ability of SnPP to inhibit HO was confirmed by incubation of spleen tissue lysate with 20 μ M SnPP, which inhibited the HO enzyme activity from 1958 ± 150 to 1083 ± 83 pmole (mg spleen tissue)⁻¹·h⁻¹ in the absence or presence of SnPP respectively.

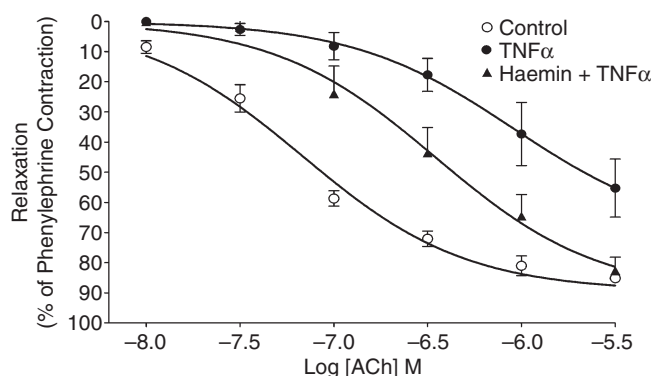


Figure 3 ACh-induced relaxation of phenylephrine-precontracted pulmonary artery rings after 2-h incubation with Krebs–Henseleit buffer (Control), TNF α (1 ng·mL⁻¹), or TNF α (1 ng·mL⁻¹) after 24 h treatment with haemin (5 μ M). Symbols indicate mean \pm SEM for $n = 8$ –9 tissues from different animals for each treatment group. TNF α , tumour necrosis factor α .

Pulmonary artery relaxation

Addition of cumulative concentrations of ACh to the organ bath resulted in concentration-related decreases in the tension of pulmonary artery rings precontracted with phenylephrine (Figure 3). Incubation of tissues with TNF α (1 ng·mL⁻¹, 2 h) or haemin (5 μ M, 24 h) had no effect on the basal tension (data not shown) and similarly these treatments had no significant effect on the contractile response to the submaximal concentration (3×10^{-7} M) of phenylephrine (2.25 ± 0.16 mN and 1.85 ± 0.25 mN compared with 2.28 ± 0.18 mN in controls respectively, $n = 7$ –13). In contrast, incubation with TNF α (1 ng·mL⁻¹; 2 h) resulted in a large reduction in pulmonary artery responsiveness to ACh, reflected by significant changes in apparent E_{\max} ($P < 0.001$) and pEC_{50} ($P < 0.001$) (Figure 3, Table 1).

The TNF α -induced hyporesponsiveness to ACh was inhibited by preincubation with haemin (5 μ M, 24 h; Figure 3 and Table 1). Similarly, HO-1 induction by curcumin (2 μ M) was also able to inhibit TNF α -induced hyporesponsiveness significantly (Table 1). Conversely, co-incubation with the HO-1 inhibitor SnPP (20 μ M) blocked the protective effect of haemin against the TNF α -induced ACh hyporesponsiveness (Table 1). When added to the organ bath 30 min before the second cumulative ACh response curve, the selective iNOS inhibitor 1400 W (1 μ M), the SOD mimetic tempol (1 mM) or the NOS cofactor BH₄ (3 μ M) inhibited TNF α -induced hyporesponsiveness (Table 1). In the absence of TNF α treatment, incubation of tissues with haemin, curcumin, SnPP (20 μ M, 24 h), 1400 W (1 μ M, 30 min), tempol (1 mM, 30 min) or BH₄ (3 μ M, 30 min) had no effect on the response of the pulmonary artery to cumulative concentrations (3×10^{-8} – 3×10^{-6} M) of ACh. Also, TNF α incubation did not affect the sensitivity of pulmonary artery to SNP (data not shown).

Determination of intracellular NO level and ROS levels

Incubation of pulmonary arterial rings with TNF α (1 ng·mL⁻¹, 2 h) was able to increase the intracellular level of NO in cryostat sections from control by about threefold (Figure 4). In the same sections, ROS levels were also increased, to about

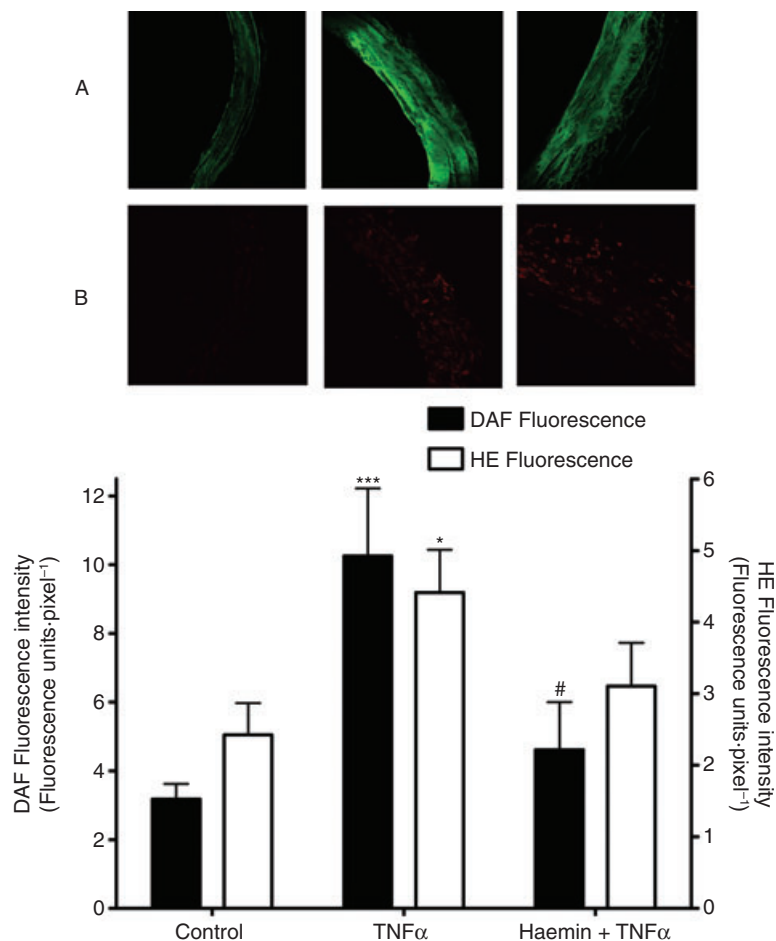


Figure 4 Effect of preincubation with haemin (5 μ M, 24 h) on TNF α (1 ng·mL⁻¹, 2 h) induced increases in A. intracellular NO and B. ROS levels. Sections are shown with the lumen on the left side of the frames. HE is oxidized by intracellular ROS forming a fluorescent complex with the DNA in the nuclei while DAF fluorescent cells were distributed throughout the endothelial and smooth muscle layers of the tissue sections. Bars indicate mean \pm SEM for $n = 3$ –5 tissues from different animals. * $P < 0.05$, *** $P < 0.001$; significantly different from control, # $P < 0.05$; significantly different from TNF α -treated tissue. DAF, 4-amino-5-methylamino-2',7'-difluorofluorescein; HE, hydroethidium; NO, nitric oxide; ROS, reactive oxygen species; TNF α , tumour necrosis factor α .

Table 1 Effect of haemin (5 μ M, 24 h), SnPP (20 μ M, 24 h), 1400 W (1 μ M, 30 min), tempol (1 mM, 30 min) or BH₄ (3 μ M, 30 min) on ACh Emax and pEC50 in TNF α -treated isolated rat pulmonary artery rings

Treatment	Δ Emax (% of PE contraction)	Δ pEC50 (log M)
Control	2.6 \pm 4.3	0.35 \pm 0.07
TNF α (1 ng·mL ⁻¹ , 2 h)	34.4 \pm 6.1***	1.03 \pm 0.17***
TNF α + curcumin	18.1 \pm 3.0†	0.35 \pm 0.07††
TNF α + haemin	9.7 \pm 2.1†††	0.63 \pm 0.17††
TNF α + haemin + SnPP	28.8 \pm 3.2***	0.45 \pm 0.11†
TNF α + 1400 W	17.3 \pm 2.7†	0.45 \pm 0.06††
TNF α + tempol	5.1 \pm 3.7†††	0.50 \pm 0.11†
TNF α + BH ₄	7.2 \pm 5.0†††	0.51 \pm 0.04†

Values are expressed as the mean \pm s.e mean change (Δ) compared with initial ACh curves prior to 2-h control or TNF α incubation in tissues from $n = 5$ –8 different animals.

*** $P < 0.001$, ** $P < 0.01$, compared with control tissue responsiveness.

† $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$; compared with tissues treated with TNF α alone.

$P < 0.05$ compared with tissues treated with TNF α plus haemin; by Student–Newman–Keuls test.

1400 W, N[[3(aminomethyl)phenyl]methyl]ethanimidamide dihydrochloride; BH₄, tetrahydrobiopterin; SnPP, tin protoporphyrin; TNF α , tumour necrosis factor α .

double control values (Figure 4). Induction of HO-1, by preincubation with haemin (5 μ M, 24 h), totally prevented this TNF α -induced increase in intracellular NO ($P < 0.05$; Figure 4) but did not have a significant effect on the TNF α -induced increase in intracellular ROS.

Real-time measurement of intracellular NO generation

Tumour necrosis factor α (1 ng·mL⁻¹) significantly increased NO generation in pulmonary artery segments (Figure 5; $P < 0.001$), and this increase was completely blocked by either 1400 W (1 μ M, 30 min; $P < 0.001$) or HO-1 induction by haemin (5 μ M, 24 h, $P < 0.001$). In addition, TNF α significantly increased the ROS generation in the pulmonary artery segments (Figure 5; $P < 0.001$), but HO-1 induction by haemin had no effect on this TNF α -induced increase in ROS generation.

Although TNF α induced NO generation (above), it inhibited NO generation stimulated by 3-min exposure to 10⁻⁴ M ACh (Figure 6; $P < 0.001$). 1400 W (1 μ M, 30 min, $P < 0.05$), BH₄ (3 μ M, 30 min, $P < 0.01$) or HO-1 induction by haemin (5 μ M, 24 h, $P < 0.05$) was able to prevent this inhibition (Figure 6). However, tempol (1 mM, 30 min) did not have a

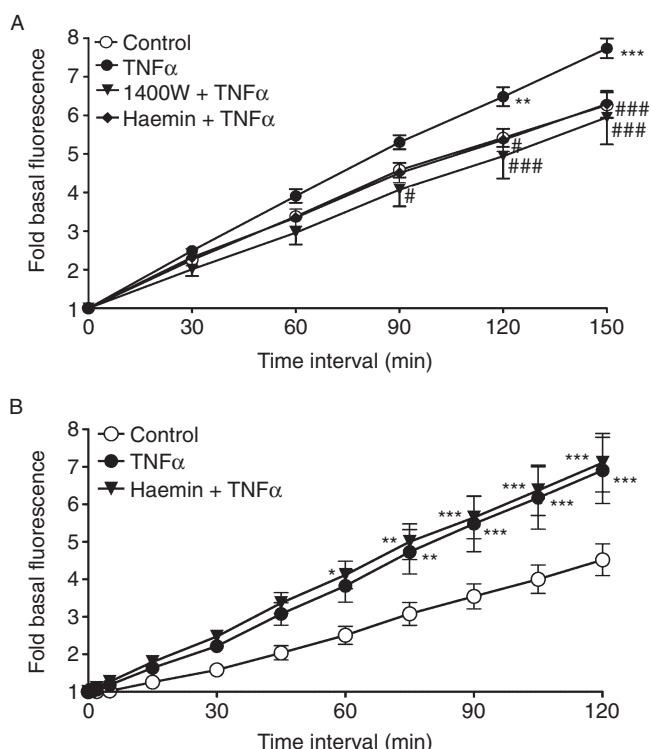


Figure 5 Effect of TNF α (1 ng·mL⁻¹) on the rate of (A) NO and (B) ROS formation in isolated rat pulmonary artery segments, and inhibition of the TNF α response by 1400 W (1 μ M) or haemin (5 μ M). * P < 0.05; ** P < 0.01; *** P < 0.001; significantly different from the corresponding control values; # P < 0.01; ### P < 0.001; significantly different from the corresponding values in TNF α -treated tissue; n = 8–12 tissues from different animals. 1400 W, N[[3(aminomethyl)phenyl]methyl]ethanimidamide dihydrochloride; NO, nitric oxide; ROS, reactive oxygen species; TNF α , tumour necrosis factor α .

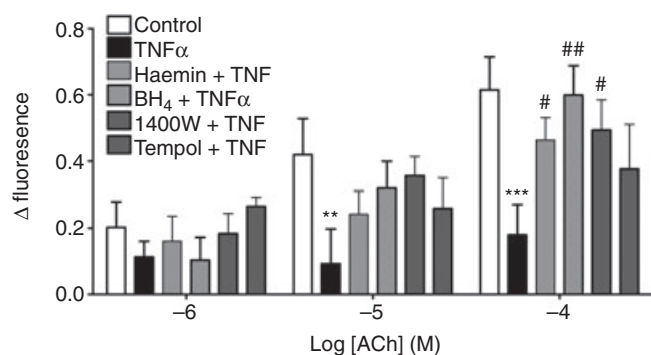


Figure 6 Effect of TNF α (1 ng·mL⁻¹, 2 h) on NO formation in isolated rat pulmonary artery segments with or without haemin (5 μ M, 24 h), BH₄ (3 μ M, 2 h), 1400 W (1 μ M, 2 h), or tempol (1 mM, 2 h) in response to cumulative concentrations of ACh (10⁻⁶ to 10⁻⁴ M). The Δ fluorescence is the fluorescence 2 min after each ACh addition after subtracting the fluorescence at 0 ACh (as assessed after 2 h cytokine pretreatment) and dividing by the basal fluorescence of each vessel for normalization. ** P < 0.01; *** P < 0.001; significantly different from the corresponding values in control tissue; # P < 0.05; ## P < 0.01; significantly different from the corresponding values after TNF α ; n = 6–10 tissues from different animals. 1400 W, N[[3(aminomethyl)phenyl]methyl]ethanimidamide dihydrochloride; BH₄, tetrahydrobiopterin; NO, nitric oxide; TNF α , tumour necrosis factor α .

significant effect on the TNF α -induced decrease in intracellular NO generation in response to ACh stimulation.

Discussion

The purpose of this study was to investigate the potentially protective effect of HO-1 against TNF α -induced impairment in pulmonary artery relaxation. We have succeeded in inducing HO-1 enzyme in rat pulmonary artery *in vitro*. We have shown, for the first time, that induction of HO-1 protein and activity was associated with protection against cytokine-induced dysfunction of pulmonary artery relaxation and that this protection was mediated by modulation of NO production in arterial tissue. These findings suggest upregulation of HO-1 as a novel potential therapy in the management of vascular disturbances associated with inflammatory lung disease.

HO-1 enzyme was induced in our system by haemin or curcumin. Haemin is a strong inducer of HO-1 expression in a variety of cell types both *in vitro* (Lang *et al.*, 2005) and *in vivo* (Attuwaybi *et al.*, 2004). The present study indicated that *in vitro* treatment of pulmonary artery with haemin leads to a time-dependent increase in HO-1 expression and activity. Curcumin has been described as a potent HO-1 inducer in bovine aortic endothelial cells (Motterlini *et al.*, 2000), astrocytes (Scapagnini *et al.*, 2002) and *in vivo* (Farombi *et al.*, 2008).

After optimizing the conditions for HO-1 induction and inhibition in our system, we investigated the potentially protective effect of HO-1 against TNF α -induced impairment of endothelium-dependent relaxation in pulmonary artery. HO-1 induction by haemin significantly inhibited the TNF α -induced hyporesponsiveness to ACh without affecting responses of the pulmonary artery to SNP. These results indicate that HO-1 induction can protect against the impairment of endothelium-dependent relaxation in pulmonary artery, without modifying the sensitivity of pulmonary artery smooth muscle to NO.

The HO-1 enzyme can be inhibited by a variety of synthetic metalloporphyrins (Ryter *et al.*, 2006), including SnPP. SnPP is a potent competitive inhibitor of HO that has been widely used *in vivo* (Gueler *et al.*, 2007) and *in vitro* (Evans *et al.*, 2007). In our study, we found that SnPP at concentration of 20 μ M was able to inhibit HO activity in spleen tissue lysate, and this concentration was employed in the pulmonary artery experiments. Co-incubation with SnPP blocked the protective effect of haemin against TNF α -induced hyporesponsiveness, while neither haemin nor SnPP had any direct effect on the relaxation when applied in control experiments. These data confirm that the observed protection was due to the HO-1 activity and was not a consequence of a direct effect of haemin on smooth muscle contraction.

In chronic hypoxia models, induction of HO-1 prevents the development of pulmonary hypertension in the rat (Christou *et al.*, 2000), and attenuates lipopolysaccharide (LPS)-induced acute lung injury in mice (Gong *et al.*, 2008). Increases of vascular HO-1 expression have been observed in several experimental models of hypertension including angiotensin II-induced hypertension (Li *et al.*, 2004), spontaneously

hypertensive rats (SHRs) (Cheng *et al.*, 2004), Dahl-Rapp salt-sensitive rats (Teran *et al.*, 2005), and deoxycorticosterone salt-sensitive rats (Johnson *et al.*, 2004). The HO-1/CO system has been associated with antihypertensive effects (Ndisang *et al.*, 2004) as well as pathological roles in the case of salt-induced hypertension models (Teran *et al.*, 2005). Administration of exogenous CO or haem derivatives reduces blood pressure in SHR (Johnson *et al.*, 1996) and human HO-1 gene delivery by vector-directed gene therapy reduces blood pressure in young SHR (Sabaawy *et al.*, 2001). HO-1 induction alleviates the high glucose-induced, endothelium-dependent, acute vascular dysfunction in rat aortic rings (Meng *et al.*, 2009). Conversely, application of metalloporphyrins (zinc proporphyrin IX, chromium mesoporphyrin) reverse the anti-hypertensive effects of haem and also elevated systolic blood pressure in SHR (Cheng *et al.*, 2004). Taken together, these findings strongly suggest that induction of HO-1 expression is a compensatory response during the development of hypertension (Ryter *et al.*, 2006).

The roles of NO and ROS in TNF α -induced impairment of endothelium-dependent relaxation in pulmonary artery were assessed to help explain the observed HO-1 protection. We found that the intracellular levels and rate of generation of both NO and ROS were significantly increased by TNF α . The highly selective iNOS inhibitor 1400 W and the superoxide scavenger, tempol, blocked the TNF α -induced attenuation of the vascular response to ACh and also ACh-stimulated NO release. These results suggest that generation of NO and ROS mediated the impairment in endothelium-dependent relaxation. Previously a role for iNOS-derived NO in the impairment of endothelium-dependent relaxation has been demonstrated in rat middle cerebral arteries following LPS treatment (Hernanz *et al.*, 2004). Also, an increase in superoxide production after LPS has been reported in rat aorta (Brandes *et al.*, 1999) and middle cerebral artery (Hernanz *et al.*, 2003). Wimalasundera *et al.* (2003) reported that preincubation with SOD prevents the TNF α attenuation of endothelium-dependent relaxation in rat mesenteric artery.

Preincubation with 1400 W completely blocked the TNF α -induced increase in the rate of NO generation, indicating a role for iNOS. We did not directly assess iNOS protein expression in these experiments, because we did not anticipate increased expression levels over the 2-h treatment period used in these experiments. Previous studies have indicated that iNOS mRNA level starts to increase only 4 h after stimulation (Lowenstein *et al.*, 1992). However, the effect of iNOS-derived NO production starts to affect the relaxation before a significant expression of the iNOS protein can be detected by immunoblotting. Hernanz *et al.* (2003) found that the iNOS inhibitor aminoguanidine increased serotonin-induced contraction from the first hour (statistically significant at hour 3) of LPS incubation while increased iNOS protein expression was only detected after 5 h.

Generated superoxide interacts with NO forming peroxynitrite (Laursen *et al.*, 2001). Peroxynitrite is a potent oxidant that can switch eNOS, via the oxidation of BH₄, from a NO-generating to a superoxide-generating enzyme (a process termed NOS uncoupling) (Landmesser *et al.*, 2003). This is supported by the observation that the NOS cofactor BH₄

blocked the TNF α -induced attenuation in the vascular response to ACh and ACh-stimulated NO generation.

The mechanism(s) by which HO-1 induction protects against the impairment in endothelium-dependent relaxation could be mediated, at least in part, by modulation of NO production. Chemical inhibition of HO activity by zinc protoporphyrin in macrophages (Turcanu *et al.*, 1998) results in increased NO production, suggesting that HO may exert an inhibitory effect on NOS. These findings are supported by the observation that induction of HO-1 in human renal epithelial cells suppressed cytokine-induced NO generation (Svensson *et al.*, 2009) and inhibited NO generation in macrophages (Kim *et al.*, 2008). As NOS is a haemoprotein, it is reasonable to postulate that CO generated by HO activity could bind to NOS, causing inactivation. CO is capable of binding to NOS (McMillan *et al.*, 1992), and exogenously administered CO inhibits NOS activity (White and Marletta, 1992). In this study, we found that HO-1 induction by haemin was able to prevent the TNF α -induced increase in the intracellular level and rate of generation of NO as well as the TNF α -induced decrease in ACh-stimulated NO generation. However, HO-1 induction did not affect the TNF α -induced increase in the intracellular ROS level or the rate of ROS generation.

In summary, HO-1 induction protects against inflammatory cytokine-induced impairment of endothelium-dependent relaxation. Inhibition of iNOS is a likely underlying mechanism of the protective effect of HO-1. These findings suggest therapeutic strategies that increase HO-1 activity are likely to be beneficial in inflammatory lung disease

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Conflict of interest

The authors state no conflict of interest.

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