Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 473 (2003) 135–141
www.elsevier.com/locate/ejphar

Heme oxygenase is expressed in human pulmonary artery smooth muscle where carbon monoxide has an anti-proliferative role

Salome J. Stanford[a,b], Matthew J. Walters[a], Alison A. Hislop[b], Sheila G. Haworth[b], Timothy W. Evans[a], Brian E. Mann[c], Roberto Motterlini[d], Jane A. Mitchell[a,*]

[a] *Unit of Critical Care, The Royal Brompton and Harefield N.H.S. Trust, Imperial College School of Medicine, Sydney Street, London SW3 6NP, UK*
[b] *Developmental Vascular Biology and Pharmacology, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK*
[c] *Department of Chemistry, Sheffield University, Sheffield S3 7HF, UK*
[d] *Vascular Biology Unit, Department of Surgical Research, Northwick Park Institute for Medical Research, Harrow, Middlesex, UK*

Received 17 April 2003; received in revised form 11 June 2003; accepted 17 June 2003

Abstract

Heme oxygenase is the rate-limiting enzyme in the catabolism of heme to carbon monoxide, bilirubin and free iron. Many cell types express heme oxygenase-2 constitutively while heme oxygenase-1 is induced at sites of inflammation and oxidative stress. In systemic blood vessels, carbon monoxide may have an important homeostatic role where, like its better-studied counterpart nitric oxide, it is emerging as a vasodilator and an inhibitor of proliferation. However, much less is known regarding the role of heme oxygenase and carbon monoxide in the pulmonary circulation where vascular responses are very different. Here, using primary cultures of human pulmonary artery smooth muscle cells, we present novel data showing that this cell type expresses heme oxygenase-2 constitutively and, in the presence of oxidants, can induce heme oxygenase-1. We also show that the carbon monoxide-releasing molecule, tricarbonyldichlororuthenium (II) dimer, potently and profoundly inhibits proliferation of human pulmonary artery smooth muscle cells. Pulmonary hypertension is a disease characterised by abnormal vascular smooth muscle cell growth and remodelling of the pulmonary vasculature. Our observations support the growing evidence that the heme oxygenase/carbon monoxide system may play a role in the pathology of pulmonary hypertension.

Keywords: Carbon monoxide; Heme oxygenase; Carbon monoxide-releasing molecule; Pulmonary artery smooth muscle cell, human; Pulmonary hypertension

1. Introduction

Heme oxygenase is the rate-limiting enzyme in the conversion of heme to carbon monoxide, free iron and biliverdin, the latter being reduced to bilirubin by biliverdin reductase. Heme oxygenase exists in two major isoforms. Heme oxygenase-2 is the constitutive form of the enzyme and is localised to the endothelium (Zakhary et al., 1996). By contrast, heme oxygenase-1 is expressed at the sites of inflammation and above all, as a consequence of oxidative stress (Willis et al., 1996). In systemic blood vessels, carbon monoxide may have an important homeostatic role where, like its better-studied counterpart nitric oxide, it is emerging as a vasodilator (Lin and McGrath, 1988) and an inhibitor of cell proliferation (Peyton et al., 2002; Durante, 2002, 2003). Recently, Motterlini et al. (2002) have reported the ability of a series of transition metal carbonyls, which they have termed carbon monoxide-releasing molecules, to liberate carbon monoxide and mimic the effects of endogenous heme oxygenase-1 induction in the systemic circulation of the rat. More specifically, carbon monoxide-releasing molecules produced vasodilation of rat aortic rings, attenuated coronary vasoconstriction and reduced acute hypertension in vivo, effects mimicked by treatment of the animals with hemin. This study firmly establishes the importance of the heme oxygenase–carbon monoxide pathway in the systemic circulation. However, much less is known regarding the role of heme oxygenase and carbon monoxide in the pulmonary circulation, where vascular responses are very different. This point is particularly important to address since our understanding of pulmonary hypertension, which is associated with abnormal vascular smooth muscle cell growth and

* Corresponding author. Unit of Critical Care, Imperial College School of Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, UK. Tel.: +44-207-351-8725, +44-797-3520149; fax: +44-171-3518270.
E-mail address: j.a.mitchell@ic.ac.uk (J.A. Mitchell).

0014-2999/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0014-2999(03)02001-6

remodelling, is incomplete. There are a limited number of studies showing that heme oxygenase-1 is induced in pulmonary vessels of the rat (Morita et al., 1995). Its role in animal models of pulmonary disease (e.g. hypertension) remains unclear (Carraway et al., 2002; Minamino et al., 2001) and the role of heme oxygenase in human lung disease is not known. While this manuscript was in preparation, Visner et al. (2003) reported that rapamycin induces heme oxygenase-1 in human pulmonary artery cells, the activity of which contributes to the anti-proliferative actions of the drug. However, the direct role of carbon monoxide in modulating proliferative responses of pulmonary artery cells, derived from any species, is not known. Thus, the aim of this study was to investigate the expression of heme oxygenase-1 and heme oxygenase-2 in primary cultures of human pulmonary artery smooth muscle cells and to study the effects of carbon monoxide on the proliferation of these cells.

2. Materials and methods

2.1. Cell culture

Human pulmonary arteries from healthy sections of the lung were obtained from patients undergoing pulmonary resection at The Royal Brompton and Harefield N.H.S. Trust. Under sterile conditions, as described previously (Wort et al., 2001), vessels were dissected clean and the endothelium removed by careful scraping with a scalpel. Vessels were cut into small pieces and placed in cell culture flasks together with Dulbecco's modified Eagle's culture medium containing sodium pyruvate (110 mg/l) and phenol red and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), amphotericin B (2.5 μg/ml), 15% foetal calf serum (FCS) and MEM nonessential amino acids. Cells were incubated in 5% carbon dioxide: 95% oxygen at 37 °C and reached confluence after approximately 4 weeks. Smooth muscle phenotype was confirmed by the characteristic hill and valley morphology of the cells and routine staining of cultures for α-actin. Cells (passages 2–6 only) were seeded onto plates for use in experiments.

2.2. Western blot analysis

Western blotting was performed to determine the level of heme oxygenase-1 and heme oxygenase-2 protein. Human pulmonary artery smooth muscle cells, in 6-well plates, were treated for 24 h with supplemented culture medium (5% foetal calf serum) alone, or in the presence of lipopolysaccharide (1 μg/ml), sodium nitroprusside (1 mM) or hemoglobin (1 mg/ml). Cells were lysed and centrifuged at 10,000 × *g*, for 10 min, at 4 °C. The supernatants were removed from samples and snap frozen for storage. Protein content of supernatants was determined by the Lowry procedure (Sigma protein assay kit). Samples were mixed 1:1 with gel loading buffer and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis performed on 12% gels. Each well was loaded with 10 μg of sample protein or 1 ng of heme oxygenase-1 or heme oxygenase-2 protein standard (Stressgen Biotechnologies, Victoria, Canada). Each gel also contained a molecular mass rainbow marker (RMN756, Amersham Pharmacia Biotech UK, Buckinghamshire, UK). The separated blots were transferred by electrophoresis onto nitrocellulose membrane, blocked overnight (phosphate buffered saline, 0.25% Tween-20, 5% milk) at 4 °C before incubation for 1 h at room temperature with an antibody directed against heme oxygenase-1 or heme oxygenase-2 (Stressgen Biotechnologies) prepared in phosphate buffered saline at concentrations of 1:1000 and 1:2000, respectively. Membranes were then washed with phosphate buffered saline containing 0.25% Tween-20 and incubated for an hour with goat anti-rabbit immunoglobulin G-horseradish peroxidase (1:2000, Sigma-Aldrich, Poole, Dorset, UK). After further washings, blots were incubated with a commercially available extended duration chemiluminescence substrate (Biowest™, UVP, Cambridge, UK) and bands visualized and captured utilising a GDS 8000 system attached to an Epi Chemi II darkroom.

2.3. Proliferation: methyl-[^{3}H]thymidine uptake

Motterlini et al. (2002) recently established the ability of a series of transition metal carbonyls to liberate carbon monoxide and elicit biological responses. Using Nuclear Magnetic Resonance Analysis, they report that for each mole of the metal carbonyl tricarbonyldichlororuthenium (II) dimer ($[Ru(CO)_3Cl_2]_2$) in dimethyl sulfoxide, approximately 0.7 mol of carbon monoxide is liberated. Dimethyl sulfoxide acts as a ligand, promoting the formation of monomers during which carbon monoxide is liberated. Based on these findings, we have used $[Ru(CO)_3Cl_2]_2$ as a tool to assess the action of carbon monoxide on human pulmonary artery smooth muscle cell proliferation.

Human pulmonary artery smooth muscles were seeded at 80% confluency (8000 cells/well) onto 96-well plates and serum-deprived for 24 h to achieve quiescence. The cells were then incubated for 24 h with serum-free, supplemented culture medium or with supplemented culture medium containing 3% foetal calf serum in the presence or absence of increasing concentrations of sodium nitroprusside (1–100 μM), the carbon monoxide-releasing molecule, $[Ru(CO)_3Cl_2]_2$ (1–100 μM) or its vehicle, dimethyl sulfoxide. In some experiments, cells were pre-treated (45 min) with 1*H*-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ; 10 μM) before the addition of the $[Ru(CO)_3Cl_2]_2$. In further experiments, cells were treated for 24 h with serum-free, supplemented culture medium or with supplemented culture medium containing 3% foetal calf serum in the presence or

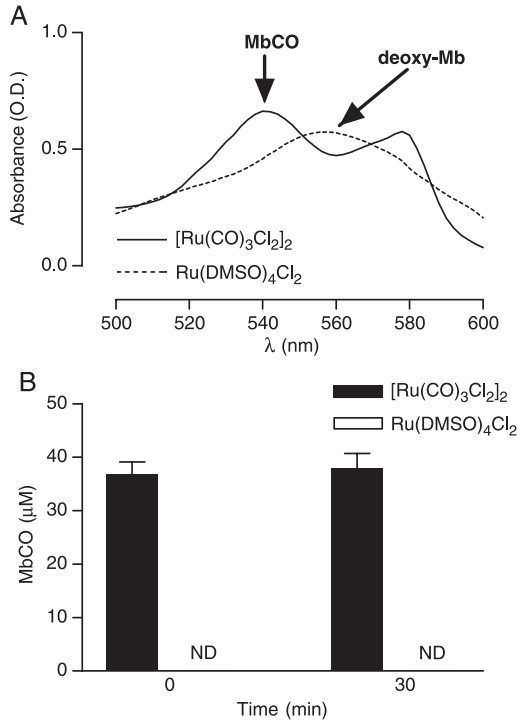

Fig. 1. (A) Absorption spectra of deoxymyoglobin (deoxy-Mb) and carbonmonoxy myoglobin (MbCO) after interaction of myoglobin with $[Ru(CO)_3Cl_2]_2$ or $Ru(DMSO)_4Cl_2$ (negative control). The metal carbonyl complex was solubilized in dimethyl sulfoxide and added directly to 1 ml of deoxymyoglobin solutions (66 μM) to reach a final concentration of 40 μM $[Ru(CO)_3Cl_2]_2$. The absorption spectra were determined immediately after mixing the samples by inversion. (B) Determination of the amount of carbonmonoxy myoglobin formed at two different time points (0 and 30 min) after the interaction of deoxymyoglobin with 40 μM $[Ru(CO)_3Cl_2]_2$ or $Ru(DMSO)_4Cl_2$.

absence of $[Ru(CO)_3Cl_2]_2$ (1–100 μM), or a chemically identical molecule lacking the carbonyl moiety (negative control), dichlorotetrakis (dimethyl sulfoxide) ruthenium(II) $(Ru(DMSO)_4Cl_2$; 1–100 μM).

In all cases, after the 24-h treatment period, methyl-[^{3}H] thymidine was added to each well (0.5 μCi/well; specific activity 25 Ci/mmol) for a further 6-h incubation period. Reactions were terminated by freezing the plates at −80 °C. Finally, cells were lysed by thawing and the DNA incorporating labelled thymidine was harvested on glass-fibre filters using a cell harvester (Packard, Meriden, CT). The incorporation of radioactivity was measured with an automatic β-scintillation counter (Packard) and expressed in counts per minute (cpm). Because primary cultures of human cells often express quite different phenotypes and 'baseline' responses, the absolute levels of cpm seen in stimulated and unstimulated cultures can vary widely.

2.4. Detection of carbon monoxide release

The release of carbon monoxide from $[Ru(CO)_3Cl_2]_2$ and its negative control, $Ru(DMSO)_4Cl_2$, was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin to carbonmonoxy myoglobin. The amount of carbonmonoxy myoglobin formed was quantified by measuring the absorbance at 540 nm. Myoglobin solutions (66 μM final concentration) were prepared fresh by dissolving the protein in 0.04 M phosphate buffer (pH 6.8). Sodium dithionite (0.1%) was added to convert myoglobin to deoxymyoglobin prior to each reading. Carbon monoxide released from $[Ru(CO)_3Cl_2]_2$ was quantified by adding an aliquot of a stock solution (10 μl) of the carbonyl complex in dimethyl sulfoxide directly to the myoglobin solution (final concentration of $[Ru(CO)_3Cl_2]_2 = 40$ μM). All the spectra were measured using a Helios spectrophotometer. Using this method, carbon monoxide release from $[Ru(CO_3)Cl_2]_2$ was confirmed (Fig. 1). Predictably, under the same conditions, no carbon monoxide release was detected from the negative control, $Ru(DMSO)_4Cl_2$ (Fig. 1).

2.5. Cell viability

The effect of $[Ru(CO)_3Cl_2]_2$ on human pulmonary artery smooth muscle cell viability was assessed by the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan (Mitchell et al., 1993). Cells were treated with supplemented culture medium (containing 3% foetal calf serum) alone or in the presence of $[Ru(CO)_3Cl_2]_2$ at concentrations of 30 and 100 μM. After 24 h, cell supernatant was removed. Cells were incubated with supplemented culture medium containing 1 mg/ml MTT for 15 min at 37 °C after which the medium was removed, cells lysed with

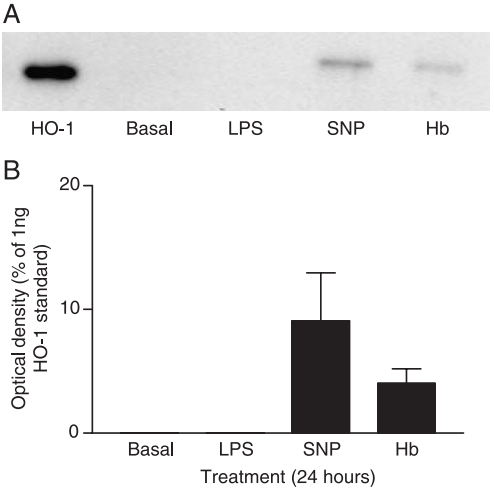

Fig. 2. Heme oxygenase-1 (HO-1) protein levels in human pulmonary artery smooth muscle cells stimulated for 24 h in the presence or absence of lipopolysaccharide (LPS, 1 μg/ml), sodium nitroprusside (SNP, 1 mM) or hemoglobin (Hb, 1 mg/ml) illustrated by (A) a representative Western blot and (B) pooled data obtained from Western blots utilising smooth muscle cells cultured from three separate patients. Data indicate mean ± S.E.M. of the absolute integrated optical density of bands expressed as a percentage of the band due to 1 ng of heme oxygenase-1 standard.

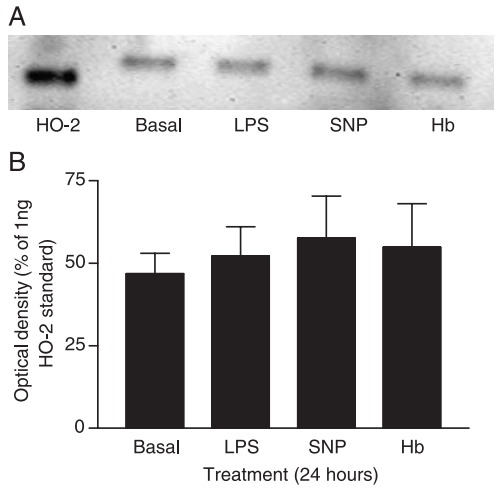

Fig. 3. Heme oxygenase-2 (HO-2) protein levels in human pulmonary artery smooth muscle cells stimulated for 24 h in the presence or absence of lipopolysaccharide (LPS, 1 μg/ml), sodium nitroprusside (SNP, 1 mM) or hemoglobin (Hb, 1 mg/ml) illustrated by (A) a representative Western blot and (B) pooled data obtained from Western blots utilising smooth muscle cells cultured from three separate patients. Data indicate mean ± S.E.M. of the absolute integrated optical density of bands expressed as a percentage of the band due to 1 ng of heme oxygenase-2 standard.

dimethyl sulfoxide and absorbance read at a wavelength of 550 nm.

2.6. Materials

All materials for cell culture were purchased from GIBCO BRL Life Technologies (Paisley, UK). Lipopolysaccharide, sodium nitroprusside, hemoglobin and $[Ru(CO)_3Cl_2]_2$ were from Sigma-Aldrich and methyl-[^{3}H]thymidine was from Amersham Pharmacia Biotech UK. ODQ was bought from Tocris Cookson (Avonmouth, UK). Dichlorotetrakis(dimethyl sulfoxide)ruthenium(II) $[Ru(DMSO)_4Cl_2]$, the negative control for carbon monoxide-releasing molecule, was synthesized as previously described (Alessio et al., 1995).

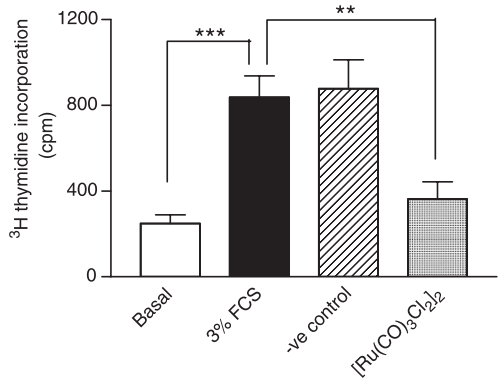

Fig. 5. Effect of the carbon monoxide-releasing molecule ($[Ru(CO)_3Cl_2]_2$, 30 μM) vs. negative (−ve) control ($Ru(DMSO)_4Cl_2$, 30 μM) on human pulmonary artery smooth muscle cell proliferation in the presence of 3% foetal calf serum (FCS). Incorporation of ^{3}H thymidine into cells was expressed in counts per minute (cpm). Data represent mean ± S.E.M., $n=9$ using cells cultured from three individual patients. $^{**}P<0.01$, $^{***}P<0.0001$ Mann–Whitney.

3. Results

3.1. Expression of heme oxygenase-1 and heme oxygenase-2 in human pulmonary artery smooth muscle cells

Under basal conditions, human pulmonary artery smooth muscle cells expressed undetectable amounts of heme oxygenase-1 protein (Fig. 2). Expression of the enzyme was not stimulated in the presence of lipopolysaccharide. Nitric oxide is a free radical species which, acting through its oxidation products, can increase oxidative stress in

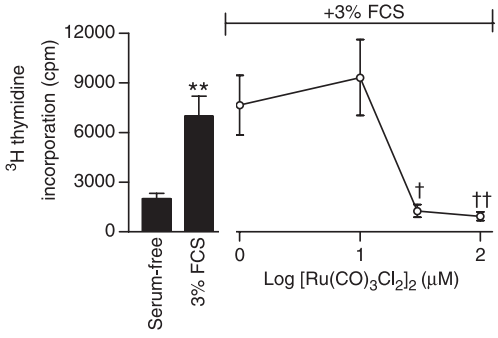

Fig. 4. Effect of foetal calf serum (FCS) and increasing concentrations of the carbon monoxide-releasing molecule (CO-RM), $[Ru(CO)_3Cl_2]_2$ (1–100 μM), on human pulmonary artery smooth muscle cell proliferation. Incorporation of ^{3}H thymidine into cells was expressed in counts per minute (cpm). Data represent mean ± S.E.M., $n=9$ using cells cultured from three individual patients. $^{**}P<0.01$ Mann–Whitney. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ Kruskal–Wallis (vs. 3% FCS), post-test Dunn's.

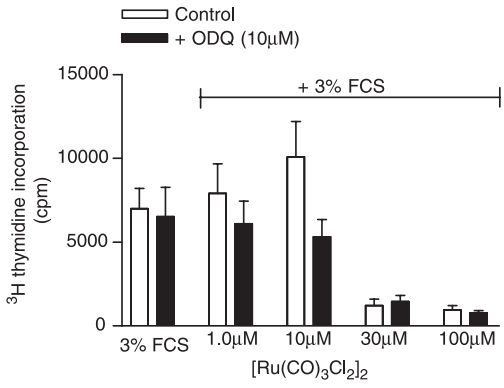

Fig. 6. The effect of the carbon monoxide-releasing molecule [Ru $(CO)_3Cl_2]_2$ on human pulmonary artery smooth muscle cell proliferation in the presence and absence of ODQ (10 μM). Incorporation of ^{3}H thymidine into cells was expressed in counts per minute (cpm). Data represent $n=9$ using cells cultured from three individual patients.

exposed cells. In the presence of the nitric oxide donor, sodium nitroprusside, heme oxygenase-1 protein expression was induced. The presence of hemoglobin in the medium also induced heme oxygenase-1 expression. In contrast to heme oxygenase-1, heme oxygenase-2 protein was detectable in cells under basal condition (Fig. 3). Heme oxygenase-2 levels were unchanged after treatment of cells with lipopolyssacharide, sodium nitroprusside or hemoglobin.

3.2. Effects of carbon monoxide on the proliferation of human pulmonary artery smooth muscle cells

Human pulmonary artery smooth muscle cell proliferation was stimulated in the presence of 3% foetal calf serum (Fig. 4). Sodium nitroprusside (1 mM) induced a significant inhibition of human pulmonary artery smooth muscle cell proliferation (data not shown). Similarly, in the presence of the carbon monoxide-releasing molecule $[Ru(CO)_3Cl_2]_2$, an anti-proliferative effect was observed (Fig. 4). The dimethyl sulfoxide vehicle had no significant effect on cell proliferation (supplemented culture medium containing 3% foetal calf serum in the presence and absence of DMSO, respectively, mean ± S.E.M., $n=9$: 9490 ± 1979 vs. 6994 ± 1210 cpm). The negative control for $[Ru(CO)_3Cl_2]_2$, $Ru(DMSO)_4Cl_2$, where the carbon monoxide component of the molecule is replaced with dimethyl sulfoxide, had no effect on foetal calf serum-induced smooth muscle cell proliferation at concentrations of 1, 3 (data not shown) or 30 μM (Fig. 5). However, at a higher concentration (100 μM), the negative control inhibited foetal calf serum-induced proliferation by 69.3 ± 10.3% (mean ± s.e.mean, $n=9$). At the same concentration, $[Ru(CO)_3Cl_2]_2$ completely abolished foetal calf serum-induced proliferation.

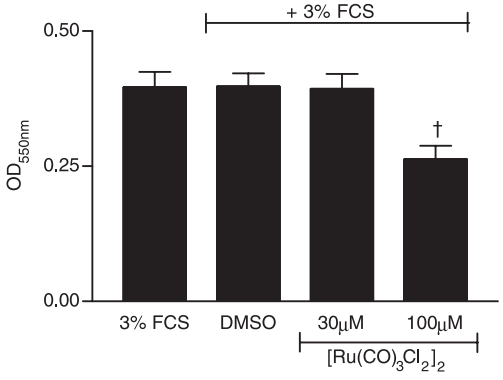

Fig. 7. Effect of the dimethyl sulfoxide (DMSO) vehicle and $[Ru(CO)_3Cl_2]_2$ (in the presence of 3% foetal calf serum, FCS) on human pulmonary artery smooth muscle cell viability at 24 h as assessed by the mitochondrial-dependent reduction of MTT to formazan. Data, shown here as optical density (OD) measured at a wavelength of 550 nm, are expressed as mean ± S.E.M., $n=9$ using cells cultured from three individual patients. $^{\dagger}P<0.05$, Kruskal–Wallis (vs. 3% FCS), post-test Dunn's.

3.3. Effects of ODQ on the proliferation of human pulmonary artery smooth muscle cells

The soluble guanylyl cyclase inhibitor ODQ (10 μM) had no effect on foetal calf serum-induced smooth muscle cell proliferation. Furthermore, ODQ had no significant effect on the inhibition of proliferation induced by the carbon monoxide-releasing molecule, $[Ru(CO)_3Cl_2]_2$ (Fig. 6).

3.4. Effects of carbon monoxide on cell viability

At a concentration of 30 μM, where foetal calf serum-induced proliferation is completely blocked, $[Ru(CO)_3Cl_2]_2$ did not effect smooth muscle cell viability (Fig. 7). However, at the higher concentration of 100 μM, cell death was evident.

4. Discussion

Pulmonary hypertension is characterised by chronic and progressive remodelling of pulmonary vessels. Treatments for pulmonary hypertension are limited and lung transplantation remains the ultimate treatment option for many affected patients. Thus, identification of novel molecules that limit or reduce remodelling in pulmonary vessels is of greatest importance in the pursuit of new treatments for pulmonary hypertension. Here we show, for the first time, that the carbon monoxide-releasing molecule $[Ru(CO)_3Cl_2]_2$ (Motterlini et al., 2002) inhibits proliferation of human pulmonary artery smooth muscle cells at a concentration where cell viability is not affected. At the same concentration, a chemically identical molecule lacking the carbonyl moiety (negative control) $Ru(DMSO)_4Cl_2$ did not affect proliferation. Our data also reveal a biological trend for carbon monoxide at lower concentrations to actually increase proliferation in these cells. This putative 'byphasic' effect of carbon monoxide on vascular remodelling is supported by literature suggesting either pro- (Carraway et al., 2002) or anti-proliferative (Taille et al., 2003) effects of heme oxygenase in other experimental systems.

Both nitric oxide and carbon monoxide are thought to exert their vascular effects primarily via the stimulation of soluble guanylyl cyclase and subsequent increase in intracellular levels of 3′,5′-Guanylate cyclic monophosphate (cyclic GMP). ODQ is an inhibitor of soluble guanylyl cyclase and has been demonstrated, by us (Stanford et al., 2001) and by others (Hussain et al., 1997; Homer et al., 1999), to inhibit the relaxant effects of nitric oxide and carbon monoxide in a range of vascular beds. Furthermore, vasodilatation elicited by $[Ru(CO)_3Cl_2]_2$ in precontracted aortic rings of the rat has been shown to be, at least in part, cyclic GMP-dependent (Motterlini et al., 2002). However, in our study, the anti-proliferative effect of the carbon monoxide-releasing molecule $[Ru(CO)_3Cl_2]_2$ was not inhibited by ODQ, suggesting that, in this setting, carbon monoxide is

acting independently of the soluble guanylyl cyclase/cyclic GMP system. This is supported in the literature by a study carried in human airway smooth muscle cells (Song et al., 2002). Taille et al. also demonstrated that the anti-proliferative effect of heme oxygenase induction in airway cells was not affected by ODQ. However, in this study, the actions of heme oxygenase appeared to be due mainly to the formation of bilirubin and not carbon monoxide (Taille et al., 2003). It should be noted that the tendency for carbon monoxide to increase proliferation was inhibited by ODQ. Whether 'low' levels of carbon monoxide stimulate proliferation in these cells via a cyclic GMP-dependent pathway remains the subject of investigation.

Carbon monoxide is released endogenously by the action of heme oxygenase on its substrate, heme. We found that human pulmonary artery vascular smooth muscle cells express heme oxygenase-2 under basal conditions. Heme oxygenase-1 was induced by both a nitric oxide-donor and hemoglobin, most likely as a result of oxidant stress. Lipopolysaccharide does not appear to induce heme oxygenase-1 in human pulmonary artery smooth muscle cells. Reports in the literature demonstrate the ability of lipopolysaccharide to induce heme oxygenase-1 in other organs and cell types including the rat lung (Carraway et al., 1998), rat glial cells (Kitamura et al., 1998) and murine macrophages (Fujiwara et al., 1996). This lipopolysaccharide-mediated induction of heme oxygenase-1 probably occurs as a result of inducible nitric oxide synthase induction and the subsequent release of nitric oxide (Kitamura et al., 1998; Fujiwara et al., 1996). However, we have shown previously (Jourdan et al., 1999) that in human pulmonary artery smooth muscle cells, lipopolysaccharide does not induce meaningful levels of inducible nitric oxide synthase (i.e. compared to those observed in cells derived from rat pulmonary artery). Thus, in this regard, it is of no surprise that lipopolysaccharide does not induce heme oxygenase-1 in this setting.

The data presented here suggest that human pulmonary artery smooth muscle cells are capable of synthesizing carbon monoxide under basal conditions and, perhaps at an increased rate during conditions of oxidative stress, such as may occur during pulmonary hypertension. This hypothesis is supported by an increasing number of studies in the literature using animal models. Heme oxygenase is expressed in pulmonary vessels of rats (Carraway et al., 2002) and mice (Minamino et al., 2001) rendered pulmonary hypertensive. Furthermore, in transgenic mice where heme oxygenase-1 is over expressed, pulmonary vascular remodelling, in response to chronic hypoxia, is reduced (Minamino et al., 2001). Thus, it seems that in animal models in vivo or in isolated human pulmonary cells in vitro, conditions of increased carbon monoxide production limit the proliferative responses associated with pulmonary hypertension. These observations lend support to others in the literature suggesting that agents that manipulate the heme oxygenase–carbon monoxide pathway may have a novel therapeutic potential in the treatment of pulmonary hypertension.

Acknowledgements

This work was funded by grants from the British Heart Foundation and the Medical Research Council. The authors would like to thank Dr. James Clark for the technical assistance and Dr. Tony Johnson for the synthesis of $Ru(DMSO)_4Cl_2$.

References

Alessio, E., Milani, B., Bolle, M., Mestroni, G., Faleschini, P., Todone, F., Geremia, S., Calligaris, M., 1995. Carbonyl derivatives of chloride-dimethyl sulfoxide-ruthenium(II) complexes: synthesis, structural characterization, and reactivity of $Ru(CO)_x(DMSO)_{4-x}Cl_2$ complexes ($x=1-3$). Inorg. Chem. 34, 4722–4734.

Carraway, M.S., Ghio, A.J., Taylor, J.L., Piantadosi, C.A., 1998. Induction of ferritin and heme oxygenase-1 by endotoxin in the lung. Am. J. Physiol. 275, L583–L892.

Carraway, M.S., Ghio, A.J., Suliman, H.B., Carter, J.D., Whorton, A.R., Piantadosi, C.A., 2002. Carbon monoxide promotes hypoxic pulmonary vascular remodelling. Am. J. Physiol., Lung Cell. Mol. Physiol. 282, L693–L702.

Durante, W., 2002. Carbon monoxide and bile pigments: surprising mediators of vascular function. Vasc. Med. 7, 195–202.

Durante, W., 2003. Heme oxygenase-1 in growth control and its clinical application to vascular disease. J. Cell. Physiol. 195, 373–382.

Fujiwara, N., Okado, A., Seo, H.G., Fujii, J., Kondo, K., Taniguchi, N., 1996. Quinazoline derivatives suppress nitric oxide production by macrophages through inhibition of NOS II gene expression. FEBS Lett. 395, 299–303.

Homer, K.L., Fiore, S.A., Wanstall, J.C., 1999. Inhibition by 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) of responses to nitric oxide-donors in rat pulmonary artery: influence of the mechanism of nitric oxide generation. J. Pharm. Pharmacol. 51, 135–139.

Hussain, A.S., Marks, G.S., Brien, J.F., Nakatsu, K., 1997. The soluble guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ) inhibits relaxation of rabbit aortic rings induced by carbon monoxide, nitric oxide, and glyceryl trinitrate. Can. J. Physiol. Pharm. 75, 1034–1037.

Jourdan, K.B., Evans, T.W., Lamb, N.J., Goldstraw, P., Mitchell, J.A., 1999. Autocrine function of inducible nitric oxide synthase and cyclooxygenase-2 in proliferation of human and rat pulmonary artery smooth muscle cells: species variation. Am. J. Respir. Cell Mol. Biol. 21, 105–110.

Kitamura, Y., Matsuoka, Y., Nomura, Y., Taniguchi, T., 1998. Induction of inducible nitric oxide synthase and heme oxygenase-1 in rat glial cells. Life Sci. 62, 1717–1721.

Lin, H., McGrath, J.J., 1988. Vasodilating effects of carbon monoxide. Drug Chem. Toxicol. 11, 371–385.

Minamino, T., Christou, H., Hsieh, C.M., Liu, Y., Dhawan, V., Abraham, N.G., Perella, M.A., Mitsialis, S.A., Kourembanas, S., 2001. Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. Proc. Natl. Acad. Sci. U. S. A. 98, 8798–8803.

Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1993. Selectivity of non-steroidal anti-inflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc. Natl. Acad. Sci. U. S. A. 90, 11693–11697.

Morita, T., Perrella, M.A., Lee, M.E., Kourembanas, S., 1995. Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. Proc. Natl. Acad. Sci. 92, 1475–1479.

Motterlini, R., Clark, J.E., Foresti, R., Sarathchandra, P., Mann, B.E., Green, C.J., 2002. Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ. Res. 90, E17–E24.

Peyton, K.J., Reyna, S.V., Chapman, G.B., Ensenat, D., Lin, X.M., Wang, H., Schafer, A.I., Durante, W., 2002. Heme oxygenase-1-derived carbon monoxide is an autocrine inhibitor of vascular smooth muscle cell growth. Blood 99, 4443–4448.

Song, R., Mahidhara, R.S., Liu, F., Ning, W., Otterbein, L.E., Choi, A.M., 2002. Carbon monoxide inhibits human airway smooth muscle cell proliferation via mitogen-activated protein kinase pathway. Am. J. Respir. Cell Mol. Biol. 27, 603–610.

Stanford, S.J., Gitlin, J.M., Mitchell, J.A., 2001. Identification of two distinct vasodilator pathways activated by ATP in the mesenteric bed of the rat. Br. J. Pharmacol. 133, 825–832.

Taille, C., Almolki, A., Benhamed, M., Zedda, C., Megret, J., Berger, P., Leseche, G., Fadel, E., Yamaguchi, T., Marthan, R., Aubier, M., Boczkows, J., 2003. Heme oxygenase inhibits human airway smooth muscle proliferation via a bilirubin-dependent modulation of ERK1/2 phosphorylation. J. Biol. Chem. (epub ahead of print).

Visner, G.A., Lu, F., Zhou, H., Liu, J., Kazemfar, K., Agarwal, A., 2003. Rapamycin induces heme oxygenase-1 in human pulmonary vascular cells: implications in the antiproliferative response to rapamycin. Circulation 107, 911–916.

Willis, D., Moore, A.R., Frederick, R., Willoughby, D.A., 1996. Heme oxygenase: a novel target for the modulation of the inflammatory response. Nat. Med. 2, 87–90.

Wort, S.J., Woods, M., Warner, T.D., Evans, T.W., Mitchell, J.A., 2001. Endogenously released endothelin-1 from human pulmonary artery smooth muscle promotes cellular proliferation: relevance to pathogenesis of pulmonary hypertension and vascular remodeling. Am. J. Respir. Cell Mol. Biol. 25, 104–110.

Zakhary, R., Gaine, S.P., Dinerman, J.L., Flavahan, N.A., Snyder, S.H., 1996. Heme oxygenase 2: endothelial and neuronal localization and role in endothelium-dependent relaxation. Proc. Natl. Acad. Sci. 93, 795–798.