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Short communication

Carbon monoxide inhibits endothelin-1 release by human pulmonary artery smooth muscle cells

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Abstract

Endothelin-1 is a potent vasoconstrictor with mitogenic properties. This 21-amino-acid protein, released in the vasculature by endothelial and smooth muscle cells, has been implicated in pulmonary hypertension. More recently, evidence has accumulated for a role of the heme oxygenase system in pulmonary hypertension. Heme oxygenase catalyses the breakdown of heme to produce carbon monoxide, biliverdin and free iron. Here we show that a carbon monoxide-releasing molecule, but not biliverdin, inhibits endothelin-1 release from serum-stimulated human pulmonary artery smooth muscle cells. Under certain conditions, carbon monoxide appears to act as an endogenous break on endothelin-1 release.

Keywords: Biliverdin; Carbon monoxide (CO); Endothelin-1; Heme oxygenase; Pulmonary artery smooth muscle cell, human; Pulmonary hypertension

1. Introduction

The endothelins are a family of three 21-amino-acid peptides. Endothelin-1, the predominant isoform expressed in the vasculature, is primarily secreted by vascular endothelial cells but also by other cell types including fibroblasts (Zeballos et al., 1991) and vascular smooth muscle cells (Resink et al., 1990). In addition to its vasoactive properties, endothelin-1 has potent mitogenic properties. We have shown previously that human pulmonary artery smooth muscle cells, stimulated in culture with fetal calf serum, release endothelin-1, mediating cell proliferation in an autocrine fashion (Wort et al., 2001). Endothelin-1 release by these cells can be further stimulated using a combination of tumour necrosis factor-α and interferon-γ (Wort et al., 2002). The endothelin system has been implicated in a number of disease states including pulmonary hypertension, a disease characterised by abnormal vascular smooth muscle cell growth and remodelling of the pulmonary vasculature.

Heme oxygenase is the rate-limiting enzyme in the catabolism of heme to carbon monoxide, biliverdin and free iron. Biliverdin is then converted to bilirubin by biliverdin reductase. Heme oxygenase exists in two major isoforms. Heme oxygenase-2 is the constitutive form of the enzyme, localised to the endothelium (Zakhary et al., 1996). Heme oxygenase-1 is expressed at the site of inflammation and as a consequence of oxidative stress (Willis et al., 1996). There exists growing evidence in the literature that the heme oxygenase system may play a role in the pathology of pulmonary hypertension. In transgenic mice where heme oxygenase-1 is overexpressed pulmonary vascular remodelling, in response to chronic hypoxia, is reduced (Minamino et al., 2001). The mechanism by which heme oxygenase provides protection is not fully understood. Biliverdin and bilirubin are well-documented antioxidants which may protect the vasculature from oxidative stress and injury (Durante, 2002). Carbon monoxide, via activation of soluble guanylyl cyclase, causes vasodilatation in the systemic and pulmonary circulations (Sylvester and McGowan, 1978). In addition, carbon monoxide has anti-inflammatory, anti-apoptotic and anti-proliferative actions. Indeed, we have recently reported that a carbon monoxide-releasing molecule

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(Motterlini et al., 2002) inhibits serum-induced proliferation of human pulmonary artery smooth muscle cells in culture (Stanford et al., 2003).

The aim of this study was to investigate the effect of two distinct products of heme breakdown, carbon monoxide and biliverdin, on serum- and cytokine-stimulated endothelin-1 release by human pulmonary artery smooth muscle cells in culture.

2. Materials and methods

2.1. Cell culture

Human pulmonary arteries from healthy sections of lung were obtained from patients undergoing pulmonary resection at The Royal Brompton Hospital. Under sterile conditions, 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 with Dulbecco's modified Eagle's 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% fetal calf serum and nonessential amino acids. Cells, incubated in 5% CO_2/95% O_2 at 37 °C, reached confluence after approximately 4 weeks. Smooth muscle phenotype was confirmed by characteristic hill and valley morphology and routine staining of cultures for α-actin.

2.2. Cell treatment

Human pulmonary artery smooth muscle cells (passage 2–8), seeded at 10,000 cells/well in 96-well plates, were serum-deprived for 24 h to achieve quiescence. The cells were then stimulated for 24 h with supplemented medium containing 10% fetal calf serum in the presence or absence of tumour necrosis factor-α (10 ng/ml) plus interferon-γ (10 ng/ml). In some experiments the carbon monoxide-releasing molecule, tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$; 10 or 30 μM), vehicle (dimethyl sulphoxide) control, a chemically identical molecule lacking the carbonyl moiety (negative control), dichlorotetrakis(dimethyl sulfoxide)ruthenium(II) ($Ru(DMSO)_4Cl_2$; 30 μM) or biliverdin (30 or 100 μM) were included in the culture medium.

After 24 h, cell supernatant was removed and stored at − 80 °C prior to measurement of endothelin-1 release. Cell viability was assessed by the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide to formazan.

2.3. Measurement of endothelin-1 release

Endothelin-1 release was measured using a commercially available human endothelin-1 ELISA in accordance with the manufacturers instructions.

2.4. Materials

All materials for cell culture were purchased from GIBCO BRL Life Technologies (Paisley, UK). Endothelin-1 ELISA kits were from R&S Systems (Abingdon, UK). $[Ru(CO)_3Cl_2]_2$ was from Sigma-Aldrich (Poole, Dorset, UK). $Ru(DMSO)_4Cl_2$, synthesized as previously described (Alessio et al., 1995), was a generous gift from Dr. R. Motterlini and Prof. B.E. Mann. Biliverdin was from Frontier Scientific (Carnforth, Lancashire, UK).

3. Results

3.1. Serum-stimulated endothelin-1 release

As observed previously (Wort et al., 2001), in the presence of serum, human pulmonary artery smooth muscle cells released endothelin-1. The carbon monoxide-releasing molecule $[Ru(CO)_3Cl_2]_2$ inhibited serum-induced endothelin-1 release in a concentration-dependent manner (Fig. 1) but had no effect on cell viability (absorbance at 550 nm; basal vs. $[Ru(CO)_3Cl_2]_2$ (30 μM): 0.27 ± 0.01 vs. 0.27 ± 0.01, $n = 7$). The negative control, $Ru(DMSO)_4Cl_2$, had no significant effect on serum-induced endothelin-1 release (Fig. 1) or on cell viability (absorbance at 550 nm; basal vs. $Ru(DMSO)_4Cl_2$ (30 μM): 0.27 ± 0.01 vs. 0.26 ± 0.01, $n = 7$). Similarly, neither the carbon monoxide-releasing molecule vehicle, nor biliverdin effected serum-induced endothelin-1 release (Fig. 1) or cell viability (absorbance at 550 nm; basal vs. vehicle and biliverdin (100 μM), respectively: 0.27 ± 0.01 vs. 0.28 ± 0.02 and 0.26 ± 0.02, $n = 7$).

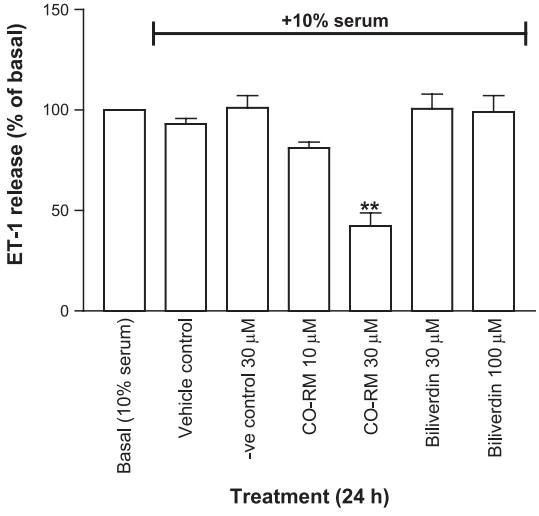

Fig. 1. Effect of the carbon monoxide-releasing molecule (CO-RM), its negative (-ve) control and biliverdin on serum-stimulated endothelin-1 release from human pulmonary artery smooth muscle cells. Data represents $n = 7$ using cells cultured from three patients. One-way ANOVA vs. vehicle control, post test Dunnett, $^{**}P < 0.01$.

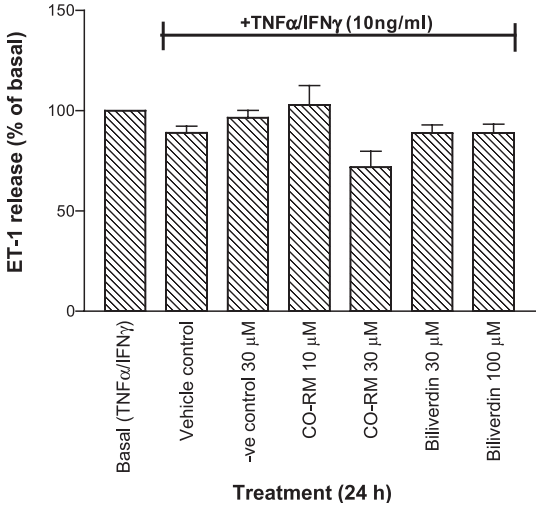

Fig. 2. Effect of the carbon monoxide-releasing molecule (CO-RM), its negative (-ve) control and biliverdin on tumour necrosis factor(TNF)-α/interferon(IFN)-γ-stimulated endothelin-1 release from human pulmonary artery smooth muscle cells in the presence of serum. Data represents $n = 7$ using cells cultured from three patients.

3.2. Serum-stimulated endothelin-1 release in the presence of tumour necrosis factor-α and interferon-γ

As demonstrated previously (Wort et al., 2002), endothelin-1 release from serum-stimulated human pulmonary artery smooth muscle cells was significantly increased in the presence of a combination of tumour necrosis factor-α and interferon-γ (5.69 ± 0.85 vs. 21.45 ± 2.13 pg/ml, $n = 7$).

The carbon monoxide-releasing molecule ($[Ru(CO)_3Cl_2]_2$) had no significant effect on tumour necrosis factor-α/interferon-γ-stimulated endothelin-1 release (one-way ANOVA vs. vehicle control, post test Dunnett) (Fig. 2). The trend indicated an inhibitory effect of the highest concentration (30 μM) of $[Ru(CO)_3Cl_2]_2$ on cytokine-induced endothelin-1 release. This might be explained by the inhibitory effect of the carbon monoxide-releasing molecule on the serum-induced component of endothelin-1 release. The carbon monoxide-releasing molecule vehicle control, negative control ($Ru(DMSO)_4Cl_2$) and biliverdin were unable to reverse the stimulatory effect of tumour necrosis factor-α/interferon-γ on endothelin-1 release.

4. Discussion

The endothelin-1 and heme oxygenase/carbon monoxide pathways have both been implicated in the pathology of pulmonary hypertension, a disease characterised by abnormal vascular smooth muscle cell growth and remodelling. Here we show that carbon monoxide, but not biliverdin, inhibits endothelin-1 release from serum-stimulated human pulmonary artery smooth muscle cells. However, neither carbon monoxide nor biliverdin was able to reverse the increased endothelin-1 release observed in the presence of a combination of tumour necrosis factor-α and interferon-γ.

Endothelin-1 is a potent vasoconstrictor and comitogen for vascular smooth muscle. We have previously shown that human pulmonary artery smooth muscle cells in culture release endothelin-1 in the presence of serum, thus mediating cell proliferation in an autocrine manner (Wort et al., 2001).

Heme oxygenase exists in two major isoforms, the constitutive heme oxygenase-2 and the inducible heme oxygenase-1. Heme oxygenase is the enzyme responsible for the breakdown of damaging heme to form carbon monoxide, biliverdin and free iron. Carbon monoxide is a vasodilator. Its effects on vascular remodelling are unclear (Morita et al., 1997; Carraway et al., 2002). Human pulmonary artery smooth muscle cells express heme oxygenase-2 under basal conditions and heme oxygenase-1 under conditions of oxidative stress (Stanford et al., 2003). We have shown that the carbon monoxide-releasing molecule, $[Ru(CO)_3Cl_2]_2$ (Motterlini et al., 2002), inhibits serum-induced proliferation of these cells (Stanford et al., 2003). In the current study we investigated the possibility that the inhibitory effects of carbon monoxide on serum-induced pulmonary artery smooth muscle cell proliferation are mediated via modulation of endothelin-1. In addition, we examined the effects of a second product of heme oxygenase, biliverdin, on endothelin-1 release from these cells.

We report that the carbon monoxide-releasing molecule $[Ru(CO)_3Cl_2]_2$, at a concentration that does not effect cell viability (this study) but completely blocks serum-induced cell proliferation (Stanford et al., 2003), inhibits serum-stimulated endothelin-1 release from human pulmonary artery smooth muscle cells. At the same concentration, the negative control for $[Ru(CO)_3Cl_2]_2$ (a chemically identical compound lacking the carbonyl moiety) had no such effect. Limited reports in the literature have demonstrated that carbon monoxide suppresses the production of endothelin-1 by endothelial cells (Kourembanas et al., 1997; Morita and Kourembanas, 1995); the authors did not examine the effects of biliverdin. We found that biliverdin, at a concentration reported to attenuate nitric oxide-induced apoptosis in smooth muscle cells (Thomas et al., 2001), had no effect on serum-stimulated endothelin-1 release from human pulmonary artery smooth muscle cells.

Serum is reported to release endothelin-1 from a number of cell types including endothelial cells (Mikkola et al., 1993), pulmonary (Wort et al., 2001) and systemic (Yu and Davenport, 1995) vascular smooth muscle cells. The active component of serum is unknown but platelet-derived growth factor is one candidate (Mikkola et al., 1993). Interestingly carbon monoxide, in addition to suppressing endothelin-1 production by endothelial cells, also decreases the expression of platelet-derived growth factor-B (Morita and Kourembanas, 1995). This may represent a mechanism by which carbon monoxide inhibits serum-induced endothelin-1 re-

lease from, and proliferation (Stanford et al., 2003) of, human pulmonary artery smooth muscle cells.

In line with previous studies (Wort et al., 2002), serum-induced endothelin-1 release from human pulmonary artery smooth muscle cells was increased in the presence of a combination of tumour necrosis factor-α and interferon-γ. Levels of endothelin-1 release are not affected by either cytokine alone (Wort et al., 2002). Neither carbon monoxide nor biliverdin was able to reverse tumour necrosis factor-α/interferon-γ-stimulated endothelin-1 release. This suggests that serum stimulates endothelin-1 release from human pulmonary artery smooth muscle cells via a different mechanism to tumour necrosis factor-α/interferon-γ. Tumour necrosis factor-α and interferon-γ are reported to induce their synergistic effect on endothelin-1 release from human pulmonary artery smooth muscle cells via the activation of nuclear factor-κB (Wort et al., 2003, unpublished observations). We would suggest that the active component in serum exerts its effect by a different pathway.

To conclude, under certain conditions, carbon monoxide appears to act as an endogenous break on endothelin-1 release from human pulmonary artery smooth muscle cells. This may underlie the anti-proliferative action of carbon monoxide on serum-stimulated cells. This study supports the idea that agents which manipulate the heme oxygenase/carbon monoxide pathway may have novel therapeutic potential in the treatment of pulmonary hypertension.

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