British Journal of Pharmacology (2008) 155, 1164–1173

0007–1188/08 $32.00
www.brjpharmacol.org

RESEARCH PAPER

Desensitization of the soluble guanylyl cyclase/cGMP pathway by lipopolysaccharide in rat isolated pulmonary artery but not aorta

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Background and purpose: To investigate the function of soluble guanylyl cyclase (sGC)/3′,5′-cyclic guanosine monophosphate (cGMP) pathway in lipopolysaccharide (LPS)-induced changes in vascular reactivity of rat isolated pulmonary artery and aorta.
Experimental approach: Nitric oxide (NO) production, contraction responses to endothelin-1 (ET-1), relaxation responses to sodium nitroprusside (SNP), 8-pCPT-cGMP, BAY412272 and T-0156, SNP-induced cGMP production and expression of $sGC_{\alpha1}$, $sGC_{\beta1}$ and 3′,5′-cyclic nucleotide phosphodiesterase-5 (PDE5) proteins were measured in LPS-treated pulmonary and aortic rings from male Wistar rats.
Key results: In both vessels, LPS (10 μg mL^{-1}, 20 h) increased NO production, which was inhibited by the selective inducible NOS (iNOS) inhibitor 1400W (1 μM). In the aorta, LPS decreased ET-1-induced contractility and this decrease was inhibited by the selective sGC inhibitor ODQ (10 μM) but not by removal of endothelium, or inhibitors of cyclooxygenase (indomethacin, 10 μM) or iNOS (1400W, 1 μM). Furthermore, aortic relaxation responses to the direct sGC activator BAY412272 were enhanced. In the pulmonary artery, SNP (1 nM to 30 μM)-induced relaxation and cGMP production, BAY412272-induced relaxation and $sGC_{\beta1}$ protein expression were decreased, whereas relaxation responses to the PDE5-specific inhibitor T-0156 (0.1–100 nM) were enhanced. Relaxation responses to the phosphodiesterase-resistant cGMP analogue, 8-pCPT-cGMP, and protein expression levels of $sGC_{\alpha1}$ and PDE5 were not altered in either vessel.
Conclusion and implications: LPS caused a selective hypocontractility of rat aorta to ET-1 mediated mainly through NO-independent sGC activation, whereas in the pulmonary artery, the effect of sGC activation was reduced by a decreased protein expression of $sGC_{\beta1}$ together with increased PDE5 activity.
British Journal of Pharmacology (2008) **155,** 1164–1173; doi:10.1038/bjp.2008.358; published online 22 September 2008

Keywords: LPS; NO; ET-1; cGMP; sGC; PDE5; pulmonary artery; aorta

Abbreviations: cGMP, 3′,5′-cyclic guanosine monophosphate; ET-1, endothelin-1; iNOS, inducible NOS; LPS, lipopolysaccharide; NO, nitric oxide; PDE5, 3′,5′-cyclic nucleotide phosphodiesterase-5; sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside

Introduction

Sepsis is a major cause of morbidity and mortality in hospitalized patients worldwide, with two main vasomotor complications, septic shock and pulmonary hypertension (Parsons *et al.*, 1989; Lorente *et al.*, 1993; Manthous *et al.*, 1993). Excessive nitric oxide (NO) production, derived mainly from inducible NOS (iNOS), has been shown to contribute to the development of acute lung injury, delayed hypotension and vasoplegia in patients with septic shock (Feihl *et al.*, 2001; Lopez *et al.*, 2004) as well as in animals injected with bacterial lipopolysaccharide (LPS) (Szabo *et al.*, 1995; Bishop-Bailey *et al.*, 1997). The most important receptor molecule for NO is NO-sensitive soluble guanylyl cyclase (sGC), which catalyses the formation of 3′,5′-cyclic guanosine monophosphate (cGMP). The amplitude and duration of a cGMP signal are determined by the activity of sGC, a heterodimeric cGMP-forming enzyme, and of the phosphodiesterases that degrade cGMP (Juilfs *et al.*, 1999). Only two sGC isoforms, α_1/β_1 and α_2/β_1, exist, with the α_1/β_1 sGC heterodimer being the predominant isoform in most tissues including lung (Friebe and Koesling, 2003). The major cGMP-degrading phosphodiesterase isoform in vascular smooth muscle is the cGMP-binding 3′,5′-cyclic nucleotide phosphodiesterase-5 (PDE5) (Maurice *et al.*, 2003).

Different strategies have been used to control excessive NO production in sepsis, such as inhibiting NO production

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Received 6 June 2008; revised 28 July 2008; accepted 11 August 2008; published online 22 September 2008

preferentially with selective iNOS inhibitors or by inhibiting NO-dependent pathways such as sGC; however, results have been conflicting. For example, the potent and highly selective iNOS inhibitor 1400W (Garvey *et al*., 1997) has been shown to prevent LPS-induced hypotension (Bachetti *et al*., 2003) and delay hypotension and circulatory failure in rats *in vivo* (Cuzzocrea *et al*., 2006), but it only partially reverses LPS-induced hyporeactivity to phenylephrine in rat superior mesenteric artery *in vitro* (O'Brien *et al*., 2001) and is not effective in rabbits (Bachetti *et al*., 2003). ODQ, an irreversible and highly selective haem-site inhibitor of sGC (Garthwaite *et al*., 1995), prevents aortic hypocontractility to norepinephrine *ex vivo* in rat (Chen *et al*., 2005) and both *in vitro* and *in vivo* in rat and mouse (Zingarelli *et al*., 1999). Conversely, ODQ is only partially effective in restoring vascular reactivity to phenylephrine in rat superior mesenteric artery *in vitro* (O'Brien *et al*., 2001) and *ex vivo* in rat aorta (Wu *et al*., 1998).

Previous studies have shown that LPS decreases sGC activity in rat lung and aorta (Fernandes *et al*., 2006), in cultured rat aortic (Papapetropoulos *et al*., 1996) and pulmonary arterial smooth muscle cells (Scott and Nakayama, 1998). PDE5 activity is increased in rat lung after LPS injection (Holzmann *et al*., 1996) and 1 h after LPS inhalation in guinea pig lungs (Toward *et al*., 2005) but decreased after 48 h in the same model. These studies have examined whole lung tissue or cultured pulmonary arterial smooth muscle cells, but there are no direct measurements published using whole pulmonary arteries. Furthermore, these changes in NO signalling pathways have not been related to vascular reactivity in the same experimental model.

Together with NO, plasma levels of the powerful vasoconstrictor endothelin-1 (ET-1) are elevated in animal models of sepsis (Curzen *et al*., 1997; Fujii *et al*., 2000) and in patients with sepsis (Pittet *et al*., 1991) and its level correlates with the severity of illness (Pittet *et al*., 1991; Piechota *et al*., 2007). Furthermore, endothelin antagonism attenuates pulmonary hypertension in porcine endotoxic shock (Wanecek *et al*., 1999) and lung injury in sheep (Kuklin *et al*., 2004), suggesting an important function of ET-1 in sepsis. However, the function of ET-1 and its relation to the NO/cGMP pathway in endotoxic shock remain unclear.

This study investigated the function of the sGC/cGMP pathway in LPS-induced changes in vascular reactivity to ET-1 in rat isolated pulmonary artery and aorta as representatives of pulmonary and systemic vascular beds, respectively. Our results suggest that, in the pulmonary artery, LPS treatment impaired sGC activation by decreasing protein expression of $sGC_{\beta 1}$ and by increasing PDE5 activity, whereas, in the aorta, LPS caused hypocontractility to ET-1 mainly through NO-independent activation of sGC.

Materials and methods

Tissue preparation

Animal housing and care was carried out in accordance with UK Home Office legislation and guidelines. In-house-bred male Wistar rats (250–300 g) were killed by cervical dislocation and the side branches of the pulmonary artery and the descending thoracic aorta were separated and placed in a cold oxygenated physiological solution composed of (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25 and glucose 11.1. The vessels were dissected free of fat and connective tissue and cut into rings (2–4 mm in length). Under sterile tissue culture conditions, vascular rings were then placed into 48-well plates, with each well containing one ring in 1 mL Dulbecco's modified Eagle medium supplemented with penicillin (100 unit mL^{-1}), streptomycin (100 $\mu g\,mL^{-1}$) and 10% foetal bovine serum. After 1 h stabilization, vascular rings were transferred to a fresh medium of the same composition (control group) or supplemented with LPS 10 $\mu g\,mL^{-1}$ (LPS-treated group) and incubated for 20 h at 37 °C in humidified atmosphere of 5% CO_2 in air. In some experiments, endothelium was removed mechanically by gentle rubbing before incubation. In other experiments, vascular rings were co-incubated with indomethacin (10 μM), 1400W (1 μM) or ODQ (10 μM). For NO measurement, phenol red-free Dulbecco's modified Eagle medium was used.

Measurement of NO release

In aqueous solutions, NO has a very short half-life and is oxidized to nitrite and nitrate, therefore NO production was determined by measuring the accumulation of these breakdown products in the culture medium (Bishop-Bailey *et al*., 1997). Nitrate was first reduced to nitrite and the total nitrite was measured spectrophotometrically by the Greiss reaction.

Pulmonary and aortic rings were divided into four groups: control, LPS, LPS + nitro-L-arginine methyl ester (100 μM) or LPS + 1400W (1 μM), with each ring incubated in 1 mL medium as described above. After 20 h incubation, 60 μL supernatant was taken from each well and samples were incubated for 60 min at 37 °C with 0.2 U mL^{-1} nitrite reductase, 50 μM NADPH and 5 μM flavin adenine dinucleotide disodium to reduce nitrate to nitrite. Any remaining NADPH, which absorbs at 550 nm, was oxidized by the addition of 10 U mL^{-1} lactate dehydrogenase and 10 mM sodium pyruvate for a further 10 min at 37 °C. A final sample volume of 100 μL was then mixed with an equal volume of modified Greiss reagent for 5 min in 96-well plates. Absorbance was measured at 550 nm by Versamax tunable microplate reader with Softmax Pro software (Molecular Devices, Wokingham, Berks, UK) and total nitrite concentration was calculated using the standard curves of sodium nitrate (0–128 nmol mL^{-1}) in culture medium run simultaneously in parallel.

In vitro *vascular reactivity studies*

Following 20 h of incubation, arterial rings from the control and the LPS-treated groups were mounted in an organ bath filled with 18 mL of the physiological salt solution at a temperature of 37 °C and bubbled with a mixture of 95% O_2 and 5% CO_2. Rings were allowed to equilibrate under 12 (aorta) and 7 (pulmonary artery) mN resting tension for 60 min, during which time the bath solution was replaced every 15 min and the resting tension was readjusted when necessary. Isometric tension generated by the vascular

smooth muscle was measured using a force displacement transducer (K30, Hugosachs Elektronik, March, Germany) and recorded with a MacLab 4S unit linked to a PC running Chart v4.2 software (ADInstruments Ltd, Chalgrove, Oxfordshire, UK).

At the beginning of each experiment, arterial ring responsiveness was assessed by measuring contraction to 80 mM KCl, and this procedure was repeated until consistent responses were obtained, and then rings were washed until tension returned to the baseline. To measure tissue contractility to ET-1, the vasoconstrictor was added cumulatively to the organ bath and concentration (0.3–100 nM)–response curves were constructed. To measure vasorelaxation, rings were first preconstricted with 30 nM ET-1, and after reaching a steady-state contraction (plateau), cumulative concentration–response curves to sodium nitroprusside (SNP) (1 nM to 30 μM), 8-pCPT-cGMP (0.1–100 μM), BAY412272 (1 nM to 10 μM) or T-0156 (0.1–100 nM) were constructed. In de-endothelialized preparations, endothelium removal was confirmed by the absence of relaxation to 1 μM acetylcholine. Appropriate vehicle control experiments were also conducted, where vehicle effects were not observed.

Assay of SNP-induced cGMP production

To assess changes in cGMP production, a NO donor (100 μM SNP) was used to stimulate cGMP production in the presence of a non-selective phosphodiesterase inhibitor (100 μM IBMX) to prevent cGMP degradation (Toward *et al*., 2005; Fernandes *et al*., 2006). Rings from control and LPS groups were transferred to a HEPES-buffered physiological solution composed of (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.5, $MgCl_2$ 1.2, HEPES 10 and glucose 11.1) and incubated for 1 h at 37 °C. Rings were then incubated with the non-selective phosphodiesterase inhibitor 100 μM IBMX for 30 min followed by incubation with 100 μM SNP for an additional 10 min. Rings were then quickly frozen and homogenized in ice-cold 6% trichloroacetic acid to give a 10% (w/v) homogenate. Homogenates were centrifuged at 2000 *g* for 15 min at 4 °C, the supernatant was recovered and the pellet was discarded. The supernatant was washed four times with 5 volumes of water-saturated diethyl ether, and the upper ether layer was discarded after each wash. The remaining aqueous extract was heated at 60 °C for 10 min to remove any traces of ether, then lyophilized, and the dried extract was dissolved in a suitable volume of assay buffer. cGMP was measured in duplicate by ELISA, using a commercially available enzyme immunoassay (R&D Systems Europe Ltd, Abingdon, UK) according to the manufacturer's instructions. Results were expressed as picomoles of cGMP per milligram of tissue weight.

Immunoblotting

After 20 h of incubation with either control or LPS, pulmonary and aortic rings were rapidly frozen in liquid nitrogen and stored at −80 °C until being used. Tissue was mechanically homogenized in 10 volumes of an ice-cold lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 7.5, 1% Nonidet P40, 10% glycerol, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethanesulphonyl fluoride and 1% protease inhibitor cocktail). Homogenates were centrifuged (18 000 *g* for 15 min at 4 °C) and supernatant protein concentration was measured by the Bradford method using BSA as a standard. Supernatant samples were mixed with 5 × SDS sample buffer (10% SDS, 200 mM Tris-HCl, pH 6.8, 50% glycerol, 5% 2-mercaptoethanol and 0.01% bromophenol blue) and heated at 100 °C for 5 min. Protein samples (40 μg per lane) were subjected to SDS-PAGE on a 7.5% gel and transferred to nitrocellulose membrane (Whatman, Maidstone, UK) by semidry transfer blot (Transblot SD cell, Bio-Rad, CA, USA). Blots were blocked by incubation for 1 h with Tris-buffered saline-Nonidet P40 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Nonidet P40) containing 5% non-fat milk with gentle shaking at room temperature to reduce non-specific binding. After blocking, membranes were subsequently incubated overnight at 4 °C with rabbit polyclonal anti-soluble guanylyl cyclase α_1 antibody (1: 10 000 dilution, Abcam, Cambridge, UK), rabbit polyclonal anti-soluble guanylyl cyclase β_1 antibody (1:4000 dilution, Abcam) or mouse monoclonal anti-phosphodiesterase 5 antibody (1:500 dilution, BD Biosciences, Oxford, UK) with gentle shaking. After washing with Tris-buffered saline-Nonidet P40 on the next day, blots were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:10 000 dilution in Tris-buffered saline-Nonidet P40 with 2% milk; Dako Cytomation, Glostrup, Denmark). Immunoreactive protein bands were detected by ECL kit (Amersham Biosciences, Little Chalfont, Bucks, UK) and visualized on an X-ray film (Fujifilm Corporation, Tokyo, Japan). The intensity of the specific bands was quantified by densitometric analysis using Labimage software (Kapelan Bio-imaging Solutions, Halle, Germany). Membranes were reprobed with rabbit polyclonal anti-β-actin antibody (1:1000 dilution, Cell Signaling Technologies, Danvers, MA, USA) to confirm equal loading of proteins.

Data analysis

Data are expressed as mean ± s.e.mean, where *n* equals the number of rats. Vascular relaxation was calculated as % of maximal steady state contraction induced by 30 nM ET-1. The highest response obtained was considered as the maximum response (E_{max}). Non-linear regression analysis (4-parameter curve fit) was carried out using Graphpad Prism software (Graphpad Software Inc., San Diego, CA, USA) and significant differences between groups were determined with paired Student's *t*-test or one-way ANOVA with Dunnett's *post hoc* test as appropriate.

Materials

Dulbecco's modified Eagle medium, penicillin–streptomycin solution and foetal bovine serum were purchased from Invitrogen (Paisley, UK), endothelin-1 (ET-1) from American Peptide Company (Sunnyvale, CA, USA), 1,2-dihydro-2-[(2-methyl-4-pyridinyl)methyl]-1-oxo-8-(2 -pyrimidinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride (T-0156), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and *N*-(3-aminomethyl)benzyl) acetamidine (1400W) from Tocris Bioscience (Avonmouth,

UK), 3-isobutyl-1-methylxanthine (IBMX), 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine (BAY412272) and reduced nicotinamide adenosine dinucleotide phosphate (NADPH) from Calbiochem (Merck Chemicals Ltd, Nottingham, UK). Nonidet P-40, LPS (*Escherichia coli* O55:B5), 8-(-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate sodium salt (8-pCPT-cGMP), protease inhibitor cocktail, modified Greiss reagent (G4410), lactate dehydrogenase from rabbit muscle, nitrate reductase (Aspergillus), flavin adenine dinucleotide disodium, indomethacin and Nω-nitro-L-arginine methyl ester were purchased from Sigma-Aldrich Co. (Gillingham, Dorset, UK). Nomenclature of all receptors, drugs, enzymes and ion channels is according to the Guide to Receptors and Channels (Alexander *et al*., 2008).

Results

Effect of LPS treatment on NO release

Incubation of the pulmonary artery and the aorta with LPS caused a significant (5- to 10-fold) increase ($P<0.01$, $n=5$) in NO release (Figure 1). The increased levels of NO production after LPS exposure were not significantly different between the two preparations. Co-incubation of pulmonary and aortic rings with LPS together with either the non-specific NOS inhibitor nitro-L-arginine methyl ester (100 μM) or the iNOS-specific inhibitor 1400W (1 μM) prevented the LPS-induced overproduction of NO in both vessels (Figure 1).

Effect of LPS treatment on vascular contractility

Incubation of arterial rings with LPS had a differential effect on ET-1-mediated contraction in the two types of blood vessels (Figure 2a). In the aorta, a significant decrease ($P<0.01$, $n=9$) in tissue contractility to ET-1 was observed in the LPS-treated group compared with the control group. This hyporeactivity to ET-1 was manifested both by a 26 ± 4% decrease in E_{max} and an increase in EC_{50} in the LPS-treated group, relative to control values. In the pulmonary artery, however, no significant changes in contraction induced by ET-1 were found between control and LPS-treated groups. Similar aortic hyporeactivity to KCl and phenylephrine was also observed, without the pulmonary artery responses being affected (data not shown). In control experiments, there were no significant differences between freshly isolated and 20-h-incubated vascular rings, indicating that tissue responsiveness was not adversely influenced by prolonged incubation.

A decreased contractility to ET-1 in LPS-treated aorta was not affected by tissue preincubation with the iNOS-specific inhibitor 1400W (1 μM), the COX inhibitor indomethacin (10 μM) or by removal of endothelium (Table 1). Also, these treatments did not significantly alter ET-1-mediated contractility in the pulmonary artery (Table 1). In contrast, inhibition of sGC by incubation of tissues with 10 μM ODQ significantly prevented the LPS-induced decrease in contraction to ET-1 in the aorta, whereas the pulmonary artery was not affected (Figure 2b).

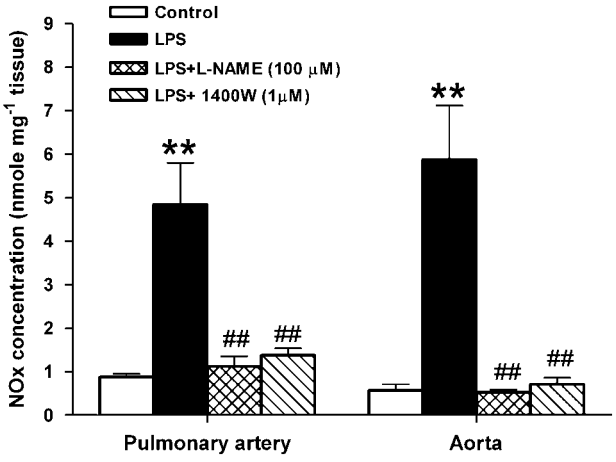

Figure 1 Lipopolysaccharide (LPS) treatment increased nitric oxide (NO) release from isolated rat pulmonary artery and aorta. NO release (total amount of nitrite plus nitrate, NOx) was determined in the supernatant spectrophotometrically using Greiss reagent after reduction of nitrate to nitrite. LPS-induced NO overproduction was inhibited by co-incubation with either nitro-L-arginine methyl ester (L-NAME, 100 μM) or 1400W (1 μM) ($n=5$). $^{**}P<0.01$ compared with control group, $^{\#\#}P<0.01$ compared with LPS group using one-way ANOVA followed by Dunnett's *post hoc* test.

Effect of LPS treatment on vascular relaxation

Vascular relaxation to SNP was significantly decreased ($P<0.01$, $n=6$) by LPS in the pulmonary artery, as manifested by a 36 ± 6% decrease in E_{max} in the LPS-treated group, relative to the control value, whereas no significant differences in SNP-induced vasorelaxation was found in the aorta (Figure 3a). In addition, vascular relaxation to the NO-independent direct sGC activator BAY412272 was significantly decreased ($P<0.01$, $n=4$) by LPS in the pulmonary artery, as manifested by a 32 ± 8% decrease in E_{max}, whereas this BAY412272-induced vasorelaxation was significantly increased ($P<0.01$, $n=4$) in the aorta as manifested by a 15 ± 7% increase in E_{max} (Figure 3b). To assess whether LPS-mediated pulmonary hyporeactivity to SNP was on account of decreased cGMP or impaired downstream cGMP-effector signalling molecules (such as protein kinase (PK)G); the vasorelaxation response to the phosphodiesterase-resistant cGMP analogue 8-pCPT-cGMP was studied. LPS did not significantly affect relaxation to 8-pCPT-cGMP either in the pulmonary artery or in the aorta (Figure 3c), suggesting that pulmonary hyporeactivity to SNP is likely to be because of either decreased cGMP synthesis by sGC or increased cGMP degradation (mainly by PDE5), or both.

Effect of LPS treatment on SNP-induced cGMP production

Incubation with LPS significantly decreased ($P<0.05$, $n=4$) the SNP-stimulated cGMP production in the pulmonary artery by 24 ± 4%, relative to the control value, whereas cGMP levels observed in the aorta were not significantly affected (Figure 4).

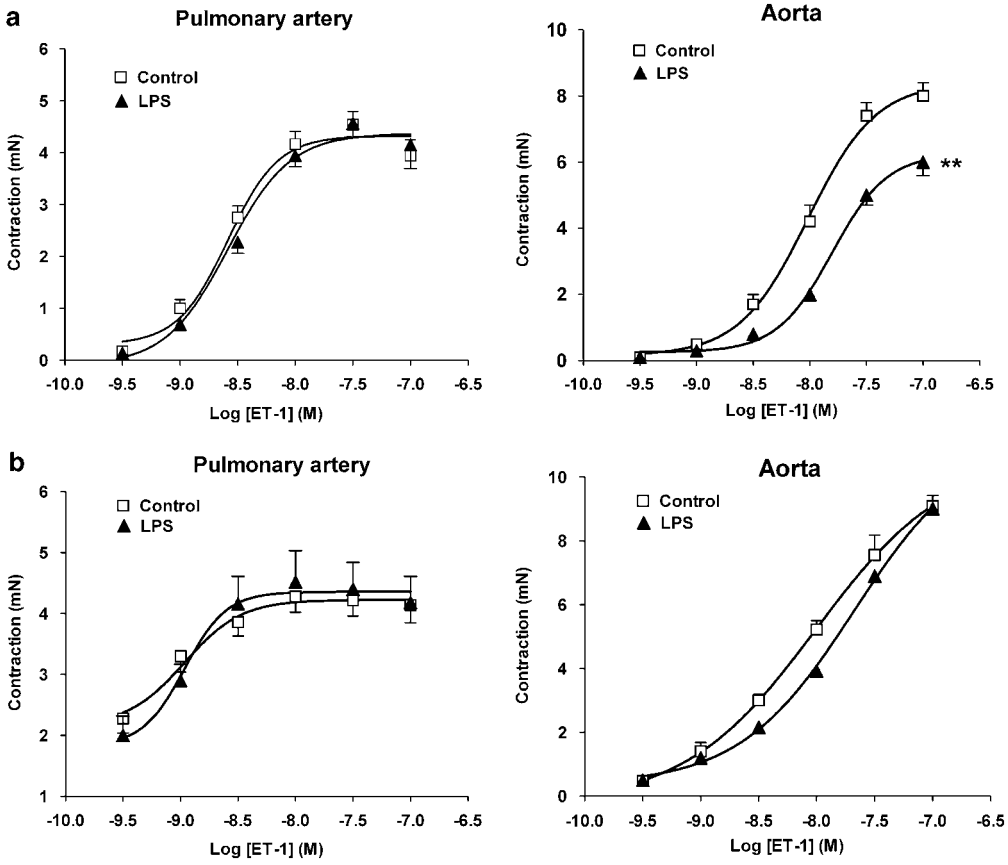

Figure 2 The specific soluble guanylyl cyclase (sGC) inhibitor ODQ prevented the lipopolysaccharide -induced decrease in ET-1-mediated contraction in the aorta. (**a**) Contraction to ET-1 (0.3–100 nM) was measured after incubation with LPS in the pulmonary and aortic arterial rings ($n=9$). (**b**) Pulmonary and aortic arterial rings were incubated with 10 μM ODQ before measuring contraction to ET-1 (0.3–100 nM) ($n=6$). **$P<0.01$ for E_{max} and pEC_{50} values compared with control group using paired Student's *t*-test.

Table 1 LPS-induced changes in vascular reactivity of isolated rat pulmonary artery and the aorta

Treatment	*Pulmonary artery*		*Aorta*	
	Control	*LPS*	*Control*	*LPS*
1400W				
pEC_{50}	8.94 ± 0.04	8.95 ± 0.05	8.19 ± 0.03	7.90 ± 0.04**
E_{max}	4.77 ± 0.65	4.97 ± 0.59	10.28 ± 0.69	8.62 ± 0.41**
Indomethacin				
pEC_{50}	8.56 ± 0.03	8.53 ± 0.02	8.01 ± 0.05	7.70 ± 0.04**
E_{max}	5.83 ± 0.51	6.00 ± 0.81	10.77 ± 0.32	8.70 ± 0.44**
Removal of endothelium				
pEC_{50}	8.86 ± 0.06	8.99 ± 0.06	8.34 ± 0.07	7.98 ± 0.03**
E_{max}	4.00 ± 0.10	4.22 ± 0.14	9.20 ± 0.60	6.20 ± 0.60**

Data are expressed as mean ± s.e.mean, $n=6$. E_{max} values are expressed in mN.
**$P<0.01$, significantly different compared with the corresponding control group using paired Student's *t*-test.

Effect of LPS on protein expression levels of sGC subunits

In the pulmonary artery, LPS pretreatment caused a significant decrease ($P<0.01$, $n=4$) in protein expression levels of $sGC_{\beta 1}$ subunit by 36 ± 7% (protein density ratio to β-actin), whereas the expression of the $sGC_{\alpha 1}$ subunit was not significantly affected (Figure 5). In contrast, LPS did not significantly change the protein expression levels of either sGC subunits in the aorta (Figure 5). As both subunits are required for sGC function, the decrease in the protein expression level of one subunit ($sGC_{\beta 1}$ in the pulmonary artery) would impair sGC activity.

Function of PDE5 in LPS-induced changes in vasorelaxation

The function of PDE5 activity in LPS-induced impairment in vasorelaxation in the pulmonary artery was investigated using the potent and highly selective PDE5 inhibitor T-0156. Vasorelaxation in response to T-0156 was significantly increased ($P<0.01$, $n=6$) by LPS in the pulmonary artery, as manifested by a 24 ± 4% increase in E_{max} in the LPS-treated group, relative to control values, but not in the aorta (Figure 6a).

For the protein expression levels of PDE5, two bands were obtained at 95 and 85 kDa as PDE5 is dimeric (Lin *et al.*, 2006). LPS treatment of the pulmonary artery and the aorta did not significantly alter PDE5 protein expression levels in either vessel (Figures 6b and c). These results therefore suggest that the LPS-induced increase in the effects of T-0156 in the pulmonary artery was likely to be due to changes in PDE5 activity.

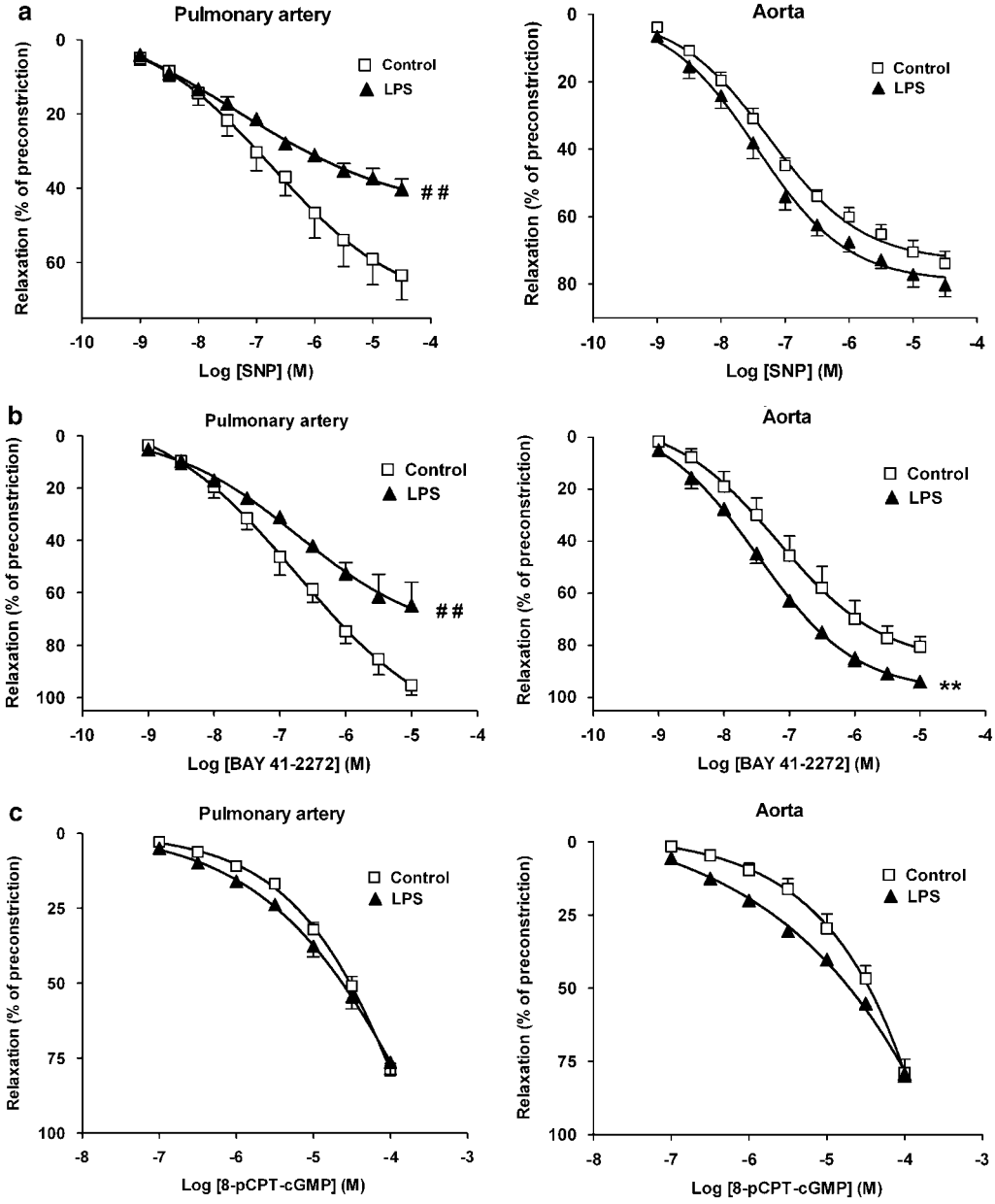

Figure 3 Sodium nitroprusside (SNP), BAY412272 and 8-pCPT-cGMP-induced relaxation responses were differentially modulated by lipopolysaccharide in pulmonary and aortic vessels. Pulmonary and aortic arterial rings were preconstricted with 30 nM ET-1 before measuring relaxation to (**a**) SNP (1 nM to 30 μM, $n=6$), (**b**) the nitric oxide-independent direct sGC activator BAY412272 (1 nM to 10 μM, $n=4$) or (**c**) the non-hydrolysable cGMP analogue 8-pCPT-cGMP (0.1–100 μM, $n=5$). $^{\#\#}P<0.01$ for E_{max} values, $^{**}P<0.01$ for E_{max} and pEC_{50} values compared with control group using paired Student's *t*-test.

Discussion and conclusion

The results presented here show that LPS activates sGC through a NO-independent pathway in the aorta. The effect of this activation is impaired in the pulmonary artery by decreasing protein expression of $sGC_{\beta 1}$ and by increasing PDE5 activity. As a consequence, LPS causes selective hypocontractility to ET-1 only in the aorta, whereas the pulmonary artery is not affected.

In both vessels, LPS significantly increased NO production through iNOS, as it was blocked by the selective iNOS inhibitor 1400W (1 μM). This NO overproduction by LPS through iNOS was reported previously in the aorta (Griffiths *et al*., 1995) and in the pulmonary artery (Bishop-Bailey *et al*., 1997). We found, however, that ET-1-mediated contraction was significantly impaired only in the aorta but not in the pulmonary artery, a finding similar to observation in clinical sepsis where systemic vasodilation and pulmonary hypertension occur at the same time (Lorente *et al*., 1993; Manthous *et al*., 1993) and to an *ex vivo* rat endotoxemic model using phenylephrine (McIntyre *et al*., 1997). We have examined LPS-induced hypocontractility at the 20 h time point only, but the expression levels of several relevant genes (ET-1, ET_A, ET_B, eNOS and iNOS) at 4, 8 and 20 h in the aorta

and pulmonary artery appear to change in parallel (data not shown). This does not exclude the possibility of other LPS effects at different time points.

Neither endothelial removal nor inhibition of COX affected vascular changes induced by LPS, consistent with previous reports (McKenna, 1990; O'Brien *et al*., 2001), suggesting that these are not involved in LPS-induced aortic hypocontractility to ET-1. Moreover, inhibition of iNOS by 1400W was unable to prevent this aortic hypocontractility to ET-1, although the same concentration of 1400W suppressed the NO overproduction in both vessels. On the other hand, incubation with the sGC inhibitor ODQ prevented aortic LPS-induced hypocontractility to ET-1, leading us to conclude that the hypocontractility is mediated through NO-independent regulators of sGC activity. In addition, the aortic relaxation responses to the new non-NO-based haem-dependent sGC activator BAY412272 (Stasch *et al*., 2001; Boerrigter and Burnett, 2007) were enhanced in the aorta, confirming that LPS treatment enhances NO-independent sGC activation in the aorta. Our experiments show that inhibition of sGC could be more effective than NOS inhibition in preventing vascular hyporeactivity induced by LPS.

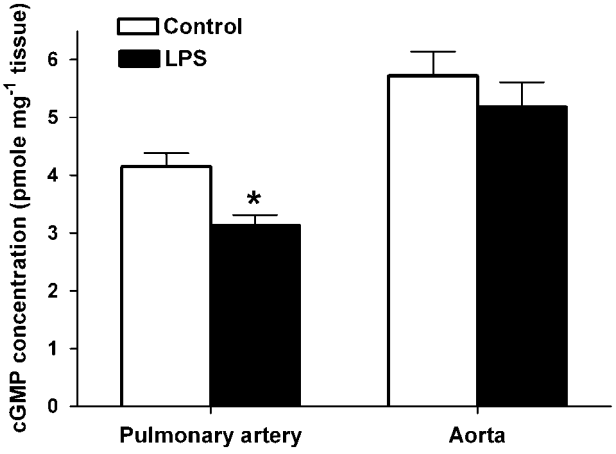

Figure 4 Lipopolysaccharide (LPS) selectively decreased sodium nitroprusside (SNP)-induced cGMP production in the pulmonary artery. Pulmonary and aortic rings from LPS-treated and control groups were stimulated by SNP (100 μM for 10 min) in the presence of the non-selective phosphodiesterase inhibitor IBMX (100 μM) and cGMP levels were determined by ELISA ($n=4$). *$P<0.05$ compared with control group using paired Student's *t*-test.

Lipopolysaccharide treatment in our study decreased pulmonary artery relaxation responses to both the NO donor SNP and the sGC activator BAY412272, suggesting that LPS treatment impaired the activation (both NO-dependent and NO-independent) of the sGC/cGMP pathway in the pulmonary artery. This impairment in sGC/cGMP pathway could be either due to an altered cGMP synthesis and/or metabolism or due to downstream changes in cGMP-effector signalling molecules such as PKG. Because the relaxation to the non-hydrolysable cGMP analogue 8-pCPT-cGMP was not affected in the LPS-treated vessels, cGMP-effector signalling molecules are not involved; leaving the possibility that LPS modified cGMP synthesis and/or degradation.

Involvement of a reduced synthesis of cGMP in the pulmonary artery was indicated by the decreased sGC activity and was confirmed by a significant decrease in the expression of the $sGC_{\beta1}$ subunit. As both sGC subunits are required for the enzyme activity, reduced levels of either subunit lead to reduced sGC activity. Importantly, $sGC_{\beta1}$ contains the major haem-binding domain and seems to be

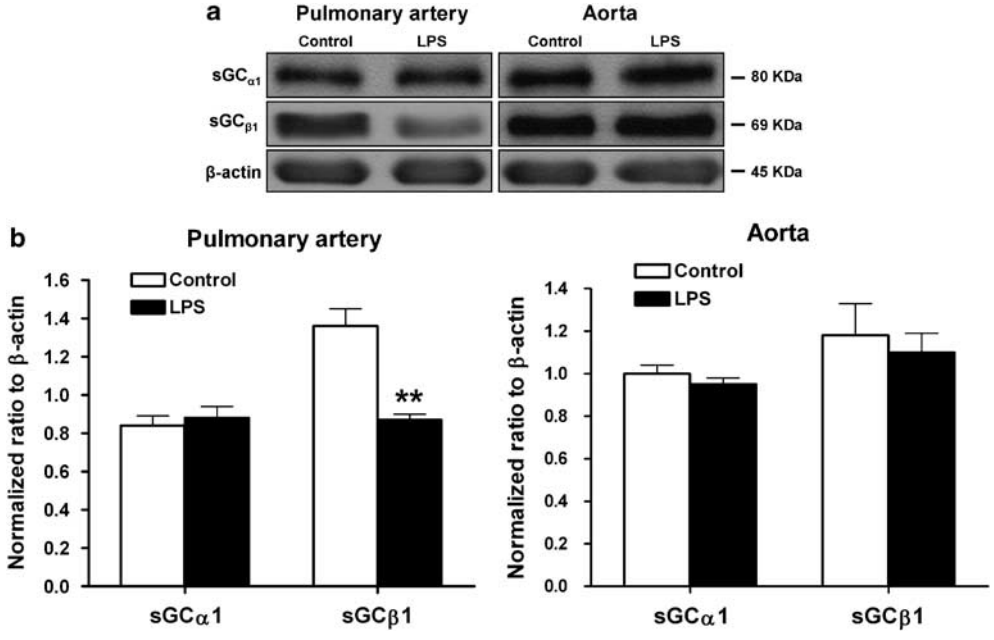

Figure 5 Lipopolysaccharide (LPS) decreased protein expression levels of soluble guanylyl cyclase-β1 ($sGC_{\beta1}$), but not $sGC_{\alpha1}$, in the pulmonary artery. (**a**) Representative immunoblots for $sGC_{\alpha1}$ and $sGC_{\beta1}$ in the pulmonary artery (left) and the aorta (right). (**b**) Densitometric ratio of sGC protein subunits normalized to β-actin in the pulmonary artery (left) and the aorta (right) ($n=4$). **$P<0.01$ compared with control group using paired Student's *t*-test.

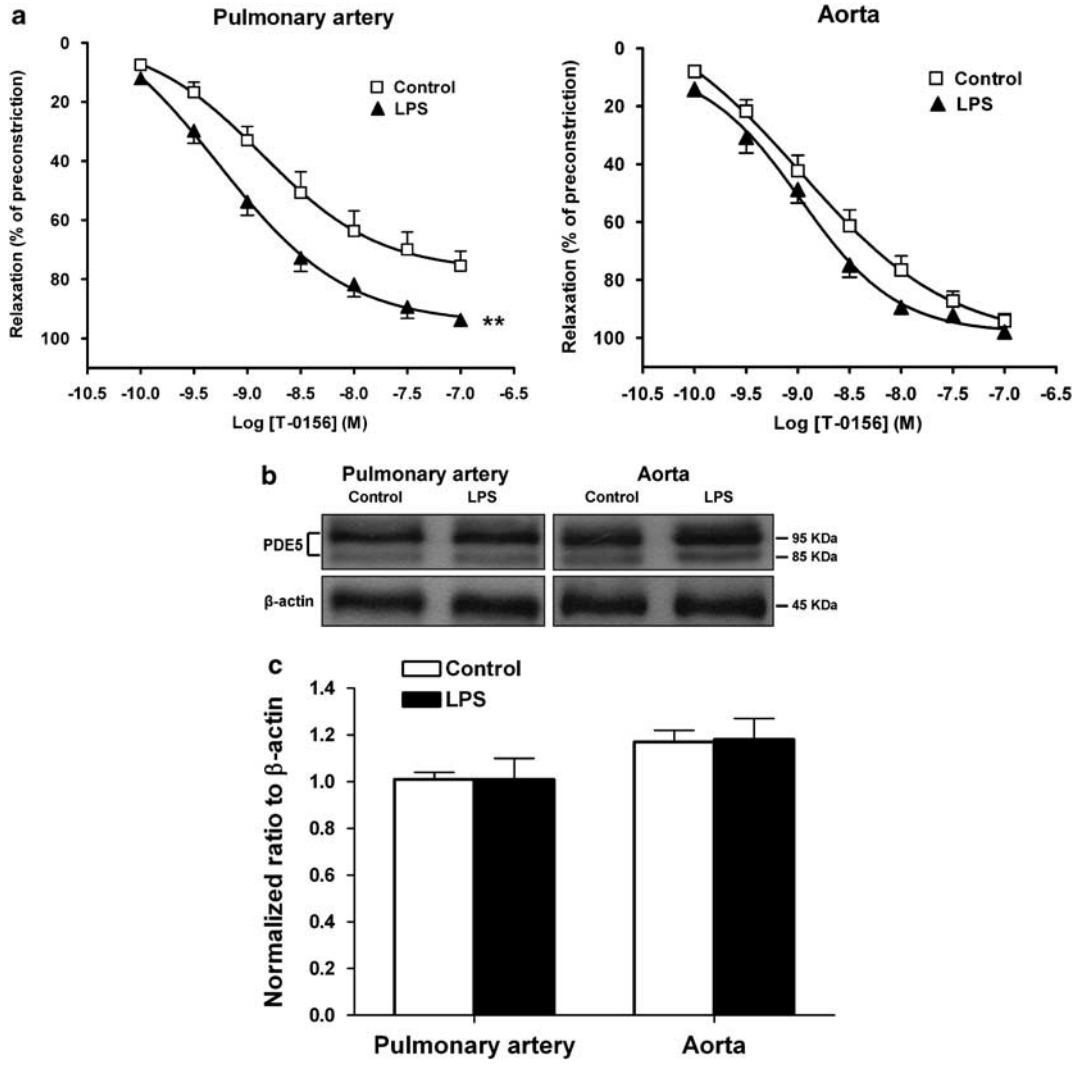

Figure 6 Selective increases in T-0156-induced relaxation by lipopolysaccharide (LPS) treatment in the pulmonary artery without changes in PDE5 protein expression levels. (**a**) Pulmonary and aortic arterial rings were preconstricted with 30 nM ET-1 before measuring relaxation to T-0156 (0.1–100 nM) ($n=6$). (**b**) Representative immunoblots for PDE5 in the pulmonary artery (left) and the aorta (right). (**c**) Densitometric ratio of PDE5 protein normalized to β-actin in the pulmonary artery (left) and the aorta (right) ($n=4$). $^{**}P<0.01$ for E_{max} and pEC_{50} values compared with control group using paired Student's *t*-test.

more rapidly and robustly regulated after inflammatory stimuli (Takata *et al*., 2001; Friebe and Koesling, 2003). Although previous studies demonstrate the involvement of sGC modulation in LPS-induced effects, these studies detected sGC in whole lung tissue or in isolated vascular smooth muscle cells, but not in isolated pulmonary arteries. For example, LPS decreased sGC activity and $sGC_{\beta1}$ protein expression in rat lung (Fernandes *et al*., 2006) and the expression of $sGC_{\beta1}$ mRNA and both sGC protein subunits in mice lung (Glynos *et al*., 2007). It is noteworthy that LPS decreased $sGC_{\alpha1}$ but not $sGC_{\beta1}$ expression in cultured rat aortic (Papapetropoulos *et al*., 1996) or pulmonary (Scott and Nakayama, 1998) smooth muscle cells, raising the possibility that expression of sGC subunits may be influenced by cell isolation or culture.

Besides reduced cGMP synthesis by sGC, an increased degradation of cGMP by PDE5 could also contribute to NO hyporeactivity in the pulmonary artery. This was directly confirmed using T-0156; a potent and highly selective PDE5 inhibitor (Mochida *et al*., 2002). As the enhanced pulmonary artery relaxation to T-0156 in LPS-treated tissue was not associated with changes in protein expression of PDE5, the most likely explanation is that the activity of PDE5 was increased. An increased PDE5 activity was demonstrated in perfused rat lungs isolated from rats 18 h after LPS injection (Holzmann *et al*., 1996) and 1 h after LPS inhalation in guinea pig lungs (Toward *et al*., 2005) but decreased after 48 h in the same model. The mechanism for increased PDE5 activity could include PKG-mediated phosphorylation and allosteric cGMP binding (either of which upregulate PDE5 activity) and a decreased PP1 phosphatase activity and/or expression (Lin *et al*., 2006). The latter will lead to a decreased dephosphorylation of PDE5, leading to maintained or enhanced PDE5 activity (Lin *et al*., 2006). As our results with the non-hydrolysable cGMP analogue suggest that downstream cGMP-effector signalling molecules are not

affected by pretreatment of vessels with LPS, downregulation of PP1 phosphatase activity and/or expression seems likely.

In sepsis, excessive vasodilation is counterbalanced by increased release of vasoconstrictors such as ET-1 and angiotensin II (Knotek *et al*., 2000), therefore reduced sensitivity to sGC activation and increased PDE5 activity in pulmonary arteries could contribute to the development of pulmonary hypertension during sepsis. These mechanisms could also explain, at least in part, why a significant fraction (up to 30%) of patients with pulmonary hypertension fail to respond to therapeutic doses of inhaled NO (Holzmann *et al*., 1996).

To summarize, we have demonstrated that LPS treatment *in vitro* causes a selective hypocontractility of rat aorta to ET-1, which is largely mediated by NO-independent activation of sGC. The pulmonary artery is not affected because LPS induces a desensitization of the sGC/cGMP-dependent pathway by decreasing protein expression levels of $sGC_{\beta 1}$, and hence sGC activity, and increasing PDE5 activity. Therefore, sGC and/or PDE5-selective inhibitors could be important in controlling systemic and pulmonary vasomotor complications in sepsis.

Acknowledgements

This study was supported by grant from the Egyptian Government (PhD studentship MM31/04 to MSHEA). We gratefully acknowledge the technical assistance of Kevin Smith.

Conflict of interest

The authors state no conflict of interest.

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