British Journal of Pharmacology (2004) 141, 488–496
www.nature.com/bjp

Iloprost inhibits superoxide formation and gp91^{phox} expression induced by the thromboxane A_2 analogue U46619, 8-isoprostane $F_{2\alpha}$, prostaglandin $F_{2\alpha}$, cytokines and endotoxin in the pig pulmonary artery

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1 Since the roles of thromboxane A_2 (TXA_2), prostacyclin (PGI_2) and 8-isoprostane $F_{2\alpha}$ in mediating vascular $O_2^{\cdot-}$ formation and its relation to adult respiratory distress syndrome (ARDS) is unknown, the effects of these eicosanoids on the expression of gp91^{phox} (catalytic subunit of NADPH oxidase) and $O_2^{\cdot-}$ release from cultured pig pulmonary artery (PA) segments, PA vascular smooth muscle cells (PAVSMCs) and PA endothelial cells (PAECs) were investigated.

2 PA segments, PAVSMCs and PAECs were incubated with the TXA_2 analogue, U46619, ($\pm$LPS, tumour necrosing factor-alpha (TNF-α) or IL-1α), 8-isoprostane $F_{2\alpha}$ and $\pm$iloprost (a stable PGI_2 analogue) for 16 h. The formation of superoxide dismutase-inhibitable $O_2^{\cdot-}$ was then measured spectrophotometrically and gp91^{phox} expression assessed using Western blotting. In parallel experiments, whole PA segments were treated with LPS, TNF-α and IL-α after which time TXA_2, PGI_2, $PGF_{2\alpha}$ and 8-isoprostane $F_{2\alpha}$ formation was measured using enzyme-linked immunoassays.

3 U46619, $PGF_{2\alpha}$ and 8-isoprostane $F_{2\alpha}$ promoted the formation of $O_2^{\cdot-}$ in PA segments, PAVSMCs and PAECs, an effect inhibited by diphenyleneiodonium and apocynin (both NADPH oxidase inhibitors) and upregulated the expression of gp91^{phox} in PAECs and PAVSMCs. These effects were augmented by LPS, TNF-α and IL-1α but inhibited by iloprost. Under identical incubation conditions, IL-1α, LPS and TNF-α all induced an increase in the formation of TXA_2, $PGF_{2\alpha}$ and 8-isoprostane $F_{2\alpha}$ but reduced the concomitant formation of PGI_2.

4 These data demonstrate that LPS and cytokines influence the relative balance of TXA_2, PGI_2, $PGF_{2\alpha}$ and 8-isoprostane $F_{2\alpha}$ in pig PA, which in turn alter NADPH oxidase expression and $O_2^{\cdot-}$ formation. These novel findings have implications in devising effective strategies for treating ARDS.

British Journal of Pharmacology (2004) **141,** 488–496. doi:10.1038/sj.bjp.0705626

Keywords: Superoxide; thromboxane; prostacyclin; isoprostane; pig pulmonary artery

Abbreviations: ARDS, adult respiratory distress syndrome; $O_2^{\cdot-}$, superoxide; OS, oxidative stress; PAECs, pulmonary artery endothelial cells; PAVSMCs, pulmonary artery vascular smooth muscle cells

Introduction

Oxidative stress (OS) plays a central role in the aetiology of adult respiratory distress syndrome (ARDS) (Chabot *et al*., 1998), a condition characterised by a time-dependent worsening of intrapulmonary inflammation and hypertension (Weinacker & Vaszar, 2001). Principal among the reactive oxygen species (ROS) generated by OS is superoxide ($O_2^{\cdot-}$), which reacts with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$), promoting not only vasoconstriction but also the adhesion of leucocytes and platelets (Stuart-Smith & Jeremy, 2001).

In sepsis-induced ARDS, endotoxins trigger the adhesion of platelets and leucocytes to the pulmonary vascular endothelium, which then release a battery of cytokines including tumour necrosing factor-alpha (TNF-α), interleukins (ILs) and thromboxane A_2 (TXA_2) (Jeremy *et al*., 1994b). TXA_2 is a potent vasoconstrictor and promoter of platelet aggregation and its involvement in ARDS is well established (Jeremy *et al*., 1994b; Ermert *et al*., 2000a, b). Other vasoconstrictors, such as angiotensin II and endothelin-1 have also been shown to upregulate NADPH oxidase that generates $O_2^{\cdot-}$ (Sorescu *et al*., 2001). OS also promotes the formation of isoprostanes, which exert similar effects as TXA_2 (Jourdan *et al*., 1997; Minuz *et al*., 1998; Audoly *et al*., 2000; Ekmekcioglu *et al*., 2002; Morrow & Roberts, 2002; Roberts & Morrow, 2002), and have been implicated in the pathophysiology of ARDS (Jourdan *et al*., 1997; Minuz *et al*., 1998; Audoly *et al*., 2000; Ekmekcioglu *et al*., 2002; Morrow & Roberts, 2002; Roberts & Morrow, 2002). It is reasonable to suggest that such a TXA_2/isoprostane-mediated effect may play an additional role in the aetiology of ARDS.

In contrast to TXA_2, vascular tissues also generate the protective prostanoid, prostacyclin (PGI_2) a vasodilator and inhibitor platelet and leucocyte adhesion (Jeremy *et al*., 1997;

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Advance online publication: 12 January 2004

Ullrich *et al*., 2001). The importance of PGI_2 in ARDS is exemplified by the therapeutic benefits of inhalational PGI_2 administration to patients with ARDS (Lowson, 2002). Cytokines also augment the formation of TXA_2 but reduce the formation of PGI_2 in vascular tissue (Jeremy *et al*., 1994b). Whether PGI_2 modulates $O_2^{\cdot-}$ formation or NADPH oxidase expression is also unknown.

In order to study this area further, the effect of endotoxin and cytokines on the formation of PGI_2, TXA_2, $PGF_{2\alpha}$ and 8-isoprostane $F_{2\alpha}$ by porcine pulmonary arteries was investigated. The effect of TXA_2 analogue, U46619, 8-isoprostane $F_{2\alpha}$ and $PGF_{2\alpha}$ on $O_2^{\cdot-}$ formation and the expression of $gp91^{phox}$, an active catalytic subunit of NADPH oxidase (Sorescu *et al*., 2001), was also investigated. The effect of the PGI_2 analogue, iloprost, on $O_2^{\cdot-}$ formation and the expression of $gp91^{phox}$ in response to cytokines, endotoxin, U46619, 8-isoprostane $F_{2\alpha}$ and $PGF_{2\alpha}$ was then studied.

Methods

Dissection and incubation of pulmonary arteries

Lungs were obtained from White Landrace male pigs of body weight ranging from 20 to 35 kg. All animals were given humane care in compliance with the rules and regulations of Bristol University and the UK Home Office. Pigs were anaesthetised with an intravenous injection of ketamine hydrochloride (10 mg kg^{-1}; Ketaset Injection, Fort Dodge Animal Health, Southampton, U.K.) and inhaled halothane (1–2% in oxygen), exsanguinated and lungs removed. Pulmonary arteries (PA; 3–4 mm diameter) were dissected out and placed in Dulbecco's minimum essential medium with Glutamax-1 (DMEM; GibcoBRL, Paisley, Scotland) and cut into 2 mm^2.

Pulmonary artery vascular smooth muscle cells (PAVSMCs) and pulmonary artery endothelial cells (PAECs) were prepared as previously described (Chaudhari *et al*., 1990; Southgate *et al*., 1992). PAECs were grown in an endothelial cell growth medium (PromoCell, Heidelberg, Germany) at 37°C in a 95% air–5% CO_2 incubator. PAVSMCs were maintained in DMEM (containing 10% foetal-calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) at 37°C in a 95% air–5% CO_2 incubator. Subconfluent cultures of pulmonary VSMCs were growth-arrested by washing in sterile phosphate-buffered saline (PBS, GibcoBRL) and incubating in a quiescing medium (serum-free DMEM supplemented with 0.5% lactalbumin hydrolysate, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) for 72 h.

Effect of LPS and cytokines on eicosanoid formation by intact PA segments

PA segments were incubated in serum-free DMEM (supplemented with 100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin) and containing LPS (1 μg ml^{-1}; *Escherichia coli*; 026:B6; Sigma Chemical Co., Poole Dorset, U.K.), human recombinant IL-1α (10 ng ml^{-1}; R&D Systems, Abingdon, U.K.) or human recombinant TNF-α (10 ng ml^{-1}; R&D Systems) for 16 h at 37°C in a 95% air–5% CO_2 incubator. It was essential to use serum-free conditions since serum contains large amounts of platelet and leucocyte release substances, including cytokines and eicosanoids, which in turn would have rendered responses to cytokines and eicosanoids indiscernible in the present study.

After washing, segments were placed in Hank's balanced salt solution (HBSS; GibcoBRL) and eicosanoid formation were stimulated with calcium ionophore A23187 (10 μM final; Sigma Chemical Co.) since basal eicosanoid release was below the lower limit of detection of the assay. A23187 elicits an increase in intracellular calcium through the formation of artificial calcium channels. Ca^{2+} then activates phosholipase A_2, which releases arachidonic acid from endogenous phospholipid stores (Jeremy *et al*., 1994a). Arachidonic acid is then converted to different eicosanoids by cyclooxygenase and different synthesising enzymes, including PGI_2 and TXA_2 synthase (Jeremy *et al*., 1994a).

After incubation for 30 min at 37°C, supernatants were removed and aliquots taken for the measurement of TXA_2 (as TXB_2) and PGI_2 (as 6-keto-$PGF_{1\alpha}$), 8-isoprostane $F_{2\alpha}$ and $PGF_{2\alpha}$ (Alexis Corporation, U.K.) using enzyme-linked immunoassay kits. The roles of $O_2^{\cdot-}$ and NO were explored using apocynin (1 μM; Sigma Chemical Co. an inhibitor of NADPH oxidase (Stolk *et al*., 1994)) diphenyleneiodonium chloride (DPI; 10 μM; Sigma Chemical Co.; another NADPH oxidase inhibitor (Griendling *et al*., 1994)), allopurinol (100 μM; Sigma Chemical Co.; a xanthine oxidase inhibitor (Greene & Paller, 1992), rotenone (10 μM; Sigma Chemical Co.; an electron transfer chain inhibitor (Meier *et al*., 1989)), L-NAME (100 μM; Sigma Chemical Co.; nitric oxide synthase inhibitor (Rees *et al*., 1990)) and aspirin (100 μM; Sigma Chemical Co.; a cyclooxygenase inhibitor (Tate *et al*., 1984)).

Effect of U46619 and 8-isoprostane $F_{2\alpha}$ ($\pm$LPS, TNF-α or IL-1α) on $O_2^{\cdot-}$ formation

PAVSMCs, PAECs or PA segments ($\pm$endothelium) were incubated with the TXA_2 analogue, U46619 or 8-isoprostane $F_{2\alpha}$ ($\pm$LPS, TNF-α or IL-1α) for 16 h at 37°C in a 95% air–5% CO_2 incubator. In order to determine the source of the $O_2^{\cdot-}$, segments or cells were incubated with DPI, rotenone, L-NAME, aspirin or allopurinol for 1 h prior to the measurement of $O_2^{\cdot-}$ using ferricytochrome *c* reduction (Muzaffar *et al*., 2003). Following incubation, segments or cells were equilibrated in DMEM without phenol red for 10 min at 37°C in a 95% air–5% CO_2 incubator (Heraeus, Hera Cell, Kandro Laboratory Products, Germany). In all, 20 μM horseradish cytochrome *c* (Sigma Chemical Co.) with or without 500 U ml^{-1} copper–zinc superoxide dismutase (SOD; Sigma Chemical Co.) was added and incubated at 37°C in a 95% air–5% CO_2 incubator for an hour. The reaction medium was removed and reduction of cytochrome *c* determined at 550 nm in an anthos *Lucy 1* spectrometer (Lab-tech International, Ringmer, East Sussex, U.K.) and converted to nanomoles of $O_2^{\cdot-}$, using $\Delta E_{550\,nm} = 21.1\,mM^{-1}\,cm^{-1}$ as the extinction coefficient. The reduction of cytochrome *c* that was inhibitable with SOD reflected actual $O_2^{\cdot-}$ release. Segments were blotted, dried and weighed, data being expressed as nanomoleses of $O_2^{\cdot-}$ mg tissue^{-1} h^{-1}. Cells were rinsed in PBS, lysed with 0.1% v v^{-1} Triton X-100 and total protein content measured using BCA-protein assay kit (Pierce, Rockford, IL, U.S.A.). The results were expressed as micromoles of $O_2^{\cdot-}$ mg protein^{-1} h^{-1}.

Effect of iloprost on LPS-, cytokine- and U46619-induced $O_2^{\cdot-}$ formation and gp91^{phox} expression

PAVSMCs or PAECs were seeded, quiesced and incubated with the TXA_2 analogue, U46619 (10 nM), $PGF_{2\alpha}$ (100 nM), 8-isoprostane $F_{2\alpha}$ (100 nM), LPS (1 μg ml^{-1}), IL-1α (10 ng ml^{-1}) and TNF-α (10 ng ml^{-1}), alone and in combination for 16 h and with or without iloprost (100 ng ml^{-1}; Schering, Berlin, Germany). Cells were then washed three times in PBS and $O_2^{\cdot-}$ formation, and then measured using the cytochrome *c* assay as described above. In order to determine whether iloprost possesses a direct $O_2^{\cdot-}$ quenching capacity, 100 ng ml^{-1} iloprost was incubated with xanthine (100 μM)/xanthine oxidase (0.15 U ml^{-1}) mixture (Sigma Chemical Co.), which generates a steady flux of superoxide radicals (Greene & Paller, 1992), and assayed for $O_2^{\cdot-}$ formation using identical conditions as above.

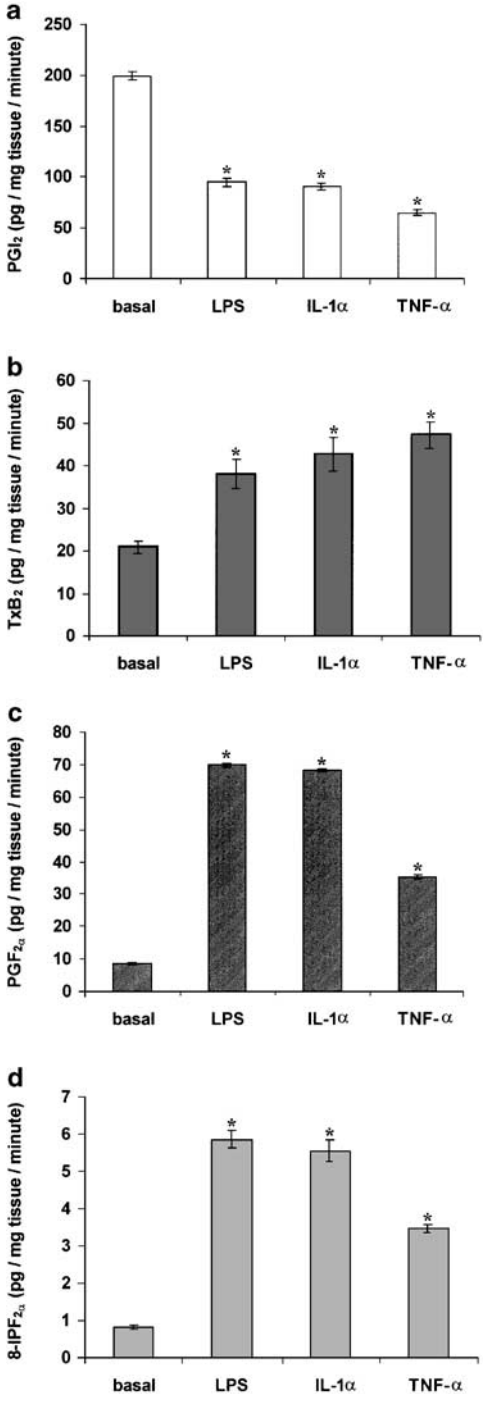

Figure 1 Effect of LPS (1 μg ml^{-1}), IL-1α (10 ng ml^{-1}) and TNF-α (10 ng ml^{-1}) on the formation of: (a) prostacyclin (PGI_2) [as 6 keto-$PGF_{1\alpha}$], (b) TXA_2 [as TXB_2], (c) $PGF_{2\alpha}$ and (d) 8-isoprostane $F_{2\alpha}$ [8-$IPF_{2\alpha}$] stimulated with calcium ionophore A23187 by intact pulmonary artery (PA) segments, following a 16-h incubation. Data = mean (pg eicosanoid^{-1}mg tissue^{-1}minute^{-1}) $\pm$ s.e.m.; $n=6$. *$P<0.05$; comparing treated with (untreated) controls.

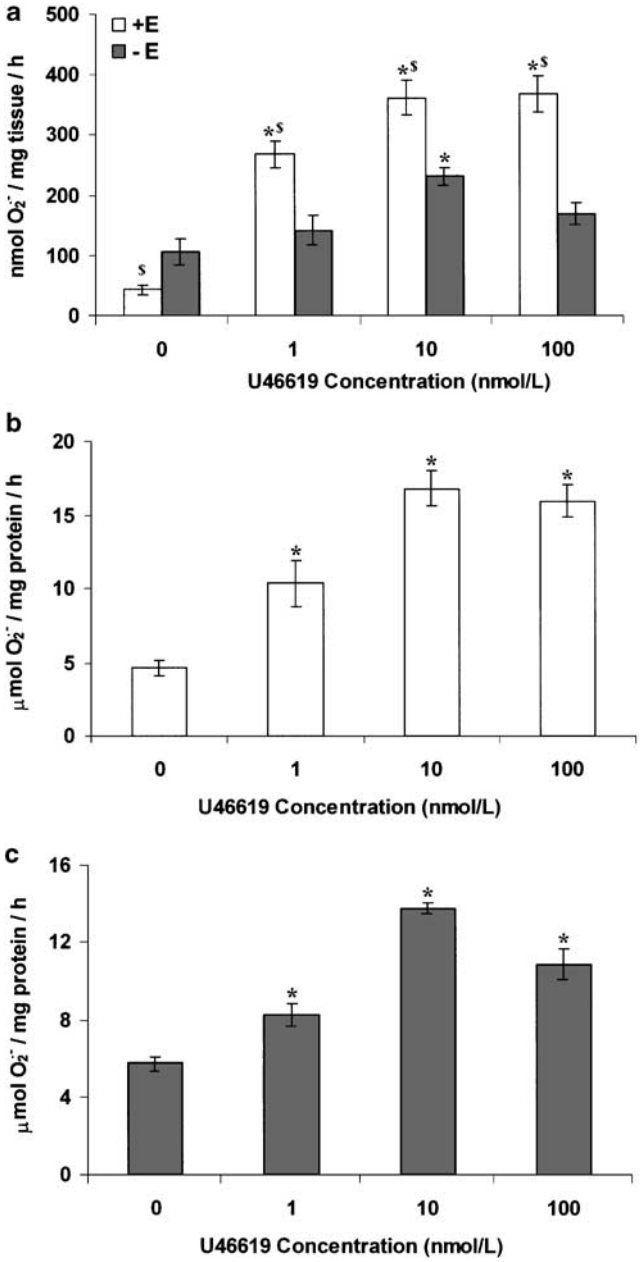

Figure 2 Effect of U46619 on $O_2^{\cdot-}$ formation by (a) whole pig PA segments (with [+E] or without [–E] endothelium), (b) cultured PAVSMCs and (c) cultured PAECs following a 16-h incubation. Data = mean $\pm$ s.e.m.; $n=6$. *$P<0.05$; comparing treated with untreated controls. $^{\$}P<0.05$; comparing with endothelium and without endothelium (in PA segments (a) only).

For Western analysis, cells were lysed with Tris buffer (50 mM pH 7.4) containing 1% v v^{-1} Triton X-100, EDTA (10 mM), PMSF (1 mM) pepstatin (0.05 mM) and leupeptin (0.2 mM). Extracts were boiled at a 1 : 1 ratio with Tris (50 mM; pH 6.8 containing 4% w v^{-1} sodium dodecyl sulphate; 10% v v^{-1} glycerol; 4% v v^{-1} 2-mercaptoethanol; 2 mg ml^{-1} bromophenol blue). Samples of equal protein (100 μg) were loaded onto 12% Tris–glycine sodium dodecyl sulphate gels and separated by electrophoresis. After transfer to nitrocellulose, the blots were primed with a specific human antineutrophil gp91^{phox} antibody (2.5 μg ml^{-1} final concentration) raised in mouse (a kind gift from Professor D. Roos, CLB, Amerstdam, The Netherlands). The blots were then incubated with goat antimouse immunoglobulin (Dako, Cambridgeshire, U.K.) conjugated to horseradish peroxidase (1 : 1000 dilution) and developed by enhanced chemiluminescence (Amersham International). Rainbow markers (14–220 kDa; Amersham) were used for molecular weight determination.

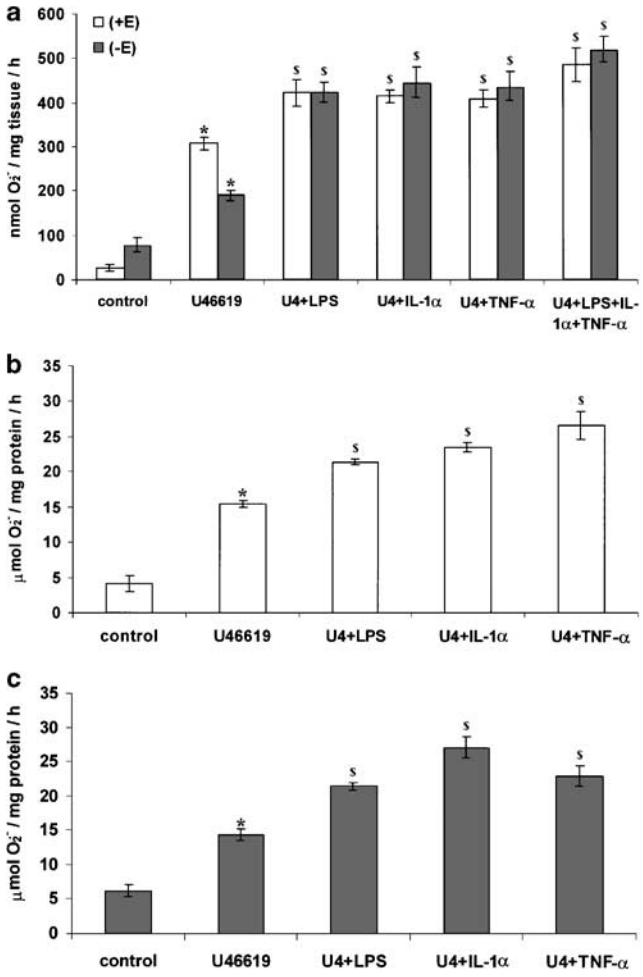

Figure 3 Effect of 10 nM U46619 (U4) in combination with LPS (1 μg ml^{-1}), IL-1α (10 ng ml^{-1}) or TNF-α (10 ng ml^{-1}) on SOD-inhibitable $O_2^{\cdot-}$ formation by: (a) pig pulmonary arterial segments with (+E) and without (–E) endothelium, (b) cultured PAECs and (c) PAVSMCs following a 16-h incubation. Data = mean ± s.e.m.; $n = 6$. $^*P < 0.05$; comparing treated with controls. $^{\$}P < 0.05$; comparing combinations with U46619 alone.

Data analysis

Data are expressed as mean ± s.e.m. and n indicates the number of animals used. Student's unpaired t-test or one-way factorial ANOVA was used to determine the difference in the data. A P-value of less than 0.05 was considered statistically significant. Multiple group comparisons were made using one-way ANOVA.

Results

Effect of LPS and cytokines on eicosanoid formation

In PA segments, LPS, IL-1α and TNF-α elicited a statistically significant increase in the formation of TXA_2 $PGF_{2\alpha}$ and

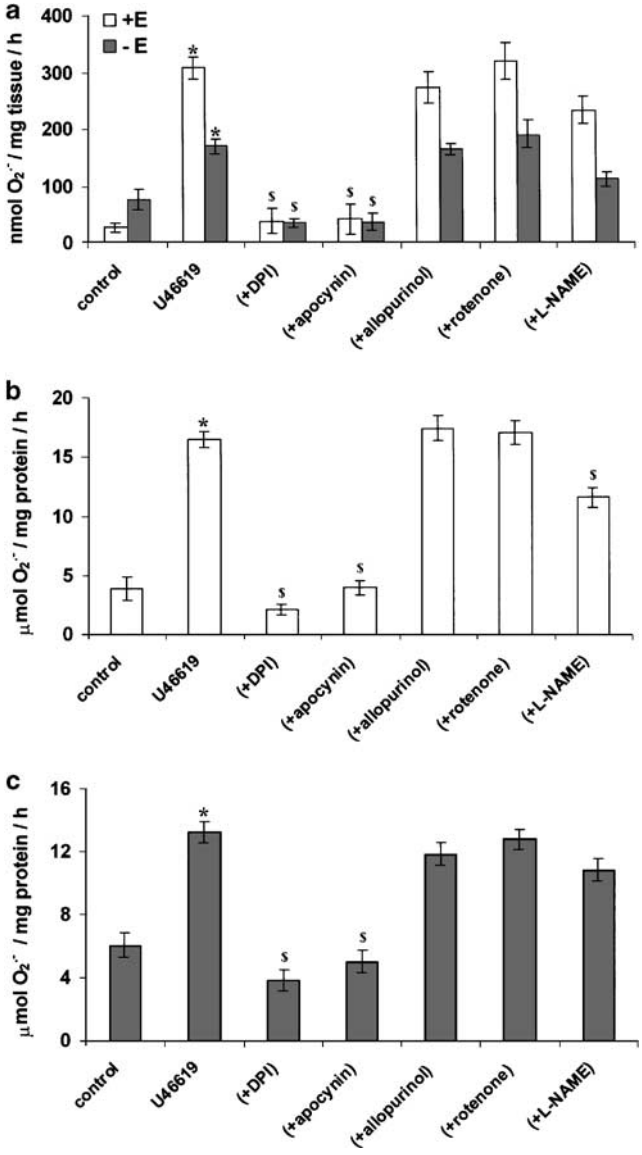

Figure 4 Effect of DPI (10 μM), apocynin (1 μM), allopurinol (100 μM), rotenone (10 μM) or L-NAME (100 μM) on U46619 (10 nM)-stimulated $O_2^{\cdot-}$ formation by: (a) PA segments (with and without endothelium), (b) PAVSMCs and (c) PAECs. Data = mean ± s.e.m.; $n = 6$. $^{\$}P < 0.05$; comparing basal to U46619-induced levels in the absence of inhibitors. $^*P < 0.05$; significantly inhibited compared to U46619-treated segments or cells.

8-isoprostane $F_{2\alpha}$ but a statistically significant decrease in PGI_2 formation (Figure 1). The amount of eicosnoids produced was substantial and all well above the lower limits of detection of the ELISAs. The $PGI_2 : TXA_2$ ratio was markedly altered from 10 : 1 in controls to the following in treated tissues: LPS, 2.4 : 1; IL-1α, 2.1 : 1; TNF-α, 1.3 : 1. The ratio of $PGF_{2\alpha}$ to 8-isoprostane $F_{2\alpha}$, both in control and treated segments was 10 : 1 (Figure 1).

Effect of eicosanoids on $O_2^{\cdot-}$ formation

U46619 promoted a statistically significant increase in the formation of $O_2^{\cdot-}$ from PA segments compared to controls (Figure 2). Removal of the endothelium caused a statistically significant reduction in the formation of $O_2^{\cdot-}$ compared to segments in which the endothelium was intact (Figure 2a). In both PAECs and PAVSMCs, U46619 promoted a statistically significant increase in the formation of $O_2^{\cdot-}$ in a concentration-dependent manner (Figure 2b and 2c). Following a 16-h incubation, the combination of U46619 with LPS, IL-1α or TNF-α, potentiated the formation of $O_2^{\cdot-}$ in PA segments, PAVSMCs and PAECs (Figure 3). Following a 16-h incubation, U46619-stimulated $O_2^{\cdot-}$ formation was inhibited by DPI, apocynin and L-NAME but not by allopurinol or rotenone in PA segments, PAVSMCs and PAECs (Figure 4).

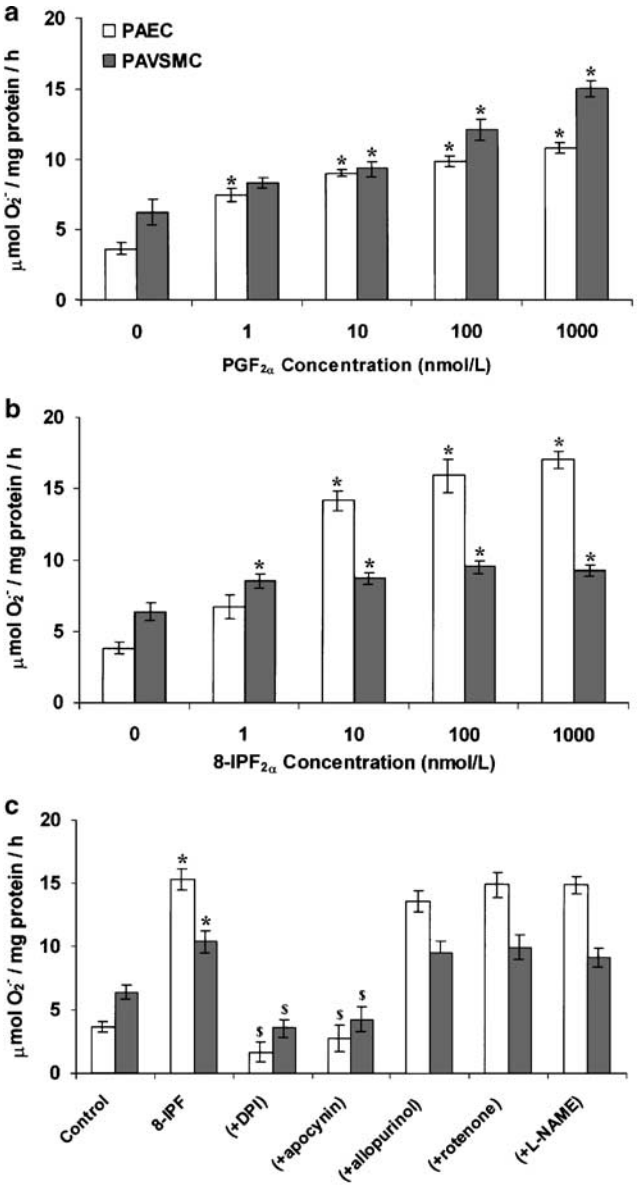

Figure 5 Effect of (a) $PGF_{2\alpha}$ (b) 8-isoprostane $F_{2\alpha}$ [8-$IPF_{2\alpha}$] and (c) 8-$IPF_{2\alpha}$ ($\pm 10\,\mu$M DPI, 1 μM apocynin, 100 μM allopurinol, 10 μM rotenone or 100 μM L-NAME) on SOD-inhibitable $O_2^{\cdot-}$ formation by cultured PAVSMCs or cultured PAECs following a 16-h culture. Data = mean $\pm$ s.e.m.; $n = 6$. $^*P < 0.05$; comparing treated with controls. $\$P < 0.05$; significantly inhibited compared to 8-$IPF_{2\alpha}$-treated cells only.

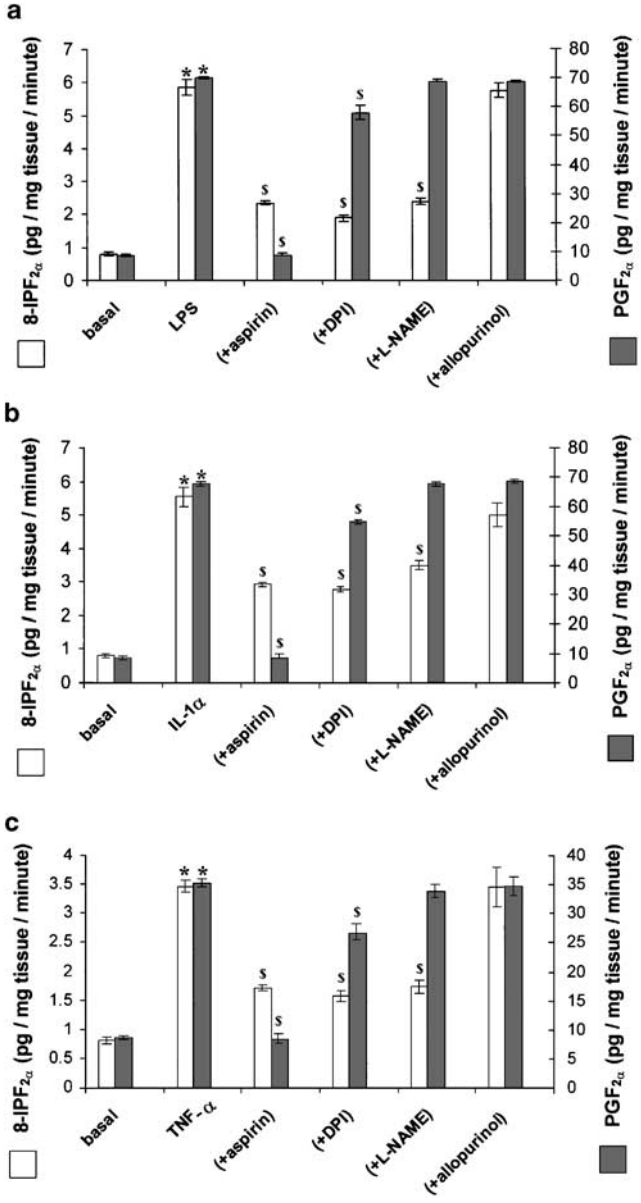

Figure 6 Effect of aspirin (100 μM), DPI (10 μM), L-NAME (100 μM) and allopurinol (100 μM) on $PGF_{2\alpha}$ and 8-isoprostane $F_{2\alpha}$ [8-$IPF_{2\alpha}$] formation induced with (a) 1 μg ml^{-1} LPS, (b) 10 ng ml^{-1} IL-1α or (c) 10 ng ml^{-1} TNF-α. Data = mean $\pm$ s.e.m.; $n = 6$. $\$P < 0.05$; comparing basal to LPS- or cytokine-induced levels in the absence of inhibitors. $^*P < 0.05$; significantly inhibited compared to LPS-/cytokine-treated only.

Similar effects as U46619 on the formation of $O_2^{\cdot-}$ were observed with 8-isoprostane $F_{2\alpha}$ and $PGF_{2\alpha}$ in PAECs and PAVSMCs (Figure 5). $O_2^{\cdot-}$ formation in response to 8-isoprostane $F_{2\alpha}$ was inhibited by DPI and apocynin but not by rotenone or, L-NAME or allopruinol (Figure 5c), indicating that NADPH oxidase mediates this effect.

The formation of 8-isoprostane $F_{2\alpha}$ was inhibited by the aspirin, DPI and L-NAME but not by allopurinol (Figure 6). In contrast, $PGF_{2\alpha}$ formation was only inhibited by aspirin (Figure 6).

Iloprost ($100\,ng\,ml^{-1}$) inhibited the formation of $O_2^{\cdot-}$ induced not only by U46619 but also by LPS, IL-1α, TNF-α following a 16-h incubation with PAECs and PAVSMCs (Figure 7). Iloprost, at the highest concentration studied ($100\,ng\,ml^{-1}$), did not directly quench $O_2^{\cdot-}$ generated by xanthine/xanthine oxidase. The $O_2^{\cdot-}$ generated by xanthine/xanthine oxidase system in the absence of iloprost was $900 \pm 30.6\,nmol\,h^{-1}$ and in the presence of iloprost was $889 \pm 75.9\,nmol\,h^{-1}$, a difference that was not statistically significant. The basal value in the absence of xanthine oxidase was $261 \pm 40.5\,nmol\,h^{-1}$.

Effect of eicosanoids (±LPS, TNF-α or IL-1α) on gp91^{phox} expression

Following a 16-h incubation, the protein expression of gp91^{phox}, the active catalytic subunit of NADPH oxidase was upregulated by U46619, $PGF_{2\alpha}$ and 8-isoprostane $F_{2\alpha}$ in PAECs and PAVSMCs (Figure 8). The combination of U46619 with LPS, IL-1α or TNF-α, further augmented gp91^{phox} protein expression in PAECs and PAVSMCs (Figure 8). Coincubation with iloprost over the 16-h incubation caused a statistically significant inhibition of gp91^{phox} expression (Figure 8).

Discussion

The primary trigger for the inflammatory cascades associated with ARDS is the release of endotoxins from bacteria destroyed by neutrophils, which then promote the adhesion of platelets and leucocytes to vessel walls (Jeremy *et al*., 1994b; Chabot *et al*., 1998; Ermert *et al*., 2000b; Stuart-Smith & Jeremy, 2001; Weinacker & Vaszar, 2001). Adherent and activated platelets and leucocytes then release a number of potent inflammogens, including cytokines and TXA_2 (Jeremy *et al*., 1994b; 1997), which may influence OS in ARDS. Thus, it was first found that the stable TXA_2 analogue, U46619, promoted the formation of $O_2^{\cdot-}$ in intact pulmonary arteries and PAVSMCs and PAECs, an effect augmented by LPS, IL-1α and TNF-α. Since elevated plasma levels of TXA_2, IL-1α and TNF-α, coexist in ARDS patients (Reines *et al*., 1982; Jeremy *et al*., 1994b), these

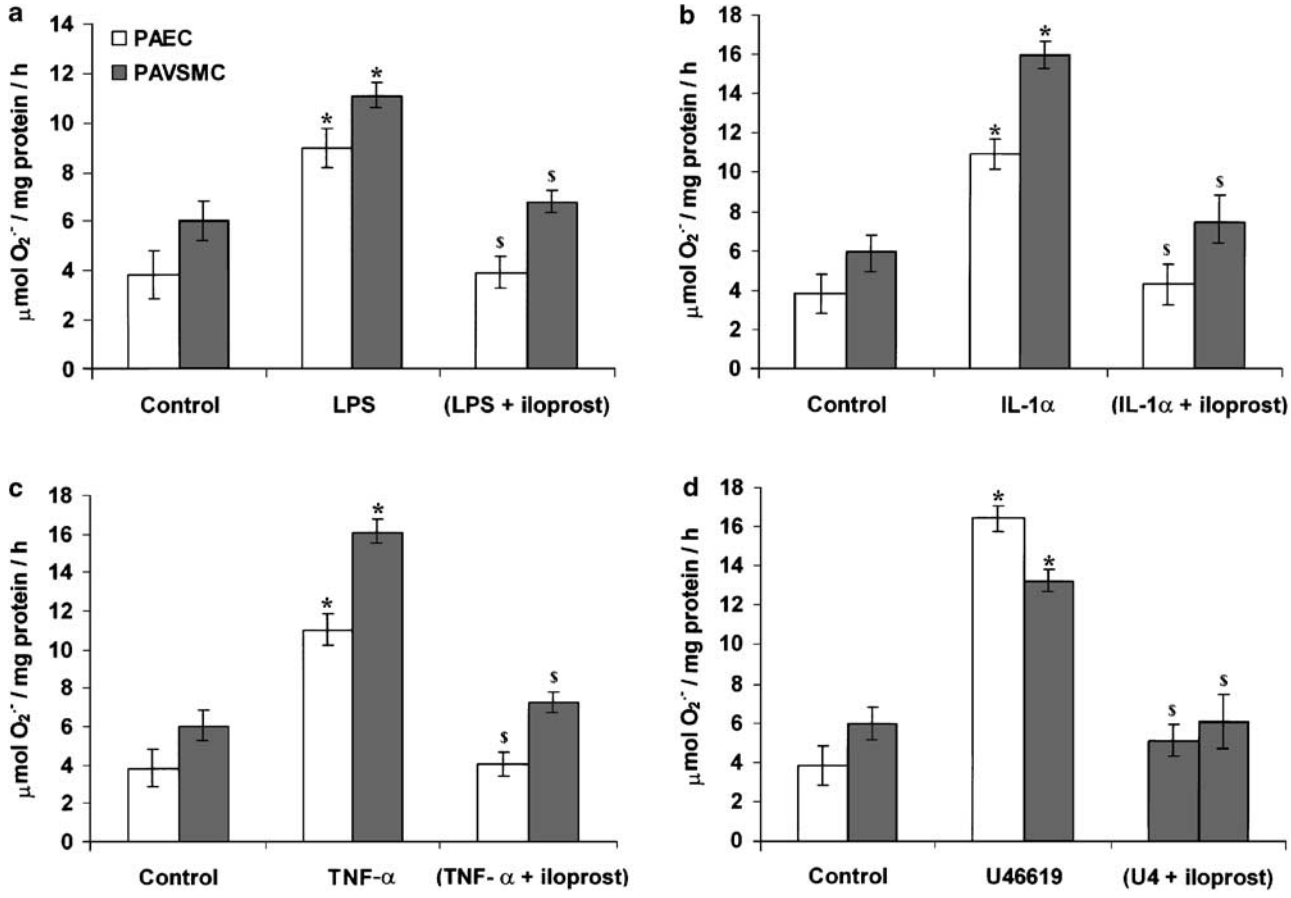

Figure 7 Effect of iloprost ($100\,ng\,ml^{-1}$) on $O_2^{\cdot-}$ formation by cultured PAVSMCs and PAECs derived from pig PA after a 16-h incubation with (a) $1\,\mu g\,ml^{-1}$ LPS, (b) $10\,ng\,ml^{-1}$ IL-1α, (c) $10\,ng\,ml^{-1}$ TNF-α or (d) 10 nM U46619. Data = mean ± s.e.m., $n = 6$. *$P < 0.05$; comparing controls to LPS, cytokine or U46619 induced levels in the absence of iloprost. $^{\$}P < 0.05$; comparing LPS, cytokines or U46619 treated in the presence of iloprost to those in the absence of iloprost.

interactions may be important in the progression and outcome of the syndrome.

Apocynin and DPI, both inhibitors of NADPH oxidase, blocked $O_2^{\cdot-}$ formation in response to U46619, both alone and in combination with LPS, IL-1α and TNF-α. In contrast, the inhibition of xanthine oxidase and mitochondrial respiration had little effect. U46619 also upregulated the expression of gp91^{phox}, an active catalytic subunit of NAPDH oxidase, in PAVSMCs and PAECs. It is concluded, therefore, that U46619 increases $O_2^{\cdot-}$ formation through an upregulation of NADPH oxidase expression/activity in pulmonary arterial cells.

Since LPS and cytokines have long been known to alter vascular eicosanoid formation (Jeremy *et al*., 1994a, b), the effect of LPS and cytokines on the formation of TXA_2 and PGI_2 in intact pig pulmonary arteries was explored. Not only did LPS and cytokines augment TXA_2 production but concomitantly diminished PGI_2 formation following a 16-h incubation. The possible importance of PGI_2 status in ARDS is exemplified by the beneficial effects of aerosolised PGI_2 to treat ARDS (Lowson, 2002). Since PGI_2 has diametrically opposite effects to TXA_2 (Ullrich *et al*., 2001) it was reasonable to speculate that PGI_2 may inhibit NADPH oxidase expression. Indeed, in the present study the stable PGI_2 analogue,

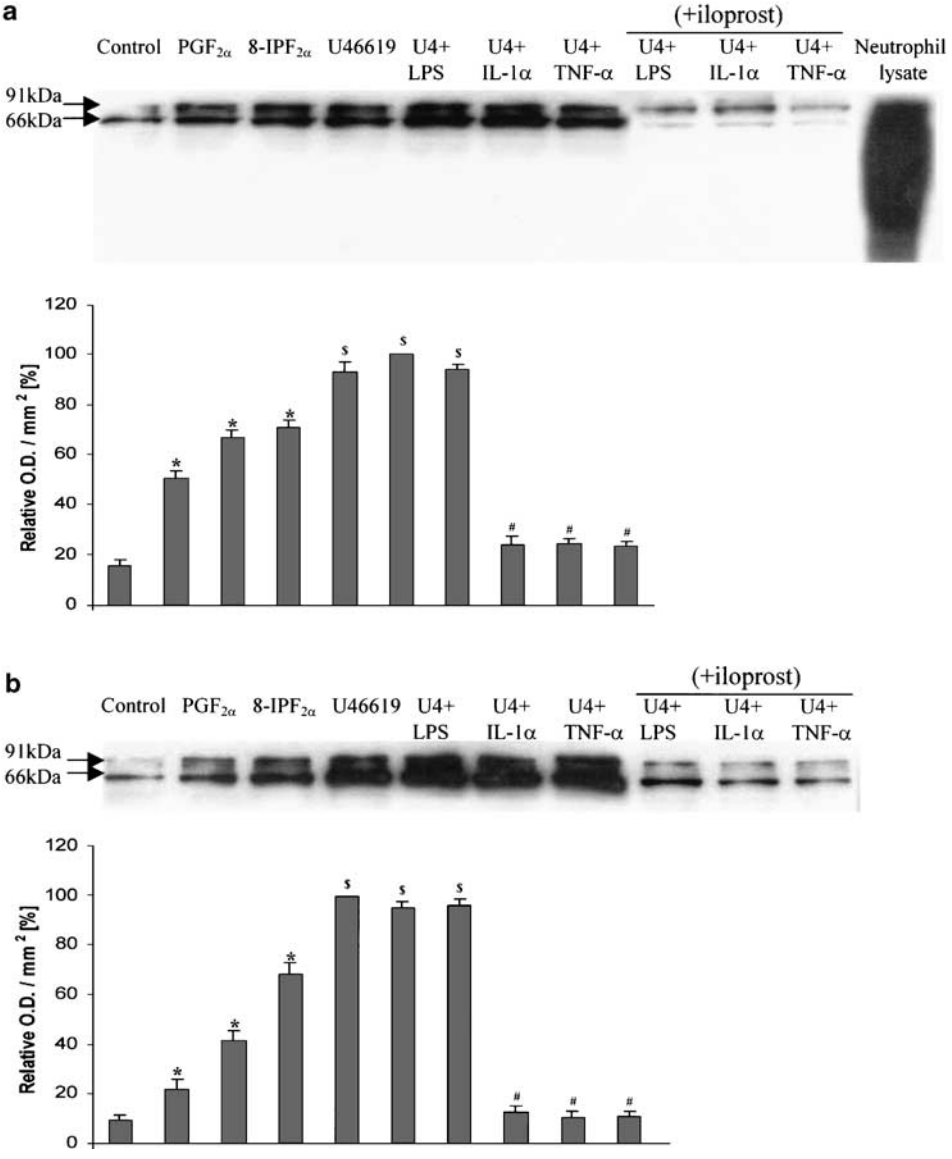

Figure 8 Western analysis of NAD(P)H oxidase in (a) PAECs (b) PAVSMCs using a monoclonal antibody directed against the gp91^{phox}-subunit of human neutrophil NAD(P)H oxidase (MoAb 48). Cells were either not treated or treated for 16 h with one of the following: $PGF_{2\alpha}$ (100 nM); 8-isoprostane $F_{2\alpha}$ (8-$IPF_{2\alpha}$; 100 nM); U46619 (as U4;10 nM) + LPS (1 $\mu g\,ml^{-1}$), IL-1α (10 $ng\,ml^{-1}$) or TNF-α (10 $ng\,ml^{-1}$) either in the absence or presence of iloprost (100 $ng\,ml^{-1}$). The bands detected are the 91 kDa for the heavily glycosylated form of gp91^{phox} and the 66 kDa for the less-glycosylated form of gp91^{phox}. The upper panels show the representative blots and the lower panels the results of the densitometric analyses of six blots (expressed as relative optical density (OD) mm^{-2}). Pig neutrophil lysates were used as positive controls. $^{*}P<0.05$; comparing treated with control. $^{\$}P< 0.05$; significantly inhibited compared to combinations of LPS or cytokines with U46619.

iloprost, inhibited the induction of gp91^{phox} expression and $O_2^{\cdot-}$ formation elicited by U46619, LPS and cytokines. A reduction of PGI_2 formation may result in diminished 'protection' against the $O_2^{\cdot-}$-promoting effects of TXA_2, LPS and cytokines.

In the present studies, LPS and cytokines also augmented the formation of 8-isoprostane $F_{2\alpha}$ and $PGF_{2\alpha}$ in intact pulmonary arteries. Isoprostanes, formed *via* oxidative pathways are potent agonists of the thromboxane/endoperoxide receptor (Morrow & Roberts, 2002; Roberts & Morrow, 2002). It was further demonstrated here that both 8-isoprostane $F_{2\alpha}$ and $PGF_{2\alpha}$, upregulated the expression of NADPH oxidase expression/activity and increased the formation of $O_2^{\cdot-}$. The formation of 8-isoprostane $F_{2\alpha}$ was inhibited not only by aspirin but also by DPI and L-NAME, whereas $PGF_{2\alpha}$ (the parent compound of 8-isoprostane $F_{2\alpha}$) was inhibited only by aspirin. This indicates that not only $O_2^{\cdot-}$ derived from NADPH oxidase but also that NO and possibly reactive nitrogen species (RNS), influence the formation of isoprostanes. It has been suggested that isoprostanes are sequestered into phospholipids and are then released by NO or that NO modifies arachidonic acid moiety in phospholipids such that when it is released and metabolised by cyclooxygenase, 8-isoprostane $F_{2\alpha}$ is formed (Morrow & Roberts, 2002; Roberts & Morrow, 2002). However, since L-NAME also indirectly inhibits RNS formation, it is possible that RNS also modulates the formation of 8-isoprostane $F_{2\alpha}$.

To summarise, the TXA_2 analogue U46619 upregulates NADPH oxidase activity and $O_2^{\cdot-}$ formation by pulmonary arterial tissues and cells. In ARDS, therefore, the initial release of TXA_2 by adherent platelets would promote intrapulmonary OS. Concomitantly, LPS and cytokines (released by adherent leucocytes) increase TXA_2-synthesising capacity but decrease that of PGI_2 in the pulmonary arterial tissue. Since TXA_2 increases $O_2^{\cdot-}$ formation through an upregulation of NADPH oxidase protein expression, which is prevented by PGI_2, this imbalance may render the vessel susceptible to further augmentation of $O_2^{\cdot-}$-mediated pathology. A self-perpetuating cascade may ensue, whereby $O_2^{\cdot-}$ and or RNS promote the release of arachidonic acid, fuelling the formation of TXA_2, which in turn would further augment the formation of $O_2^{\cdot-}$ *via* upregulation of NADPH oxidase. Finally, $O_2^{\cdot-}$ (and/or NO or RNS) also increases the formation of 8-isoprostane $F_{2\alpha}$, which also engenders NADPH oxidase protein expression, and $O_2^{\cdot-}$ formation. Together, these events constitute a novel self-perpetuating pathway that would amplify and worsen the progress of ARDS.

From a clinical perspective, ARDS is an intractable condition to treat and many drugs, including NSAIDS and thromboxane formation inhibitors, have proved ineffective in treating the condition (The ARDS Network, 2000; Tasaka *et al*., 2002). An innate problem with treating ARDS is its rapidity of onset (Jeremy *et al*., 1994b; Stuart-Smith & Jeremy, 2001; Weinacker & Vaszar, 2001), such that by the time ARDS has been diagnosed and interventions implemented, the upregulation of NAPDH oxidase may already have occurred. Thus, one may be 'shutting the therapeutic door after the pathological horse has bolted' when one administers drugs such as ketoconazole. Furthermore, since inflammation associated with ARDS is promulgated by many disparate factors, then the inhibition of any one factor (e.g. TXA_2 formation) may not prevent the progress of the disease. To exemplify this point, several disparate factors have been shown in this study to promote the upregulation of NAPDH oxidase. However, since this is a common denominator effect of all these factors, it is reasonable to suggest that the pharmacological inhibition of NAPDH oxidase activity and/or inhibition of further upregulation of the enzyme may constitute a possible effective approach to treating ARDS. In support of this and in the context of the present study, inhalational PGI_2 and NO (Klinger, 2002; Lowson, 2002) have proved effective in treating ARDS and are both inhibited by $O_2^{\cdot-}$-mediated mechanisms.

This research was funded by the British Heart Foundation (Grant No. FS/2001041).

References

AUDOLY, L., ROCCA, B., FABRE, J., KOLLER, B., THOMAS, D., LOEB, A., COFFMAN, T. & FITZGERALD, G. (2000). Cardiovascular responses to the isoprostane iPF2-III and iPE2-III are mediated *via* the thromboxane A2receptor. *Circulation,* **101,** 2833–2840.

CHABOT, F., MITCHELL, J.A., GUTTERIDGE, J.M.C. & EVANS, A.M. (1998). Reactive oxygen species in acute lung injury. *Eur. Respir. J.,* **11,** 745–757.

CHAUDHARI, A., PEDRAM, A. & KIRSCHENBAUM, M.A. (1990). Prostanoid biosynthesis in cultured rabbit renal microvascular smooth muscle cells. Effect of arachidonic acid, calcium, and A23187. *Lab. Invest.,* **63,** 30–37.

EKMEKCIOGLU, C., SCHWEIGER, B., STRAUSS-BLASCHE, G., MUNDIGLER, G., SIOSTRZONEK, P. & MARKTL, W. (2002). Urinary excretion of 8-iso-PGF(2 alpha) in three patients during sepsis, recovery and state of health. *Prostaglandins Leukotr. Essent. Fatty Acids,* **66,** 441–442.

ERMERT, L., EMRERT, M., DUNCKER, H.R., GRIMMINGER, F. & SEEGER, W. (2000a). *In situ* localisation and regulation of thromboxane A_2 synthase in normal and LPS-primed lungs. *Am. J. Physiol.,* **278,** L744–L753.

ERMERT, M., MERKLE, M., MOOTZ, R., GRIMMINGER, F., SEEGER, W. & ERMERT, L. (2000b). Endotoxin priming of the cyclooxygenase-2-thromboxane axis in isolated rat lungs. *Am. J. Physiol.,* **278,** L1195–L1203.

GREENE, E.L. & PALLER, M.S. (1992). Xanthine oxidase produces superoxide in posthypoxic injury of renal epithelial cells. *Am. J. Physiol.,* **263,** F251–F255.

GRIENDLING, K.K., MINIERI, C.A., OLLERENSHAW, J.D. & ALEXANDER, R.W. (1994). Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cellls. *Circ. Res.,* **74,** 1141–1148.

JEREMY, J.Y., MEHTA, D., BRYAN, A.J., LEWIS, D. & ANGELINI, G.D. (1997). Platelets and saphenous vein graft failure following coronary artery bypass surgery. *Platelets,* **8,** 295–309.

JEREMY, J.Y., MIKHAILIDIS, D.P., KARATAPANIS, S., HARRY, D., BURROUGHS, A.K., MCINTYRE, N., STANSBY, G., JACOBS, M. & MCCORMICK, A. (1994a). Altered prostacyclin synthesis by aortae from hepatic portal vein-constricted rats: evidence for effects on protein kinase C and calcium. *J. Hepatol.,* **21,** 1017–1022.

JEREMY, J.Y., NYSTROM, M.L., BARRADAS, M.A. & MIKHAILIDIS, D.P. (1994b). Eicosanoids and septicaemia. *Prostaglandins Leukotr. Essent. Fatty Acids,* **50,** 287–297.

JOURDAN, K.B., MITCHELL, J.A. & EVANS, T.W. (1997). Release of isoprostanes by human pulmonary artery in organ culture: a cyclooxygenase and nitric oxide dependent pathway. *Biochem. Biophys. Res. Commun.,* **233,** 668–672.

KLINGER, J.R. (2002). Inhaled nitric oxide in ARDS. *Crit. Care Clin.,* **18,** 45–68.

LOWSON, S.M. (2002). Inhaled alternatives to nitric oxide. *Anesthesiology,* **96,** 1504–1513.

MEIER, B., RADEKE, H.H., SELLE, S., YOUNES, M., SIES, H., RESCH, K. & HABERMEHL, G.G. (1989). Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-alpha. *Biochem. J.,* **263,** 539–545.

MINUZ, P., ANDRIOLI, G., DEGAN, M., GAINO, S., ORTOLANI, R., TOMMASOLI, R., ZULIANI, V., LECHI, A. & LECHI, C. (1998). The F2-isoprostane 8-epiprostaglandin F2alpha increases platelet adhesion and reduces the antiadhesive and antiaggregatory effects of NO. *Arterioscl. Thromb. Vasc. Biol.,* **18,** 1248–1256.

MORROW, J.D. & ROBERTS, L.J. (2002). The isoprostanes: their role as an index of oxidant stress status in human pulmonary disease. *Am. J. Respir. Crit. Care Med.,* **166,** S25–30.

MUZAFFAR, S., JEREMY, J.Y., ANGELINI, G.D., STUART-SMITH, K. & SHUKLA, N. (2003). Role of the endothelium and nitric oxide synthases in modulating superoxide formation induced by endotoxin and cytokines in porcine pulmonary arteries. *Thorax,* **58,** 598–604.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.,* **101,** 746–752.

REINES, H.D., HALUSHKA, P.V., COOK, J.A., WISE, W.C. & RAMBO, W. (1982). Plasma thromboxane concentrations are raised in patients dying with septic shock. *Lancet,* **2,** 174–175.

ROBERTS II, L.J. & MORROW, J.D. (2002). Products of the isoprostane pathway: unique bioactive compounds and markers of lipid peroxidation. *Cell Mol. Life Sci.,* **59,** 808–820.

SORESCU, D., SZOCS, K. & GRIENDLING, K.K. (2001). NAD(P)H oxidases and their relevance to atherosclerosis. *TCM,* **11,** 124–131.

SOUTHGATE, K.M., DAVIES, M., BOOTH, R.F. & NEWBY, A.C. (1992). Involvement of extracellular-matrix-degrading metalloproteinases in rabbit aortic smooth-muscle cell proliferation. *Biochem J.,* **288,** 93–99.

STOLK, J., HILTERMANN, T.J. & DIJKMAN, J.H.V.A.J. (1994). Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am. J. Respir. Cell Mol. Biol.,* **11,** 95–102.

STUART-SMITH, K. & JEREMY, J.Y. (2001). Microvessel damage in acute respiratory distress syndrome: the answer may not be NO. *Br. J. Anaesth.,* **87,** 272–279.

TASAKA, S., HASEGAWA, N. & ISHIZAKA, A. (2002). Pharmacology of acute lung injury. *Pulm. Pharmacol. Ther.,* **15,** 83–95.

TATE, R.M., MORRIS, H.G., SCHROEDER, W.R. & REPINE, J.E. (1984). Oxygen metabolites stimulate thromboxane production and vasoconstriction in isolated saline-perfused rabbit lungs. *J. Clin. Invest.,* **74,** 608–613.

THE ARDS NETWORK AUTHORS FOR THE ARDS NETWORK (2000). Ketoconazole for early treatment of acute lung injury and acute respiratory distress syndrome: a randomized controlled trial. *JAMA,* **283,** 1995–2002.

ULLRICH, V., ZOU, M.H. & BACHSCHMID, M. (2001). New physiological and pathophysiological aspects on the thromboxane A(2)-prostacyclin regulatory system. *Biochim. Biophys. Acta,* **1532,** 1–14.

WEINACKER, A.B. & VASZAR, L.T. (2001). Acute respiratory distress syndrome: physiology and new management strategies. *Annu. Rev. Med.,* **52,** 221–237.

(Received August 18, 2003
Revised November 6, 2003
Accepted November 11, 2003)