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Superoxide auto-augments superoxide formation and upregulates gp91^{phox} expression in porcine pulmonary artery endothelial cells: Inhibition by iloprost

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Abstract

Central to the aetiology of Acute Respiratory Distress Syndrome (ARDS) is superoxide, the principal source of which is nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase). To test whether superoxide may influence NADPH oxidase expression directly, the effect of incubation of superoxide with porcine pulmonary arterial endothelial cells on the expression of gp91^{phox} (a catalytic subunit of NADPH oxidase) and superoxide formation was investigated. Since iloprost has been purported to be potentially effective in treating ARDS, the effect of iloprost on superoxide-mediated effects was also studied.

Pulmonary artery endothelial cells were incubated with xanthine/xanthine oxidase which generates superoxide, or tumour necrosis factor α (TNFα) or thromboxane A_2 analogue, U46619 (± superoxide dismutase [SOD] or catalase or iloprost) for 16 h. Cells were then washed and superoxide formation assessed spectrophometrically and gp91^{phox} expression using Western blotting. The role of NADPH oxidase was also studied in the above settings using apocynin, an NADPH oxidase inhibitor.

Superoxide, TNFα and U46619 elicited an increase in the formation of superoxide and induced gp91^{phox} expression in pulmonary artery endothelial cells following a 16 h incubation an effect blocked by the continual presence of SOD and apocynin but not catalase. Apocynin completely inhibited superoxide formation induced with xanthine/xanthine oxidase after the 16 h incubation. Rotenone and allopurinol were without effect. Iloprost inhibited the formation of superoxide and gp91^{phox} expression.

These data demonstrate that superoxide upregulates gp91^{phox} expression in pulmonary artery endothelial cells and thus augments superoxide formation, an effect blocked by iloprost. This constitutes a novel mechanism by which vascular superoxide creates a self-perpetuating cascade that may be of importance to the etiology of ARDS and other vasculopathies.

Keywords: Superoxide; NADPH oxidase [nicotinamide adenine dinucleotide phosphate oxidase]; Endothelial cell; Pulmonary artery

1. Introduction

Acute respiratory distress syndrome (ARDS), a severe form of acute lung injury, is a common complication in critically ill patients and is associated with significant morbidity and mortality (Chabot et al., 1998; Metnitz et al., 1999; Stuart-Smith and Jeremy, 2001; Weinacker and Vaszar, 2001). Although ARDS can be initiated by a number of causal factors that include sepsis, shock, trauma and multiple transfusions, the pathological and clinical manifestations of the syndrome are very similar (Chabot et al., 1998). ARDS remains a difficult syndrome to treat and as such further insights into the pathobiology is required in order to develop effective therapeutic strategies.

It has become apparent that oxidative stress, in particular superoxide formation, is involved in the aetiology of ARDS (Chabot et al., 1998; Weinacker and Vaszar, 2001; Metnitz et al., 1999; Stuart-Smith and Jeremy, 2001), a condition characterised by a rapid and time-dependent worsening of intrapulmonary inflammation and hypertension (Stuart-Smith and Jeremy, 2001). Apart from directly eliciting vasoconstriction (Metnitz

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et al., 1999), superoxide reacts with nitric oxide (NO) to form peroxynitrite and other reactive nitrogen species (Folkerts et al., 2001), effectively reducing NO bioavailability (Dweik, 2005). Since NO is protective in the lung, this reaction has been suggested as a key mechanism in the progression of ARDS (Muzaffar et al., 2003, 2005a).

It has become increasingly apparent that the principal source of intra-pulmonary superoxide in ARDS may be nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), the expression of which is rapidly upregulated by diverse factors associated with ARDS. These factors include cytokines, thromboxane A_2, isoprostanes $F_{2\alpha}$, hypoxia and re-oxygenation following hypoxia (Muzaffar et al., 2003, 2004a,b, 2005a,b). By contrast, NADPH oxidase expression is inhibited by prostacyclin (Muzaffar et al., 2004a), which when administered to patients can ameliorate the severity of ARDS indicating a protective role for these substances. However, the endogenous formation of PGI_2 is reduced by superoxide (Muzaffar et al., 2004a), thus adding to the vasculopathic repertoire of the anion.

Apart from direct chemical reactions, another facet of superoxide is that it upregulates the expression of proteins and activates signal transduction systems that control gene expression (Li and Shah, 2004). These effects include calcium mobilisation, nuclear factor kappa β expression, tyrosine kinase activation and activation of the mitogen activated protein kinases (Baas and Berk, 1995; Greene et al., 2000; Li et al., 1999; Marumo et al., 1997; Su et al., 2001; Yeh et al., 1999). It is not unreasonable to suggest, therefore, that superoxide may itself increase the expression of NADPH oxidase, which would constitute a self-amplifying positive feedback mechanism that may augment ARDS.

Our hypothesis, therefore, is that superoxide may autoaugment the formation of superoxide through upregulation of NADPH oxidase expression. In order to test this possibility, the effect of a superoxide-generating system, xanthine/xanthine oxidase (Greene and Paller, 1992) on superoxide formation and the expression of $gp91^{phox}$, an active catalytic subunit of NADPH oxidase in endothelial cells (Muzaffar et al., 2004a,b) was studied in porcine pulmonary artery endothelial cells. Since superoxide dismutates to form hydrogen peroxide, studies were also carried out with catalase. Furthermore, since TNFα and the thromboxane A_2 analogue, U44619, have been shown to augment superoxide formation through an uregulation of $gp91^{phox}$ (Muzaffar et al., 2003, 2004a, 2005b) the direct role of superoxide on this pathway was also examined. Finally, the effects of iloprost were studied since iloprost has been shown to inhibit NAPDH oxidase expression in response to cytokines and other inflammatory factors (Muzaffar et al., 2004a). Incubations were undertaken over a 16 h time course since this represents the time scale of onset of inflammation in ARDS.

2. Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

2.1. Drugs

Tumour-necrosis factor-alpha (TNFα) was purchased from RandD Systems (Abingdon, UK). 9, 11-Dideoxy-9α, 11α-methanoepoxyprostaglandin $F_2\alpha$ (U46619), protein kinase A inhibitor, 14–22 amide peptide, and protein kinase G inhibitor, DT-3 peptide, were purchased from Calbiochem (Nottingham, UK). Iloprost was purchased from Schering (Berlin, Germany). All the other drugs were purchased from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated.

2.2. Preparation of pulmonary arterial endothelial cells

Endothelial cells were prepared as previously described (Muzaffar et al., 2004a,b) Lungs were obtained from White Landrace male pigs of body weight ranging from 20–35 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; Ketaset Injection, Fort Dodge Animal Health, Southampton, UK) and inhaled halothane (1–2% v/v/ in oxygen), exsanguinated and lungs removed. Pulmonary arteries (3–4 mm diameter) were dissected out immediately and rinsed in Dulbecco's Minimum Essential Medium (DMEM, GibcoBRL; Paisley, Scotland). Pulmonary artery endothelial cells were cultured by the explant method as previously described (Muzaffar et al., 2004a,b). Pulmonary artery endothelial cells were grown and maintained in Endothelial Cell Growth Medium (PromoCell, Heidelberg, Germany) at 37 °C in a 95% air–5% CO_2 incubator. When confluent, cells were harvested by trypsinisation. All subsequent experiments were carried out using cells at passage 4, since this allows for the build up of cell numbers. We have also previously demonstrated that pulmonary artery endothelial cells used at passage 4 behave in much the same way as endothelial cells in fresh, intact, pulmonary arteries (Muzaffar et al., 2003).

2.3. Measurement of superoxide

The measurement of superoxide formation and release by cultured cells was performed by detection of ferricytochrome *c*

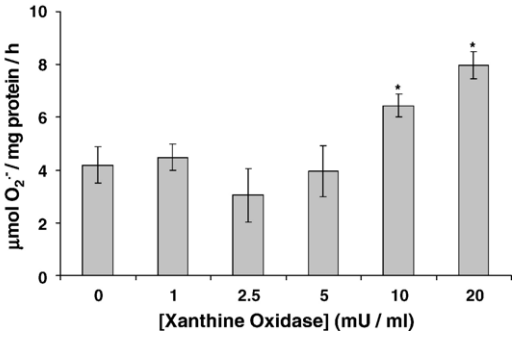

Fig. 1. Effect of incubation of pulmonary arterial endothelial cells for 16 h with xanthine (100 µM)+different concentrations of xanthine oxidase, followed by extensive washing, on the formation of $O_2^{\bullet-}$. Each bar represents the mean± S.E.M. ($n=6$ separate experiments). *$P<0.01$; significantly increased compared to controls.

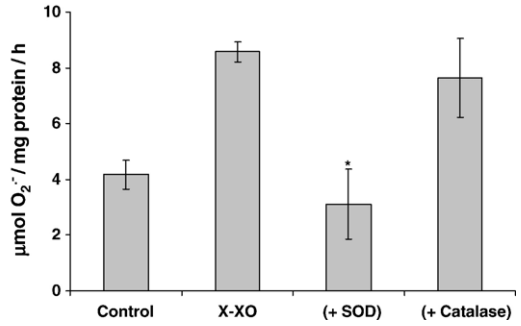

Fig. 2. Effect of incubation of pulmonary arterial endothelial cells for 16 h with xanthine (100 µM)+xanthine oxidase (20 mU/ml) with or without SOD (500 U/ml) or catalase (100 U/ml) followed by extensive washing, on the formation of $O_2^{\bullet-}$. Each bar represents the mean±S.E.M. (n=6 separate experiments). *P<0.01; significantly reduced compared to cells treated with xanthine/xanthine oxidase alone.

reduction, as previously described (Muzaffar et al., 2004a,b). We opted for this method rather than the lucigenin method since, even though more sensitive, lucigenin itself undergoes auto-oxidation and thereby act as a source of superoxide generation (Li et al., 1998; Spasojevic et al., 2000) We also found that cytochrome *c* was adequate as sensitive in detecting superoxide release in our system. For this reason and due to inaccessibility to a dedicated luminometer we have been using ferricytochrome *c* method to measure superoxide. Following incubation, cells were washed three times with phosphate buffered saline (PBS) and 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). 20 µM horseradish cytochrome *c* with or without 500 U/ml copper–zinc SOD was added to the cells and incubated at 37 °C in a 95% air–5% CO_2 incubator for an hour. The final volume of the reaction mixture was 0.5 ml per well. After 1 h, the reaction medium was removed and maximum rate of reduction of cytochrome *c* was determined at 550 nM on a temperature controlled *Anthos Lucy 1* spectrometer (Lab-tech International, Ringmer, East Sussex, UK) and converted to µmoles of superoxide, using $\Delta E_{550\ nM}$=21.1 mM/cm/min as the extinction coefficient for (reduced–oxidized) cytochrome *c*. The reduction of

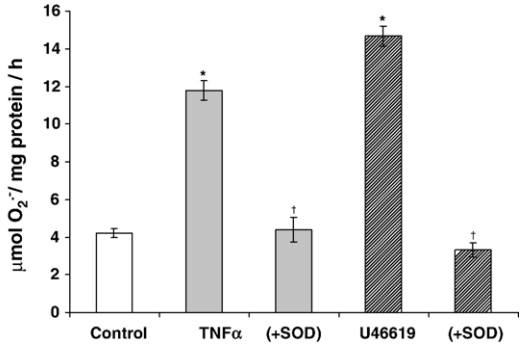

Fig. 3. Effect of incubation of pulmonary arterial endothelial cells for 16-h with TNFα (10 ng/ml) or U46619 (10 nM), with or without SOD (500 U/ml) followed by extensive washing on the formation of $O_2^{\bullet-}$. Each bar represents the mean±S.E.M. (n=6 separate experiments). *P<0.01; comparing treated with control cells. †P<0.01; significantly reduced compared to cells treated with TNFα or U46619.

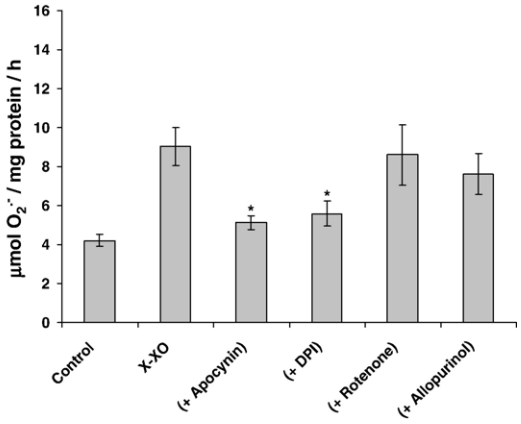

Fig. 4. Effect of apocynin (1 µM), DPI (10 µM), rotenone (10 µM) or allopurinol (100 µM) on $O_2^{\bullet-}$ formation, following a 16 h incubation of pulmonary artery endothelial cells with xanthine (100 µM)+xanthine oxidase (20 mU/ml). Data=mean±S.E.M.; n=6. *P<0.05; significantly inhibited compared to xanthine/xanthine oxidase-treated cells.

cytochrome *c* that was inhibitable with SOD reflected actual formation release. Cells were rinsed in PBS, lysed with 0.1% v/v Triton-X 100 and total protein content measured using BCA-protein assay kit.

2.4. Effect of superoxide on superoxide formation, role of NADPH oxidase

Pulmonary artery endothelial cells were incubated with xanthine (100 µM)+xanthine oxidase (1–20 mU/ml) system which generates superoxide (Greene and Paller, 1992) or 10 ng/ml tumour necrosis factor α (TNF α) or 10 nM thromboxane A_2 analogue, U46619 with or without the continual presence of SOD or catalase for 16 h at 37 °C in a 95% air–5% CO_2 incubator. Following incubation the cells were washed 3 times with phosphate buffered saline and superoxide measured as described above. The final wash supernatant was tested for residual xanthine/xanthine oxidase, by measuring $O_2^{\bullet-}$ as described above. No superoxide was detected indicating that all the xanthine/xanthine oxidase had been removed and as such could not interfere with the subsequent assay.

In order to determine the source of superoxide in response to superoxide, pulmonary artery endothelial cells were pre-incubated for 16 h with the xanthine/xanthine oxidase system. The cells were then rinsed repeatedly in PBS and incubated for further 2 h with one of the following, 1) diphenylene iodonium (DPI; 10 µM; a NADPH oxidase inhibitor), 2) apocynin (1 µM; a selective NADPH oxidase inhibitor), 3) allopurinol (100 µM; a xanthine oxidase inhibitor) or 4) rotenone (10 µM; mitochondrial electron transfer chain inhibitor) (Bayraktutan et al., 1998; Jiang et al., 2004; Muzaffar et al., 2004a) and superoxide measured by ferricytochrome *c* reduction assay as above. In studies investigating the long-term effect of apocynin, pulmonary artery endothelial cells were exposed to xanthine/xanthine oxidase system in the continual presence of apocynin for 16 h. The cells were then thoroughly rinsed in PBS before superoxide measurements were performed.

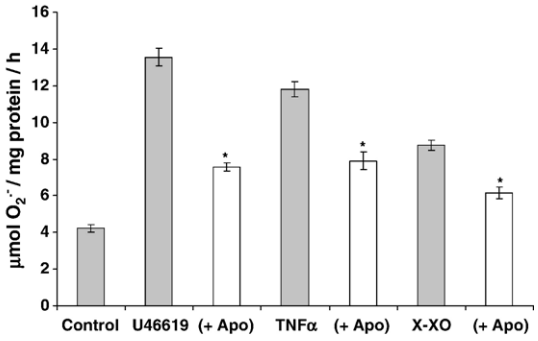

Fig. 5. Effect of co-incubation of pulmonary arterial endothelial cells for 16 h with apocynin (1 µM) and TNFα (10 ng/ml), U46619 (10 nM) or xanthine (100 µM)+xanthine oxidase (20 mU/ml) followed by extensive washing on the formation $O_2^{\bullet-}$. Data=mean±S.E.M.; $n=6$. *$P<0.05$; significantly inhibited comparing effects of apocynin with controls.

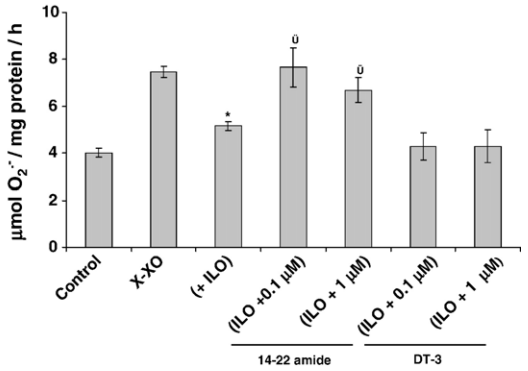

Fig. 7. Effect of iloprost (ILO; 100 ng/ml) in the presence or absence of PKA inhibitor (14–22 amide) or PKG inhibitor (DT-3) on xanthine (100 µM)+xanthine oxidase (20 mU/ml)-induced O_2^- formation in pulmonary artery endothelial cells. Data=mean±S.E.M.; $n=6$. *$P<0.05$; significantly inhibited compared to xanthine/xanthine oxidase-treated cells. †$P<0.01$; significantly increased compared to iloprost treated cells.

2.5. Western blotting

For Western analysis, pulmonary artery endothelial cells were washed and lysed with Tris buffer (100 mM, pH 6.8) containing 1% glycerol and 1% sodium dodecyl sulfate (SDS) (Muzaffar et al., 2004a,b). Extracts were boiled at a 1:1 ratio with the loading buffer containing Tris (125 mM, pH 6.8); 4% w/v SDS; 10% v/v glycerol; 4% v/v 2-mercaptoethanol and 2 mg/ml bromophenol blue. Total cell lysates of equal protein (40 µg) were loaded onto 10% Tris-glycine sodium dodecyl sulfate gels and separated by electrophoresis. After transfer to nitrocellulose, the blots were primed with a specific $gp91^{phox}$ monoclonal antibody (1:500 dilution; BD Biosciences, Oxford, UK) (Yu et al., 1998). The blots were then incubated with goat anti-mouse antibody conjugated to horseradish peroxidase (1:2000 dilution) and developed by enhanced chemiluminescence (Amersham International). Rainbow markers (10–250 kDa; Amersham) were used for molecular weight determination. Membranes were re-probed with anti-GAPDH monoclonal antibody (Chemicon International, CA) as an internal control for equal protein loading.

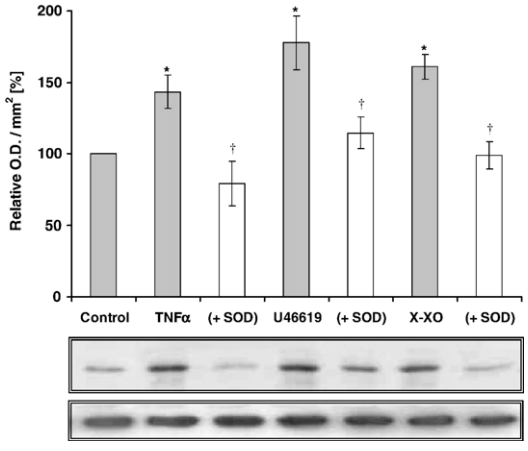

Fig. 6. Western analysis of NADPH oxidase in PAEC lysates using a monoclonal antibody directed against the extracellular epitope of $gp91^{phox}$ subunit of mouse NADPH oxidase. Cells were incubated with TNFα (10 ng/ml) or U46619 (10 nM) or xanthine (100 µM)+xanthine oxidase (20 mU/ml) for 16 h either in the presence or absence of SOD (500 U/ml). The middle panel shows the representative blot and the upper panel the results of the densitometric analyses of 6 blots (expressed as relative optical density (O.D.)/mm^2). GAPDH expression was used as a loading control (lower panel). *$P<0.05$; significantly increased compared to untreated controls. †$P<0.01$; significantly inhibited compared to corresponding minus SOD values.

2.6. Effect of iloprost on superoxide release and NADPH oxidase expression elicited by superoxide, role of protein kinase A

Pulmonary artery endothelial cells were incubated with xanthine (100 µM)+xanthine oxidase (20 mU/ml) as described above

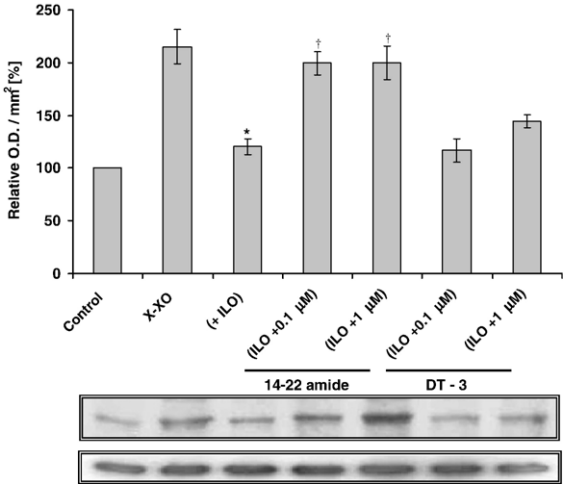

Fig. 8. Western analysis of NADPH oxidase in PAEC lysates using a monoclonal antibody directed against the extracellular epitope of $gp91^{phox}$ subunit of mouse NADPH oxidase. Cells were incubated with xanthine (100 µM)+xanthine oxidase (20 mU/ml) for 16 h either in the presence or absence of iloprost (100 ng/ml). In some experiments cells were pre-treated with either a PKA inhibitor (14–22 amide) or a PKG inhibitor (DT-3) for an hour. The middle panel shows the representative blot and the upper panel the results of the densitometric analyses of 6 blots (expressed as relative optical density (O.D.)/mm^2). GAPDH expression was used as a loading control (lower panel). *$P<0.05$; significantly inhibited compared to xanthine/xanthine oxidase-treated cells. †$P<0.01$; significantly increased compared to iloprost treated cells.

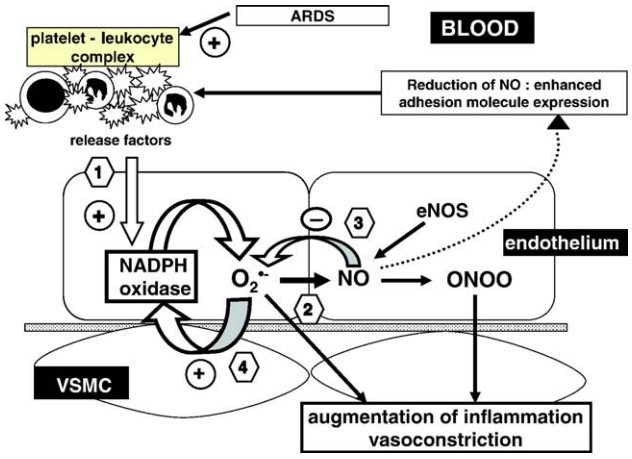

Fig. 9. Model of the pathological events leading to increased NADPH oxidase expression. 1. ARDS promotes the adhesion of blood cells to endothelium. These then release a battery of factors (including cytokines and eicosanoids) which upregulate the expression of NADPH oxidase in blood vessels, thereby increasing the endogenous formation of superoxide ($O_2^{\bullet-}$). 2. $O_2^{\bullet-}$ promotes inflammation and vasoconstriction in its own right but also reduces NO derived from eNOS to form reactive nitrogen species, including peroxynitrite ($ONOO^-$). Since NO is a potent endogenous inhibitor of blood cell adhesion and activity and is a vasodilator, this reduction augments the inflammatory and hypertensive effects of $O_2^{\bullet-}$. 3. Since NO also inhibits the expression of NADPH oxidase, this reduction of NO would also result in further augmentation of NADPH oxidase expression and activity and therefore greater $O_2^{\bullet-}$ formation. 4. $O_2^{\bullet-}$ itself induces the expression of NADPH oxidase thereby increasing endogenous $O_2^{\bullet-}$ formation by a self-amplifying positive feedback mechanism, which in turn would worsen the progression of ARDS.

for 16 h either in the presence or absence of iloprost (100 ng/ml). In order to determine whether the effect of iloprost on superoxide formation and NADPH oxidase expression was mediated by the cyclic AMP (cAMP), pulmonary artery endothelial cells were incubated with the heat-stable and cell-permeable inhibitors of protein kinase A, 14–22 amide peptide sequence, or protein kinase G, DT-3 peptide. Following incubation, cells were washed and superoxide formation assessed as described above. Protein expression was analysed by Western blotting.

2.7. Data analysis

The data were tested for normality by inspecting histograms and by applying the Kolmogorov–Smirnov test (automatically applied by Sigma Stat™ as part of the procedure for producing ANOVA results). In all cases the data did not deviate sufficiently from normality to warrant non-parametric statistics. The data were expressed as mean±S.E.M. Both one-way and two-way analysis of variance (ANOVA) was used to determine statistical significance. Two-way ANOVA tests were employed where two conditions existed and one-way ANOVA was used when comparing effects of drug treatments with untreated controls.

3. Results

Incubation of pulmonary artery endothelial cells for 16 h with 100 μM xanthine+different concentrations of xanthine oxidase, followed by extensive washing of the cells with PBS, elicited a concentration-dependent increase in the formation of superoxide in pulmonary artery endothelial cells (maximal response at 20 mU/ml of xanthine oxidase) (Fig. 1). Superoxide was not detectable in the supernatants after washing, indicating that that the xanthine/xanthine oxidase cocktail had been completely removed from the pulmonary artery endothelial cells and as such could not interfere with the assay. In subsequent studies, therefore, 20 mU/ml of xanthine oxidase+100 μM xanthine was used to generate superoxide.

The continual presence of SOD, which dismutates superoxide, when co-incubated with 20 mU/ml of xanthine oxidase+100 μM xanthine for 16 h, again followed by extensive washing of the cells with PBS reduced the increased superoxide formation in pulmonary artery endothelial cells (Fig. 2). By contrast, catalase had no effect when incubated with xanthine/xanthine oxidase over the same time-course (Fig. 2), indicating that the effects could not be ascribed to hydrogen peroxide (dismutation product of superoxide). The formation of superoxide induced with 16 h of incubation with 10 ng/ml TNFα or 10 nM U46619 was also inhibited by the continual presence of SOD, again followed by washing (Fig. 3).

DPI and apocynin, the inhibitors of NADPH oxidase activity, completely abolished xanthine+xanthine oxidase-stimulated superoxide formation and release from pulmonary artery endothelial cells (Fig. 4). By contrast, rotenone and allopurinol were without effect when co-incubated with xanthine/xanthine oxidase (Fig. 4). Furthermore, apocynin also blocked the increase in xanthine/xanthine oxidase-stimulated superoxide formation in pulmonary artery endothelial cells, when co-incubated with xanthine/xanthine oxidase for 16 h, again followed by extensive washing of the cells with PBS (Fig. 5). Xanthine/xanthine oxidase, TNFα or U46619 all induced $gp91^{phox}$ expression in pulmonary artery endothelial cells after a 16-h incubation period, an effect blocked by the continual presence of SOD (Fig. 6).

The continual presence of iloprost (100 ng/ml) over the 16-h incubation period blocked the increased superoxide formation in pulmonary artery endothelial cells when co-incubated with 20 mU/ml of xanthine oxidase+100 μM xanthine, again followed by extensive washing of the cells with PBS (Fig. 7) as well $gp91^{phox}$ expression (Fig. 8). This effect was blocked by the protein kinase A inhibitor peptide, 14–22 amide, but not by the protein kinase G inhibitor peptide, DT-3 (Figs. 7 and 8).

4. Discussion

The present study demonstrates that incubation of endothelial cells derived from pig pulmonary artery for 16 h with xanthine/xanthine oxidase, a combination that generates superoxide (Greene and Paller, 1992; Greene et al., 2000), induces an increase in the endogenous formation of superoxide. This augmentation of superoxide formation in pulmonary artery endothelial cells was blocked by the continual presence of SOD over this 16-h time course. Since SOD accelerates the dismutation of superoxide this indicates that superoxide auto-augments the formation of superoxide in these cells. By contrast, this effect was not blocked by catalase over the 16-h incubation phase.

Following a 16-h incubation period, xanthine/xanthine oxidase-enhanced superoxide formation was completely inhibited by both apocynin and DPI, inhibitors of NADPH oxidase activity (Jiang et al., 2004). Rotenone and allopurinol, inhibitors of mitochondrial respiration and xanthine oxidase, respectively, were without effect. These data indicate that the auto-augmentation of superoxide by superoxide in pulmonary artery endothelial cells is mediated by an increase in the activity or expression of NADPH oxidase in these cells. In the present study, xanthine/xanthine oxidase system also upregulated the expression of $gp91^{phox}$, an active catalytic subunit of NADPH oxidase (Cai et al., 2003) in pulmonary artery endothelial cells, an effect again blocked by the continual presence of SOD in the 16-h incubation phase. Taken together, therefore, these data indicate that the auto-augmentation of superoxide by superoxide in endothelial cells is mediated by an upregulation of NADPH oxidase ($gp91^{phox}$) expression.

Previous studies have demonstrated that a wide range of inflammatory factors upregulate the expression of $gp91^{phox}$ and superoxide formation in pulmonary artery endothelial cells, including endotoxin, cytokines, thromboxane A_2 (TXA_2) and isoprostanes (Muzaffar et al., 2005b). In the present study, co-incubation of SOD with TNFα or the TXA_2 analogue, U46619, over 16 h also blocked the upregulation of $gp91^{phox}$ and superoxide formation in pulmonary artery endothelial cells. This indicates that an a priori upregulation of $gp91^{phox}$ (NADPH oxidase) by TNFα and the TXA_2 analogue, U46619 results in an increase in superoxide formation in pulmonary artery endothelial cells, which in turn further augments the expression of $gp91^{phox}$. This would constitute a self-amplifying positive feedback loop. In support of this proposal, apocynin, an NADPH oxidase inhibitor, also blocked the superoxide formation in response not only to xanthine/xanthine oxidase but also to TNFα or U46619 when co-incubated over the 16-h time course. It should be noted, too that the responses to TNFα or U46619 appear to be more robust than to xanthine/xanthine oxidase alone, indicating perhaps that other signal transduction factors may come into play in term of upregulation of the expression of $gp91^{phox}$.

Thus, it is suggested that inflammogens released from adherent blood cells initially upregulate the expression of NADPH oxidase which increases the amounts of superoxide generated, intravascularly. In turn, superoxide augments the expression of NAPDH oxidase, which would amplify and augment the progression of ARDS (Fig. 9). Etiologically, superoxide not only elicits vasoconstriction and inflammation in its own right but also reacts with NO to form peroxynitrite and other reactive nitrogen species, effectively reducing NO bioavailabilty (Folkerts et al., 2001; Dweik, 2005). Since NO is a vasodilator and prevents inflammation, a reduction of NO would render the pulmonary vasculature susceptible to ARDS (Fig. 9). Indeed, in a recent multicentre clinical trial in which the NOS inhibitor 546C88 was studied for safety and efficacy in 797 patients with septic shock, it was found that the inhibitor caused a marked increase in mortality (Lopez et al., 2004). This trial indicates that the protective effect of endogenous NO is important in mediating the progression of ARDS. In support of this proposal, recombinant human superoxide dismutase has been shown to enhance the effect of inhaled nitric oxide in persistent pulmonary hypertension (Steinhorn et al., 2001).

Therapeutically, therefore, a reduction in oxidative stress, especially the quenching of superoxide, appears to be a potentially effective strategy in treating ARDS. Indeed, several studies in patients with ARDS and clinical studies and experiments in animal models have indicated that antioxidants may be beneficial in reducing the impact of oxidant stress in ARDS and pulmonary hypertension (Baboolal et al., 2002; Irukayama-Tomobe et al., 2000; Nathens et al., 2002; Pacht et al., 2003; Quinlan et al., 2004).

Finally, the present study also demonstrates that superoxide mediated upregulation of NAPDH oxidase and superoxide formation is inhibited by iloprost, a stable analogue of prostacyclin (Muzaffar et al., 2004b). These effects of iloprost were reversed by inhibition of protein kinase A but not of protein kinase G. Since prostacyclin classically activates cyclic AMP, which in turn activates protein kinase A, these data indicate that iloprost inhibits upregulation of NADPH oxidase and therefore subsequent superoxide release through cAMP-PKA pathway. In previous studies it was shown that iloprost inhibits the upregulation of NAPDH oxidase elicited by other inflammogenic agents, including thromboxane A_2, isoprostanes and cytokines (Muzaffar et al., 2004a). The data therefore indicate that the inhibitory mechanisms of iloprost are similar for all those factors that upregulate the expression of $gp91^{phox}$ and possibly other components of the NADPH oxidase complex. It also consolidates that iloprost may be effective in treating ARDS through suppression of NAPDH oxidase expression and activity. Indeed, several trials have now indicated that inhalational prostacylin is effective in treating ARDS (Lowson, 2002).

To summarise, the present study demonstrates that in isolated pulmonary artery endothelial cells, superoxide auto-augments the formation of superoxide through an upregulation of NAPDH oxidase activity. Since superoxide elicits a number of pathogenic effects that promote ARDS, including the negation of NO availability, the quenching of superoxide coupled with the delivery to NO would seem to be a potentially effective strategy in treating ARDS. This novel positive feedback mechanism also has implications for the etiology and treatment of other cardiovascular diseases and syndromes. It is accepted that the main limitation of this study is that it was carried out using in vitro systems and that further studies using in vivo models are required to expand these observations.

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