Nitroaspirins and Morpholinosydnonimine but Not Aspirin Inhibit the Formation of Superoxide and the Expression of gp91phox Induced by Endotoxin and Cytokines in Pig Pulmonary Artery Vascular Smooth Muscle Cells and Endothelial Cells

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Background—Although nonsteroidal antiinflammatory drugs (NSAIDs) are ineffective in treating acute respiratory distress syndrome (ARDS), inhalational NO has proved to be useful. NO-donating NSAIDs may therefore be more effective in treating ARDS than NSAIDs alone. Because oxidant stress is central to the pathophysiology of ARDS, the effect of nitroaspirins (NCX 4016, NCX 4040, and NCX 4050) compared with morpholinosydnonimine (SIN-1; an NO donor) and aspirin (ASA) on superoxide (O₂⁻) formation and gp91phox (an active catalytic subunit of NADPH oxidase) expression in pig pulmonary artery vascular smooth muscle cells (PAVSMCs) and endothelial cells (PAECs) was investigated.

Methods and Results—Cultured PAVSMCs and PAECs were incubated with lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, and interleukin (IL)-1α (with or without NO-ASA, SIN-1, or ASA) for 16 hours, and O₂⁻ release was measured by use of the reduction of ferricytochrome c. The expression of gp91phox was assessed by use of Western blotting. LPS, TNF-α, and IL-1α all stimulated the formation of O₂⁻ and expression of gp91phox in both PAVSMCs and PAECs, an effect inhibited by NADPH oxidase inhibitors, diphenyleneiodonium, and apocynin. SIN-1, NCX 4016, and NCX 4050 but not ASA alone inhibited the formation of O₂⁻ and expression of gp91phox.

Conclusions—LPS and cytokines promote the formation of O₂⁻ in PAVSMCs and PAECs through an augmentation of NADPH oxidase activity, which in turn is prevented by NO. Thus, NO may play a protective role in preventing excess O₂⁻ formation, but its negation by O₂⁻ may augment the progress of ARDS. The inhibitory effect of nitroaspirins suggests that they may be therapeutically useful in treating ARDS through the suppression of NADPH oxidase upregulation and O₂⁻ formation. (Circulation. 2004;110:1140-1147.)

Key Words: superoxides ■ NADPH oxidase ■ nitric oxide ■ respiratory distress syndrome, adult ■ pulmonary artery

Oxidative stress plays a central role in the pathogenesis of acute respiratory distress syndrome (ARDS), a condition characterized by a time-dependent worsening of intrapulmonary inflammation and hypertension. Principal among the reactive oxygen species (ROS) generated by oxidative stress is superoxide (O₂⁻), which reacts with nitric oxide (NO) to produce peroxynitrite (ONOO⁻), thereby reducing bioavailable NO. A reduction of NO availability promotes not only vasoconstriction but also the adhesion of leukocytes and platelets, which in turn release a battery of vasoconstrictors and cytokines, thereby exacerbating ongoing inflammatory cascades and the development of pulmonary hypertension. Adherent neutrophils and monocytes release tumor necrosis factor-α (TNF-α) and interleukins (ILs), the blood levels of which are markedly elevated in patients with ARDS. In turn, TNF-α and ILs and lipopolysaccharide (LPS) upregulate enzymes that generate O₂⁻ in cultured vascular tissues, in particular, NADPH oxidase. A self-perpetuating inflammatory cascade ensues.

ARDS is a difficult, if not intractable, condition to treat. Because inflammation is the pathological hallmark of ARDS, it would be reasonable to expect that nonsteroidal antiinflammatory drugs (NSAIDs) would be effective in treating ARDS. However, NSAIDs, in particular ibuprofen, have proved ineffective in reducing mortality associated with ARDS despite eliciting a profound inhibition of proinflammatory eicosanoids, such as thromboxane A₂ and prostaglandin E₂. Because NSAIDs also inhibit the formation of prostacyclin (PGI₂), and inhaled PGI₂ has also proved effective in treating ARDS, it is possible that NSAIDs may be...
intrinsically “self-defeating” in this particular scenario. Notably, we have recently demonstrated that PGI₂ inhibits the formation of \( \text{O}_2^- \) via a downregulation of NADPH oxidase activity induced by cytokines and endotoxin in pulmonary arterial cells.\(^{11}\) NSAIDs would therefore remove this protective system, thereby augmenting oxidative stress and the depletion of NO as described above. In this context, inhaled NO has proved more effective in treating ARDS and reducing leukocyte activity and oxidant stress in ARDS.\(^{12}\) NO acts at multiple sites to limit inflammation, including the inhibition of leukocyte and platelet adhesion and of release substances, including that of cytokines.\(^{13}\) Because NO and PGI₂ have similar properties, including vasodilatation, the inhibition of adhesion molecule expression, inhibition of platelet and leukocyte activity, and a reduction in oxidative stress,\(^{13,14}\) it is reasonable to suggest that NO may also inhibit NADPH oxidase expression.

A novel class of NSAIDs that may be effective in treating ARDS is the NO-donating aspirins (NO-ASA). NO-ASA releases NO in vivo while retaining the antiinflammatory capacity of aspirin.\(^{15}\) The donation of NO may also compensate for the inhibition of PGI₂ by NSAIDs. Indeed, drugs of this class have proved effective in preventing gastropathy associated with aspirin by virtue of their NO-donating capacity.\(^{15}\) The NO moiety of NO-ASA may also act by preventing excess \( \text{O}_2^- \) formation, which, as proposed above, would be of potential therapeutic benefit in treating ARDS. To test these proposals, the effect of the NO-ASA adducts NCX 4016, NCX 4040, and NCX 4050 compared with an NO donor alone (morpholinosydnonimine, SIN-1) and ASA alone on PGI₂ activity induced by cytokines and endotoxin in pig pulmonary artery vascular smooth muscle cells and endothelial cells was investigated.

**Methods**

**Dissection of Pulmonary Arteries**

Lungs were obtained from White Landrace male pigs of body weight ranging from 20 to 35 kg. All animal experiments were conducted in accordance with the rules and regulations of Bristol University and the Home Office for the care and use of experimental animals. Pigs were anesthetized with an intravenous injection of ketamine hydrochloride (10 mg/kg; Ketaset Injection, Fort Dodge Animal Health) and inhaled oxygenated halothane. The internal carotid artery was cannulated and preequilibrated in DMEM without phenol red for 10 minutes at 37°C in a 95% air–5% CO₂ incubator. Then 20 μg/mL LPS (1 μg/mL; Escherichia coli; 026:B6; Sigma Chemical Co), human recombinant IL-1α (10 ng/mL; R&D Systems) or human recombinant TNF-α (10 ng/mL; R&D Systems) at 37°C in a 95% air–5% CO₂ incubator. The measurement of \( \text{O}_2^- \) release by cells was performed by detection of ferricytochrome c reduction.\(^{7}\) Thus, after incubation for 16 hours, pulmonary arterial cells were rinsed 3 times in PBS to remove drugs and stimulators and preequilibrated in DMEM without phenol red for 10 minutes at 37°C in a 95% air–5% CO₂ incubator. Then 20 μmol/L horseradish cytochrome c (Sigma Chemical Co) with or without 500 μmol/L copper-zinc superoxide dismutase (SOD; Sigma Chemical Co) was added to the segments or cells and incubated at 37°C in a 95% air–5% CO₂ incubator for 1 hour. The reaction medium was then removed, and the maximum rate of reduction of cytochrome c was determined at 550 nm by use of a temperature-controlled Anthos Lucy 1 spectrometer (Laboratory-tech International and converted to nanomoles of \( \text{O}_2^- \) by use of \( \Delta E_{550 \text{ nm}} = 21.1 \text{ mmol · L}^{-1} · \text{cm}^{-1} \) as the extinction coefficient for (reduced-oxidized) cytochrome c. The
reduction of cytochrome c that was inhibitable with SOD reflected actual O$_2^·$ release. Segments were blotted, dried, and weighed, data being expressed as nmol of O$_2^·$ mg tissue $^{-1}$ h$^{-1}$. Cells were rinsed in PBS and lysed with 0.1% vol/vol Triton-X100, and total protein content was measured by use of a BCA-protein assay kit (Pierce). Data are expressed as mol O$_2^·$ mg protein $^{-1}$ h$^{-1}$.

To determine the source of the O$_2^·$, PAECs and PAVSMCs were preincubated with diphenyleneiodonium chloride (DPI; 10 μmol/L) or apocynin (1 μmol/L; NADPH oxidase inhibitors; Sigma Chemical Co), rotenone (10 μmol/L; an inhibitor of mitochondrial respiration; Sigma Chemical Co), and allopurinol (100 μmol/L; an inhibitor of xanthine oxidase; Sigma Chemical Co) for 2 hours before the measurement of O$_2^·$. Effect of Nitroaspirins, SIN-1, and Aspirin on O$_2^·$ Formation and gp91 phox Expression

The NO-ASA adducts studied were NCX 4016, NCX 4040, NCX 4050, SIN-1 alone, and aspirin alone on (A) LPS- (1 μg/mL), (B) IL-1α- (10 ng/mL), and (C) TNF-α (10 ng/mL)–induced SOD-inhibitable superoxide (O$_2^·$) formation by PAECs after a 16-hour incubation. Each point indicates mean±SEM, n=6. *P<0.01, significantly inhibited vs LPS- or cytokine-treated (0) cells.

Figure 2. Effect of nitroaspirins NCX 4016, NCX 4040, NCX 4050, SIN-1 alone, and aspirin alone on (A) LPS- (1 μg/mL), (B) IL-1α- (10 ng/mL), and (C) TNF-α (10 ng/mL)–induced SOD-inhibitable superoxide (O$_2^·$) formation by PAECs after a 16-hour incubation. Each point indicates mean±SEM, n=6. $P<0.01$, zero (0) vs basal level. *$P<0.01$, significantly inhibited vs LPS- or cytokine-treated (0) cells.

Effect of Nitroaspirins, SIN-1, and Aspirin on O$_2^·$ Formation and gp91phox Expression

The NO-ASA adducts studied were NCX 4040 (2-acetoxybenzoate 2-(2-nitroxy)-butyl ester), NCX 4050 (2-acetoxybenzoate 2-(2-nitroxy-methyl)-phenyl ester, and NCX 4016 (2-acetoxyloxy)benzoic acid 6-(nitrooxymethyl)-2-pyridinylmethy1 ester chloride), which were supplied by NiCox SA. PAVSMCs or PAECs were incubated with LPS (1 μg/mL), IL-1α (10 ng/mL), or TNF-α (10 ng/mL) in either the absence or the presence of (1) nitroaspirins: NCX 4016, NCX 4040, and NCX 4050 (all 0 to 10 μmol/L), (2) 3-morpholinosydnonimine hydrochloride (SIN-1; 0 to 10 μmol/L; Sigma Chemical Co), and (3) aspirin (0 to 10 μmol/L; Sigma Chemical Co) for 16 hours. O$_2^·$ formation was measured by ferricytochrome c assay as described above.

For Western analysis, the medium was removed after 16-hour incubation and the cells lysed with Tris buffer (50 mmol/L, pH 7.4) containing 1% vol/vol Triton X-100, EDTA (10 mmol/L) PMSF (1 mmol/L), pepstatin (0.05 mmol/L), and leupeptin (0.2 mmol/L). Extracts were boiled at a 1:1 ratio with Tris (50 mmol/L, pH 6.8, containing 4% wt/vol sodium dodecyl sulfate, 10% vol/vol glycerol, 4% vol/vol 2-mercaptoethanol, and 2 mg/mL bromophenol blue). Samples of equal protein (100 μg) were loaded onto 12% Tris-glycine SDS gels and separated by electrophoresis. After transfer to nitrocellulose, the blots were primed with a specific human anti-neutrophil gp91phox antibody (2.5 μg/mL final concentration) raised in mouse (a kind gift from Professor D. Roos, CLB, Amsterdam, the Netherlands). The blots were then incubated with goat anti-mouse immunoglobulin (Dako) conjugated to horseradish peroxidase.
Maximum inhibition by nitroaspirins and SIN-1 was achieved with nitroaspirin, aspirin, or SIN-1. After incubation, cells were washed with PBS, incubated for 16 hours with various concentrations of the nitroaspirins, and TNF-α (10 ng/mL) and (C) TNF-α (10 ng/mL). Each point indicates mean±SEM, n=6. *P<0.05, nitroaspirin-treated vs LPS- or cytokine-treated cells only. P<0.05, significantly increased vs nitroaspirin-treated cells.

Effect of Drugs on PGI2 Formation
As mentioned in the introduction, the nitroaspirins possess the potential to compensate for the intrinsic negative effect of aspirin on PGI2 formation. To confirm that nitroaspirins inhibit PGI2 formation, PAECs and PAVSMCs were incubated with various concentrations of the nitroaspirins, aspirin, or SIN-1. After incubation, cells were washed with PBS, and PGI2 formation was stimulated with calcium ionophore A23187 (10 μmol/L; Sigma).11 After incubation for 30 minutes at 37°C, supernatants were removed and aliquots taken for the measurement of PGI2 (as 6-keto-PGF1α; R&D Systems) by use of enzyme-linked immunosassay kits.11

Statistical Analysis
Statistical analysis was performed by use of Instat (GraphPad Software Inc.). Before the study was undertaken, power analysis was performed, from which it was determined that a number of n=6 was required for statistical assurance. Thus, data are expressed as mean±SEM, n=6. Statistical analysis was performed by use of 1-way ANOVA or post hoc unequalled, 2-sided Student’s t test with a Bonferroni adjustment. Statistical significance was assumed at a value of P<0.05.

Results
Source of O2− Formation Elicited by LPS, IL-1α, and TNF-α by PAECs and PAVSMCs
LPS (1 μg/mL) and TNF-α and IL-1α (both at 10 ng/mL) augmented O2− release from PAECs and PAVSMCs after a 16-hour time course (Figure 1). DPI and apocynin, both inhibitors of NADPH oxidase (but not rotenone or allopurinol) significantly inhibited O2− formation and release from PAECs and PAVSMCs after a 16-hour incubation with LPS, IL-1α, and TNF-α (Figure 1).

Effect of Nitroaspirins, NO Donor (SIN-1), Aspirin, and Guanylyl Cyclase Inhibitor (ODQ) on O2− Release and gp91phox Expression
The nitroaspirins (NCX 4016, NCX 4040, or NCX 4050) and SIN-1 but not aspirin alone inhibited LPS- and cytokine-induced O2− release (Figures 2 and 3). In both cell types, the maximum inhibition by nitroaspirins and SIN-1 was achieved at 0.1 μmol/L, which was reversed by the addition of NO-sensitive guanylyl cyclase inhibitor, ODQ, in a dose-dependent manner (Figure 4), indicating that the cGMP-protein kinase G axis mediates this inhibitory effect of NO. Furthermore, nitroaspirins (NCX 4016 and NCX 4050) and SIN-1 also inhibited any increase in LPS-, IL-1α-, and TNF-α-induced gp91phox protein expression in PAVSMCs (Figure 5) and PAECs (Figure 6). Aspirin alone, however, had no significant effect on LPS- or cytokine-induced gp91phox expression in both cell types (Figures 5 and 6).

Effect of Nitroaspirins, SIN-1, and Aspirin on Quenching of O2− and PGI2 Formation
The nitroaspirins, aspirin, and SIN-1 had no significant quenching effect on O2− generated by the xanthine/xanthine oxidase system (Table, top). Nitroaspirins and aspirin all inhibited the formation of PGI2 (as 6-keto-PGF1α) in a dose-dependent manner (Table, bottom). In contrast, SIN-1 alone significantly elevated the PGI2 levels produced by PAVSMCs (Table, bottom).

Discussion
The present study demonstrates, first, that TNF-α, IL-1α, and LPS promote the formation of O2− in both PAVSMCs and...
PAECs in a time-dependent manner and at concentrations that have been reported to appear in the blood of patients with ARDS. Furthermore, apocynin and DPI, both inhibitors of NADPH oxidase activity, completely inhibited the generation of \( \text{O}_2^- / \text{H}_2 \text{O}_2 \) in response to TNF-\( \alpha \), IL-1\( \alpha \), and LPS, indicating that increased NADPH oxidase activity/levels mediate these effects, a conclusion confirmed by the upregulation of gp91phox by TNF-\( \alpha \), IL-1\( \alpha \), and LPS. These data consolidate the proposition that the pathogenic effect of endotoxins and cytokines in ARDS may be mediated, in part, through an induction of intrapulmonary arterial oxidative stress.

In a previous study, we demonstrated that the enhancement of \( \text{O}_2^- \) formation in intact pig pulmonary arteries by TNF-\( \alpha \), IL-1\( \alpha \), and LPS resulted in the formation of peroxynitrite (\( \text{ONOO}^- / \text{H}_2 \text{O}_2 \)) \(^\text{4} \) by the reaction between \( \text{O}_2^- \) and NO. \(^\text{20} \) The lowering of NO bioavailability may result in vasoconstriction and pulmonary hypertension as well as a local increase in cytokines, because NO is a vasodilator \(^\text{20} \) and prevents the adhesion of platelets and neutrophils. \(^\text{20} \) \( \text{ONOO}^- \) itself may be proinflammatory in this scenario, because it promotes adhesion molecule expression in leukocytes \(^\text{21} \) and inhibits PGI\(_2\) synthase activity. \(^\text{22} \) In addition, in the present study, the NO donor SIN-1 inhibited gp91phox expression and \( \text{O}_2^- \) formation through a guanylyl cyclase–dependent pathway, because the effects were reversed by ODQ. This effect could not be ascribed to a direct quenching effect, because the drugs were washed from of the system before \( \text{O}_2^- \) or gp91phox expression was measured, and the nitroaspirins and SIN-1 had no effect on \( \text{O}_2^- \) generated by xanthine/xanthine oxidase in the absence of cells. Thus, endogenous NO formation may protect the pulmonary vasculature by preventing the expression of NADPH oxidase induced by LPS and cytokines.
verse is that the loss of NO availability by the overproduction of $O_2^-$ would render the vasculature susceptible to augmented NADPH oxidase activity and therefore to increased NO destruction, thereby worsening the inflammatory cascades associated with ARDS.

In terms of treating ARDS, inhalational NO has been shown to be beneficial. In the present study, not only SIN-1 but also the NO-donating aspirins inhibited $O_2^-$ formation and NADPH oxidase upregulation, again via a cGMP-dependent mechanism. In contrast, aspirin had no effect, indicating that the effects of the nitroaspirins are mediated by the NO rather than the aspirin moiety of the drug. Several studies have demonstrated that nitroaspirins exert both NO-mediated effects and the inhibition of cyclooxygenase and therefore of the generation of proinflammatory eicosanoids, such as thromboxane $A_2$ (TXA$_2$) and PGE$_2$.

Although many clinical trials have demonstrated that NSAIDS are relatively ineffective in treating ARDS, they have been shown to inhibit the formation of proinflammatory eicosanoids in patients with sepsis. One possible explanation for the lack of efficacy of NSAIDs in ARDS is that they also inhibit the formation of the antiinflammatory eicosanoid PGI$_2$. The possible importance of endogenous PGI$_2$ in protecting against ARDS is exemplified by the therapeutic benefits of inhalational PGI$_2$ to treat the condition. In a recent study, we also demonstrated that the PGI$_2$ analogue iloprost inhibits the expression of NADPH oxidase and $O_2^-$ formation in pig PAVSMCs and PAECs, again induced by LPS and cytokines. Thus, NSAIDs may intrinsically negate their therapeutic potential through this “double-edged” effect on PGI$_2$. In the present study, the nitroaspirins inhibited PGI$_2$ formation but still blocked the gp91phox expression and $O_2^-$ formation.

**Figure 6.** Protein expression of NAD(P)H oxidase in PAECs as measured by Western blot using a monoclonal antibody directed against gp91phox-subunit of human neutrophil NAD(P)H oxidase (MoAb 48). Cells were either not treated or treated with (A) LPS (1 $\mu$g/mL), (B) IL-1$\alpha$ (10 ng/mL), or (C) TNF- $\alpha$ (10 ng/mL) for 16 hours with one of the following: NCX 4016 (100 nmol/L), NCX 4050 (100 nmol/L), SIN-1 (1 $\mu$mol/L), or aspirin (10 $\mu$mol/L). Bands detected are 91 kDa for heavily glycosylated form of gp91phox and 66 kDa for less glycosylated form of gp91phox. Top, Representative blots and bottom, results of densitometric analyses of 6 blots (expressed as relative optical density [O.D.]/mm$^2$). Pig neutrophil lysates were used as positive controls. *$P<0.05$, significantly inhibited vs LPS- or cytokine-treated cells.
formation. Thus, the NO-donating aspirins, by virtue of possessing an NO-donating moiety, may overcome this limitation of NSAIDs, because NO has properties similar to those of PGI₂, including vasodilatation and the inhibition of adhesion molecule expression and platelet and leukocyte activity.13,20

Notwithstanding NO and the lack of effect of ASA on NADPH oxidase activity, the aspirin moiety of NO-ASA may reduce vascular O₂⁻ formation through an indirect effect on platelet TXA₂ formation. We found that the TXA₂ analogue U46619 promoted O₂⁻ formation and NADPH oxidase expression in pig PAECs and PAVSMCs.11 Because hyperactive platelets and increased TXA₂ formation is a hallmark of ARDS,23 this inhibition may also be beneficial in attenuating oxidative stress and reducing the prohypertensive actions of TXA₂.14

One potential drawback of orally administered nitroaspirins, however, is their possible contraindication in sepsis, which etiologically accounts for 40% of ARDS patients.21 In contrast to the pulmonary hypertension seen in ARDS, sepsis is associated with systemic hypotension, which is evoked by endotoxin-induced inducible NO synthase expression22 and as such may be worsened by an NO donor. Indeed, it has been widely advocated that sepsis can be treated with inhibitors of NO synthase.24 Nevertheless, in the remainder of patients with non–sepsis-mediated ARDS, nitroaspirins may still be appropriate.

In conclusion, this study demonstrates that LPS and cytokines promote the formation of O₂⁻ in pig pulmonary arterial cells via an upregulation of NADPH oxidase activity. This would lead to the negation of NO availability and the generation of ONOO⁻, which may render the vasculature susceptible to oxidative attack. The administration of nitroaspirins may be therapeutically useful in treating ARDS through (1) inhibition of leukocyte and platelet adhesion, (2) inhibition of release of cytokines from these blood cells, (3) suppression of NADPH oxidase upregulation and therefore O₂⁻ release, (4) replacement of NO depleted by excess O₂⁻ formation, and (5) inhibition of TXA₂ formation, a promotor of both oxidative stress and vasoconstriction. Apart from ARDS, these data indicate that the therapeutic action of NO donors, including nitroaspirins, may be mediated, in part, through the suppression of NADPH oxidase expression and therefore excess O₂⁻ formation. Nitroaspirins may also be effective in treating vascular diseases associated with oxidative stress.

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