Effect of exogenous nitric oxide and superoxide on interleukin-8 from human polymorphonuclear leucocytes

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Summary

Patients with acute inflammatory lung injury are commonly treated with inhaled nitric oxide. Nitric oxide has profound immunoregulatory effects. Increased concentrations of the cytokine interleukin-8 (IL-8) in bronchoalveolar lavage fluid has been associated with disease severity. We have investigated the effects of a nitric oxide donor and a combined nitric oxide–superoxide donor on lipopolysaccharide-mediated accumulation of IL-8 from cultured human neutrophils. Interleukin-8 was measured in culture supernatant after 20 h using enzyme immunoassay. The combined nitric oxide–superoxide donor, 3-morpholinosydnonimine (SIN-1), dose-dependently decreased lipopolysaccharide-mediated IL-8 accumulation (P<0.01). SIN-1 also decreased IL-8 accumulation from unstimulated neutrophils (P<0.001). In contrast, the pure nitric oxide donor, 1,2,3,4-oxatriazolium 5-amino chloride (GEA-3162), increased stimulated IL-8 accumulation (P<0.01) and also increased IL-8 accumulation in unstimulated cells (P<0.002). Nitric oxide and superoxide have profound effects on IL-8. These results have important implications for the treatment of patients with acute lung injury with inhaled nitric oxide. (Br. J. Anaesth. 1997; 78: 714–717).

Key words


Nitric oxide has been found to have many biological roles within the body and nitric oxide gas is used as a selective pulmonary vasodilator in critically ill patients with acute respiratory distress syndrome (ARDS). A variety of mediators have been shown to reflect the severity of lung inflammation in ARDS. Concentrations of the cytokine interleukin-8 (IL-8) in bronchoalveolar lavage fluid correlated with the development of ARDS and increased IL-8 concentrations early in the disease process correlated with mortality. In addition, inhaled nitric oxide therapy modulates increased cytokine concentrations, including IL-8, in patients with ARDS.

Nitric oxide and its metabolite peroxynitrite are known to have a profound effect on the inflammatory process as it is now known that activated white cells release nitric oxide in addition to superoxide anion during the respiratory burst. There is however, little information on the possible interaction of nitric oxide and the metabolites of nitric oxide on the regulation of the inflammatory process. Such regulation is governed by circulating inflammatory mediators which are released from immune cells and affect the functioning of other cells. Information on this interaction is important because of the increasing use of exogenous nitric oxide in the treatment of refractory hypoxia and pulmonary hypertension in ARDS.

We have therefore investigated the effects of nitric oxide and superoxide on cytokine production by polymorphonuclear leucocytes both with and without lipopolysaccharide and in the presence of either the nitric oxide donor 1,2,3,4-oxatriazolium 5-amino chloride (GEA-3162) or the combined nitric oxide and superoxide donor 3-morpholinosydnonimine (SIN-1).

Materials and methods

Polymorphonuclear leucocytes were isolated from a single volunteer for all experiments using a single step density gradient procedure. Arterial blood was added to sterile preservative-free heparin (10 u/ml of blood) and layered onto Polymorphprep separation medium (Nycomed UK Ltd, Birmingham, West Midlands, UK). After centrifugation at 475 g for 35 min at 20°C, the mononuclear cell band was discarded and polymorphonuclear leucocytes (PMN) were retrieved and washed in Dulbecco's modification of Eagle's basal medium (DMEM, ICN Biomedicals Ltd, Thame, Oxon, UK). Contaminating erythrocytes were removed by hypotonic shock and cells were washed three times in DMEM before being resuspended, counted and adjusted to 3 × 10⁶ cells/ml.

Replicate samples (1.5 × 10⁶ cells) were placed into 24-well tissue culture plates and lipopolysaccharide to a final concentration of 1.7 µg ml⁻¹ (LPS, from E. coli strain 0111.B4, Sigma-Aldrich Chemical Co. Ltd, Poole, Dorset, UK) or phosphate-buffered...
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saline blank was added as appropriate. To each well was also added varying concentrations of the nitric oxide donors. The agents used were 3-morpholinosydnonimine hydrochloride (SIN-1) (Alexis Corporation, Nottingham, UK) which is a metabolite of the vasodilator molsidomine and is a nitric oxide and superoxide anion donor and thus considered a peroxynitrite releasing compound. In addition, to some cells was added 1,2,3,4-oxatriazolium-5-amino-3-(3,4-dichlorophenyl)-chloride (GEA-3162) (Alexis Corporation, Nottingham, UK) which is a water soluble nitric oxide donor. The concentrations used were 10, 100 and 1000 μmol litre⁻¹ for SIN-1 and 0.01, 0.1 and 1.0 μmol litre⁻¹ for GEA-3162 in phosphate-buffered saline. Non-LPS stimulated neutrophils were also incubated with SIN-1 100 μmol litre⁻¹ or GEA-3162 1 μmol litre⁻¹ as controls.

Tissue culture plates were incubated at 37°C in an atmosphere of 5% carbon dioxide–95% air for 20 h. Culture medium was removed for measurement of IL-8 using solid phase sandwich enzyme-linked immunosorbent assay (ELISA, R&D Systems Europe Ltd, Abingdon, Oxon, UK). Results are expressed as pg/10⁶ cells. The assay is highly specific for IL-8 and shows no cross-reactivity for any of the other human cytokines tested. Within-assay precision was 3.2% (n=20) and between-assay precision 9.5% (n=20). Cells were detached for viability count using the vital stain trypan blue.

STATISTICAL ANALYSIS
Data are presented as median (range) and represent 5–6 separate experiments. Statistical analysis was performed using Microsoft Excel with Astute add-in and data were analysed using Kruskal–Wallis analysis of variance or Mann–Whitney U test as appropriate, with P<0.05 being considered statistically significant.

Results
Unstimulated polymorphonuclear leucocytes produced detectable basal concentrations of IL-8 which were increased when cells were incubated with LPS (P<0.0001; figs 1, 2). Incubation with SIN-1 caused concentration-dependent depression of IL-8 accumulation in culture medium of LPS-stimulated cells (P<0.01; fig. 1). Addition of SIN-1 1000 μmol litre⁻¹ to cells in the absence of stimulation with LPS also caused a reduction in basal IL-8 accumulation (P<0.001; fig. 1).

In contrast, GEA significantly increased IL-8 accumulation from both LPS-stimulated and unstimulated cells (P<0.01 and P<0.002 respectively; fig. 2).

Trypan blue staining showed that cell viability in SIN-1 and GEA-3162 treated cells after 20 h incubation was similar to that of cells in the absence of these agents.

Discussion
We have shown that exogenous nitric oxide and a combination of nitric oxide-superoxide has a marked effect on the accumulation of IL-8 from LPS-stimulated human polymorphonuclear leucocytes. IL-8 is a potent neutrophil chemotactic and activating factor released by neutrophils in a self-amplifying process in response to inflammatory stimuli. IL-8 has been implicated in the pathogenesis of ARDS both as a marker of onset and severity of ARDS,⁻² and concentrations in bronchoalveolar lavage fluid correlate with mortality.⁻³ It is a potent chemoattractant and has an important role in the recruitment and activation of neutrophils into the lung. Activated neutrophils via the release of cytokines, proteinases, hypochlorous acid, oxygen-derived free radicals and nitric oxide are important in the pathophysiology of ARDS as these inflammatory mediators may result in tissue damage. Further release of these chemoattractant factors potentiates this inflammatory process. Factors which regulate the production and release of IL-8 may, therefore, have a major role in altering the regulation of inflammation. The release of IL-8 by the same cell type as that on which it acts has important implications as it so acts to amplify the inflammatory process occurring

Figure 1 Effect of SIN-1 on interleukin-8 accumulation from non-stimulated and lipopolysaccharide-stimulated human neutrophils. LPS = lipopolysaccharide, SIN-1 = 3-morpholinosydnonimine hydrochloride.

Figure 2 Effect of GEA-3162 on interleukin-8 accumulation from non-stimulated and lipopolysaccharide-stimulated human neutrophils. LPS = lipopolysaccharide, GEA-3162 = 1,2,3,4-oxatriazolium-5-amino-3-(3,4-dichlorophenyl)-chloride.
at that particular site. For these reasons the study of isolated neutrophils in vitro provides a model of the pathophysiological processes in ARDS. The dedicated nitric oxide donor GEA and the combined nitric oxide–superoxide anion donor SIN-1 had markedly different effects on IL-8. Such changes have clear implications for patients receiving inhaled nitric oxide for the acute inflammatory process of ARDS.

Exogenous nitric oxide has been shown to have various effects on the activity of neutrophils. Production of the arachidonic acid product leukotriene B\textsubscript{4} is dose-dependently reduced in the presence of either SIN-1 or GEA–3162 in calcium ionophore-stimulated neutrophils.\textsuperscript{6} In addition, there is a dose-dependent reduction in leukotriene B\textsubscript{4} formation in formyl-methionyl-leucyl-phenylalanine- (FMLP) stimulated neutrophils in the presence of SIN-1.\textsuperscript{7} There is some evidence of a biphasic effect of SIN-1 on the oxidative burst activity (superoxide production) of opsonised zymosan-activated neutrophils.\textsuperscript{8} Other investigators have shown dose-dependent reduction in superoxide production by SIN-1 in stimulated and non-stimulated neutrophils.\textsuperscript{9} Nitric oxide can inhibit the enzyme NADPH oxidase which produces superoxide.\textsuperscript{10} Nitric oxide has also been shown to reduce neutrophil adherence and migration and thus accumulation.\textsuperscript{11}

We have found a different effect of dedicated nitric oxide donor compared with the combined nitric oxide–superoxide anion donors on human polymorphonuclear leucocytes. Exogenous nitric oxide alone increased the accumulation of IL-8 in both LPS-stimulated and unstimulated neutrophils. The combined nitric oxide–superoxide anion donor (effectively a peroxynitrite donor) caused a concentration-dependent reduction in IL-8 production. This is in contrast with a previous study of human melanoma cells in which both SIN-1 and the dedicated nitric oxide donors S-nitroso-L-glutathione and S-nitroso-N-acetylpenicillamine caused an increase in IL-8 at the transcriptional level.\textsuperscript{12} Another study using a human endothelial cell line showed that exogenous nitric oxide increased IL-8 release.\textsuperscript{13} These effects may represent different actions on the transcription factor nuclear factor κB (NFκB) in the cell types described. This transcription factor is activated by external cell signals and allows rapid production of immunologically active compounds such as cytokines and nitric oxide synthase. It is known that activation of NFκB is stimulated by hydrogen peroxide and possibly other free radicals\textsuperscript{14} and that nitric oxide also activates NFκB in some cell types.\textsuperscript{12,15} although in human endothelial cells NFκB is inhibited by nitric oxide.\textsuperscript{16}

The effect of nitric oxide and superoxide on NFκB activation or inhibition in human neutrophils is not known. It is also possible that nitric oxide and superoxide could affect transcriptional, translational, post-translational and stability or excretion of IL-8 after its production. Further research to elucidate the role of free radicals on inflammatory responses is required.

The relative levels of nitric oxide and peroxynitrite could determine the recruitment of neutrophils into the lung either through a direct effect of nitric oxide on neutrophil adherence or through IL-8 induced chemotaxis. During inhaled nitric oxide therapy in ARDS this balance may be disturbed thereby resulting in alteration of the inflammatory process. Inhaled nitric oxide therapy has been shown to decrease the high IL-6 and IL-8 concentrations in bronchoalveolar lavage fluid in patients with ARDS.\textsuperscript{4}

These results contradict our findings but could be explained by the high availability of superoxide in inflammatory lung diseases leading to peroxynitrite production.\textsuperscript{17} This would agree with work that showed the presence of nitrotyrosin residues (a marker of peroxynitrite damage) within the lung of all patients with ARDS and would explain decreased IL-8 concentrations in bronchoalveolar lavage fluid in these patients.\textsuperscript{4,18}

The precise role of exogenously administered nitric oxide in the regulation of the inflammatory process still needs to be established in the intact human.

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References


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