The physiological role and pharmacological potential of nitric oxide in neutrophil activation

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Abstract

There is contention over whether human neutrophils produce physiologically significant levels of nitric oxide (NO) during inflammatory reactions. Nevertheless, regardless of its cell source, NO does exert regulatory effects on neutrophil function. Depending on experimental conditions, NO can either inhibit or enhance neutrophil activation, in both cases probably acting through cyclic GMP. The explanation for these apparently contradictory findings may be that the effect depends upon the concentration of NO: low concentrations of NO being stimulatory and high concentrations inhibitory. Nitrite, produced at high concentrations from NO during inflammation, can react with neutrophil myeloperoxidase-derived hypochlorous acid (HOCl) to form the active oxidant nitryl chloride, a species capable of nitrating tyrosine and tyrosyl residues on proteins. Whether nitryl chloride acts to limit or amplify the oxidant effects of myeloperoxidase is not yet clear, although formation of nitrotyrosine has been linked with nitration of phagocytosed bacteria. Clearly, a better understanding of the inflammatory effects of NO on neutrophils is needed before the therapeutic potential of NO donors or inhibitors in inflammation can be realised.

1. Introduction

Neutrophils comprise a fundamental component of the non-specific immune response to bacterial infection. The neutrophil response is characterised by adhesion to the vascular endothelium, followed by migration into tissues, oxygen radical-dependent killing of microbes, and elimination of microbes and tissue debris by phagocytosis. Since the agents released by activated neutrophils are potentially toxic to host tissues, neutrophils are subsequently removed by the process of apoptosis and are engulfed by macrophages to resolve the inflammatory response. Thus, modulation of the activation status of the neutrophil is of key importance in determining the balance between immune defense and host injury.

The aim of this review is to examine the role of nitric oxide (NO) in modulating neutrophil function. Effects of NO on the neutrophil functions detailed above will be discussed to elucidate the physiological role of endogenous NO, as well as its pharmacological potential in clinical situations.

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2. NO production by neutrophils

Several studies have reported NO production by neutrophils, but others have claimed little or no NO production by these cells. For example, NO synthesis coincident with neutrophil chemotaxis has been detected by functional NO activity [1,2], chemically [3–5] and by ADP ribosylation of proteins [6]. Human blood neutrophils stimulated in vitro with monocyte-derived cytokines and neutrophils from inflamed exudates are reported to express inducible NO synthase (iNOS) [7,8]. Furthermore, neutrophil-enriched urine fractions from patients with urinary tract infections were found to have 43-fold more NOS activity than similar fractions from non-infected controls [9].

In spite of these reports, there is still controversy as to whether human neutrophils can produce functionally significant levels of NO [10–12]. For example, Miles et al. [12] reported that rat peritoneal neutrophils express iNOS, whereas circulating and extravasated human neutrophils, derived from peritoneal dialysis of peritonitis patients, did not express iNOS mRNA, iNOS protein or enzymatic activity.

Synthesis of NO by human neutrophils is certainly low compared to that by endothelial cells [6,11]. This may be explained by the presence of an endogenous inhibitory factor synthesised by neutrophils that inhibits both constitutive NOS (NOS1) and iNOS [13]. There is also evidence for enzymatic generation of NO in neutrophils from other nitrogen-containing compounds [14].

In experiments from our own laboratory, we could not detect significant increases in total nitrate/nitrite production in response to fMLP stimulation of human neutrophils (Fig. 1A), whereas a significant increase in cyclic GMP levels was observed in neutrophils activated by either NO donors or fMLP (Fig. 1B). Thus, although we could not detect NO production by human neutrophils in the absence of a NO donor, these cells did appear to be responsive to fMLP and NO donors in terms of cGMP production.

Fig. 1. Production of nitrate/nitrite (A) and cyclic GMP (B) by human neutrophils. The cells were incubated with the neutrophil activating agent fMLP or the NO donors GEA 3162, GEA 5024 and SIN-1 (modified from Wanikiat et al. [21]). fMLP was used over the concentration range 0.1–0.3 μM, since this is optimal for neutrophil activation, and NO donors were used at concentrations that inhibit neutrophil activation. (A) No significant production of nitrate/nitrite was observed with fMLP. The expected concentration-dependent increases in nitrate/nitrite were observed with the NO donors. (B) All four agents significantly increased cyclic GMP in the neutrophils, although the response to fMLP was smaller than that induced by the NO donors. Experiments were carried out in the presence of the non-selective phosphodiesterase inhibitor, isobutylmethylxanthine (0.25 mM).
Rat neutrophils express the constitutive neuronal type NOS (nNOS or NOS1) and NOS1 protein. Accordingly, these cells generate basal levels of nitrate/nitrite. On the other hand, human neutrophils express NOS1 mRNA, but NOS1 enzyme and NO production are not detected [15]. Clearly it is important to understand the signals required to translate NOS1 mRNA into NOS protein in human neutrophils.

Overall, it is evident that there are differences between human and rodent neutrophils in terms of their capacity to produce NO, and possibly differences in the isoforms of NOS that may be involved.

3. Role of NO in neutrophil chemotaxis

There is considerable evidence that NO and cyclic GMP act as endogenous mediators of the chemotactic response of neutrophils. NOS inhibitors such as \( N^G \)-monomethyl \( L \)-arginine (L-NMMA), \( N^G \)-nitro-\( L \)-arginine methyl ester (L-NAME) and canavanine (L-CAN) all inhibited neutrophil chemotaxis induced by the bacterial peptide \( n \)-formyl-methionyl-leucyl-phenylalanine (fMLP) (Table 1), supporting a role for NO as a mediator of chemotaxis. \( N^\omega \)-nitro \( L \)-arginine (L-NNA) was ineffective in this respect, perhaps because there is no block of neutrophil NOS by this compound [11]. It is likely that NO induces chemotaxis through the subsequent synthesis of cyclic GMP, because inhibition of guanylate cyclase activity with LY-83583 or inhibition of cyclic GMP signaling through protein kinase G with KT 5823 or Rp-8-pCPT-cGMPS inhibited neutrophil chemotaxis (Table 1). The role of iNOS-derived NO in neutrophil migration has also been examined in gene knockout studies. Injection of zymosan into the peritoneal cavity of both wild-type and iNOS knockout mice cavity led to neutrophil recruitment and chemokine production. The response was clearly not dependent on NO but subtle differences in kinetics were observed between the two types of mice [16]. Despite the balance of results from studies with NOS inhibitors indicating that endogenous NO acts

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<tr>
<td>L-NMMA and L-CAN</td>
<td>Inhibition of neutrophil NO release, assayed functionally as inhibition of platelet aggregation. Reversed by ( L )-arginine.</td>
<td>[1]</td>
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<tr>
<td>L-NMMA</td>
<td>Inhibition of fMLP-induced neutrophil chemotaxis. Reversed by cyclic GMP.</td>
<td>[92]</td>
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<td>L-NMMA, L-CAN but not L-NAME</td>
<td>Inhibition of chemotaxis of LPS-primed or unprimed neutrophils. Reversed by ( L )-arginine or 8 bromo cyclic GMP.</td>
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<td>L-NMMA</td>
<td>Inhibition of fMLP-induced neutrophil chemotaxis. Mimicked by NO scavenger, carboxy PTIO.</td>
<td>[21]</td>
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<tr>
<td>L-NNA, N-iminoethyl-L-ornithine and diphenyleneiodonium</td>
<td>No effect on neutrophil chemotaxis measured by orientation or trajectory.</td>
<td>[94]</td>
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<td>L-NNA</td>
<td>No block of fMLP or IL-8-induced neutrophil chemotaxis.</td>
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<td>Guanylate cyclase</td>
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<td>LY-83583</td>
<td>Inhibition of fMLP-induced increase in neutrophil cyclic GMP levels and G kinase phosphorylation of vimentin.</td>
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<td>LY-83583</td>
<td>Marked inhibition of fMLP-induced neutrophil chemotaxis.</td>
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<td>LY-83583 and methylene blue</td>
<td>Inhibition of the enhancement of neutrophil migration observed with NO.</td>
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<td>Protein kinase G</td>
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<td>KT8807</td>
<td>Directly induced neutrophil shape change.</td>
<td>[98,99]</td>
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<td>Rp-8-pCPT-cGMPS and S</td>
<td>Inhibition of the enhancement of neutrophil migration observed with NO.</td>
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<td>Rp-8-bromo-cGMPS</td>
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<tr>
<td>KT8807 but not Rp-8-pCPT-cGMPS</td>
<td>Inhibition of fMLP-induced neutrophil chemotaxis.</td>
<td>[21]</td>
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as a mediator of neutrophil chemotaxis, paradoxically NO donors inhibit various aspects of neutrophil activation, including chemotaxis [17–21]. This inhibitory effect is mediated by cyclic GMP [22,23]. The simplest explanation for these contradictory findings is that the effect depends upon the concentration of NO, whereby low concentrations stimulate chemotaxis and high concentrations are inhibitory. It may be that the increase in NO and cyclic GMP observed concomitantly with neutrophil activation represents a negative feedback pathway. NO may inhibit neutrophil chemotaxis through peroxynitrite which is produced under conditions of high NO and superoxide.

4. NO in neutrophil adhesion

The migration of neutrophils from the blood though the endothelium to the site of inflammation requires highly coordinated cell–cell adhesive interactions between neutrophils and the endothelium. Selectins mediate the initial, low affinity adherence of neutrophils when rolling along the endothelium [24], whereas activation of neutrophil integrin adhesion molecules, and their interaction with endothelial immunoglobulins, enables transmigration of neutrophils to occur [25].

Neutrophil aggregation can be used as a model of homotypic adhesion that is dependent on upregulation of β2 integrin adhesion molecules [26]. It has recently been shown that L-arginine increases and prolongs fMLP-induced neutrophil aggregation through a NO-dependent mechanism involving ADP-ribosylation of actin [27]. However, as with chemotaxis, the effect of endogenous cell-synthesised NO is the opposite of that observed with exogenous NO, in the form of NO chemical donors. The latter inhibit adherence also by a mechanism associated with ADP ribosylation of actin [28].

Again, this discrepancy may relate to the small amount of NO synthesised by neutrophils compared with the larger amounts added exogenously as NO donors. NO donors inhibit neutrophil adhesion to endothelial cells induced by lipopolysaccharide (LPS) [29,30] or tumour necrosis factor α (TNFα) [31]. Measurement of neutrophil adhesion to human umbilical vein endothelial cells in culture demonstrated that LPS treatment upregulated expression of E-selectin and intercellular adhesion molecule-1 (ICAM-1) as assessed by flow cytometry [29]. NO donors reduced expression of endothelial E-selectin without affecting ICAM-1 expression. Similarly, NO treatment of blood in an extracorporeal circuit reduced the induced expression of neutrophil integrin receptors and subsequent adhesion to endothelial cells in culture [32]. However, the opposite result was found with a high concentration of the NO donor spermine NONO-ate, which promoted neutrophil adhesion to endothelial cells in culture by a mechanism related to mobilisation of P-selectin and increased endothelial synthesis of platelet activating factor [33].

Most research, however, has focused on the role of NO released by endothelial cells, rather than neutrophils, in regulation of neutrophil–endothelium adhesion. The inhibitory role of endothelium-derived NO on neutrophil adhesion was first demonstrated by Kubes et al. [19], and has since formed the basis for the hypothesis that reduced endothelial NO is largely responsible for neutrophil-dependent myocardial damage in reperfusion injury through increased neutrophil adhesion and activation [34]. It has been postulated that the ability of NO to inhibit neutrophil adhesion to the endothelium results from its ability to inactivate superoxide anions [103]. Reduced endothelial NO and the increased generation of neutrophil reactive oxygen species is thought to account for the damage observed in reperfusion injury. Since cardiac myocytes express ICAM-1 on their surface [35] and this adhesion molecule has been shown to be upregulated by cytokines when the endothelium is dysfunctional [36], this will promote the binding and activation of neutrophils.

This hypothesis is supported by observations that endogenous NO [37], its substrate L-arginine [38–40] and NO donors [41–44] are protective in animal models of reperfusion injury. However, the usefulness of NO donors in conditions where neutrophil damage have been implicated is not likely to be restricted to reperfusion injury.

5. NO and generation of reactive oxygen species by neutrophils

Generation of superoxide (O$_2^-$) during the respiratory burst results from the assembly and activation of
the NADPH oxidase-dependent transmembrane electron transport chain. This oxidase activation is essential for the bactericidal function of neutrophils [45] and consequently, their toxic potential. Several studies have looked at the effects of NOS inhibitors on the neutrophil respiratory burst (Table 2) but only one [104] revealed the involvement of NO. This cannot relate to lack of NOS inhibitory activity by these agents in human neutrophils because the same compounds were effective inhibitors of neutrophil chemotaxis (Table 1). Fewer studies have been carried out with agents that affect the cyclic GMP pathway, but substantial blockage of superoxide anion generation was observed with the protein kinase G inhibitors KT 5823 and Rp-8-pCPT-cGMPS [21].

There is better evidence that NO added exogenously can inhibit neutrophil superoxide generation, but there are considerable methodological problems in demonstrating this. Although NO has been shown to inhibit production of superoxide measured spectrophotometrically by reduction of ferricytochrome C [46], NO rapidly reacts with cytochrome C to form nitrosyl cytochrome C [47] and so this assay cannot prove inhibition of the NADPH oxidase reaction. NO and superoxide can also react together to form peroxynitrite (ONOO\textsuperscript{−}) [48], which is both an oxidant and nitrating species [49] and so will itself alter neutrophil activation.

Luminol-dependent chemiluminescence can be used to assess the generation of reactive oxygen species (ROS) by neutrophils [50]. However, this assay measures both myeloperoxidase [51] and NADPH oxidase products [52]. The NO donors GEA 3162 and GEA 5024 (0.3–3 μM) markedly inhibited luminol-dependent chemiluminescence induced by opsonized zymosan [23]. They were much more effective than SIN-1, which itself stimulated chemiluminescence. However, SIN-1 releases both NO and superoxide anions and so, unlike the GEA compounds, forms peroxynitrite (ONOO\textsuperscript{−}) [53,54], which may be responsible for this stimulatory action. SIN-1 has also been shown to stimulate PMA-induced respiratory burst [47]. The same study showed that NO reversibly inhibited oxygen consumption and superoxide formation induced by PMA [47], as does nitroprusside [55]. Similarly NO, but not peroxynitrite, inactivates NADPH oxidase in pig neutrophils by inhibiting the assembly process [56]. This is consistent with NO-inhibition of NADPH oxidase in human neutrophils resulting from a direct effect on the enzyme occurring before the assembly of the activated complex [57].

Although superoxide (O\textsubscript{2}\textsuperscript{−}) has some antibacterial action, it is rapidly converted to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), which is the precursor for the generation of more potent oxidising radicals [58] such as hypo-

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Table 2

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<th>Inhibitor</th>
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<tr>
<td>NO synthase</td>
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<tr>
<td>1-CAN</td>
<td>No effect on fMLP-induced chemiluminescence.</td>
<td>[100]</td>
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<td>1-NMMA and N-iminoethyl-L-ornithine</td>
<td>Block of luminol-dependent chemiluminescence induced by phorbol ester-activated neutrophils.</td>
<td>[104]</td>
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<td>1-NAME and 1-NMMA</td>
<td>No effect on arachidonic acid-induced chemiluminescence or superoxide generation.</td>
<td>[101]</td>
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<tr>
<td>1-NMMA or 1-CAN</td>
<td>No effect on fMLP-induced superoxide generation, but this was blocked by the NO scavenger, carboxy PTIO.</td>
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<td>Guanylate cyclase</td>
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<td>LY 83583</td>
<td>Inhibited the production of hydrogen peroxide and superoxide by PMA-stimulated neutrophils.</td>
<td>[102]</td>
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<tr>
<td>LY-83583</td>
<td>Enhanced rather than inhibited neutrophil superoxide generation.</td>
<td>[21]</td>
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<td>Protein kinase G</td>
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<td>KT5823 and Rp-8-pCPT-cGMPS</td>
<td>Inhibited neutrophil superoxide generation.</td>
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chlorous acid (HOCI) formed by the action of myeloperoxidase. Although NO donors are likely to reduce production of ROS by neutrophils (see above), there is new evidence indicating that myeloperoxidase can oxidise nitrite, the breakdown product of NO, to a species capable of nitrating tyrosine and tyrosyl residues on proteins [59–61]. Kinetic analysis of the reaction between nitrite and human myeloperoxidase demonstrated that both myeloperoxidase intermediates oxidised nitrite in one-electron steps [62]. However, nitrite was a poor substrate for myeloperoxidase, making these reactions more likely to occur in inflammatory conditions, such as rheumatoid arthritis, in which high levels of nitrite are generated subsequent to induction of iNOS [63,64]. In inflammatory conditions, additional co-substrates for myeloperoxidase II, like tyrosine, will be available to facilitate this reaction [65].

It has also been postulated that nitrite will compete with taurine for reaction with neutrophil HOCI [105] (Fig. 2). These authors demonstrated that high concentrations of nitrite diminish the cytotoxic effects of HOCI, whereas others have shown that nitrite potentiates the bactericidal activity of ROS [66]. Clearly, the impact of nitrite on the HOCI system depends on the relative concentrations of taurine and nitrite. Since it has recently been demonstrated that iNOS is co-localised with myeloperoxidase in neutrophil primary granules [60], NO formation may regulate the bactericidal system of neutrophils. This competing action of nitryl chloride with taurine should be investigated carefully when identifying the clinical potential of taurine in conditions such as angina [67].

6. NO and neutrophil phagocytosis

Less work has been carried out looking at NO and neutrophil phagocytosis. An early study suggested that endogenous NO could play a role in human neutrophil phagocytosis since anucleate, granule-poor neutrophil cytoplasts decreased their uptake and killing of staphylococci from supernatants in the presence of the NOS inhibitor l-NMMA, an effect that was reversed by l-arginine [68]. Subsequently l-arginine supplementation was shown to increase phagocytosis of staphylococcus by human neutrophils [69]. However, although the NOS inhibitors l-CAN, aminoguanidine and l-NNA reversed the effect of l-arginine, they did not suppress phagocytosis of unsupplemented cells, thus questioning the physiological role of NO. Again results differ depending on whether human or rat neutrophils are used and whether circulating or elicited neutrophils are studied. Rat peritoneal neutrophils have been shown to spontaneously release NO in vitro, which presumably promoted killing of the yeast Candida albicans because killing is decreased in the presence of a NOS blocker [70]. However, rat blood neutrophils produced much lower levels of NO and exhibited weaker killing activity. Furthermore, stimulation of human neutrophils by the cytokines IL-1, TNFα, and IFNγ, which induces iNOS, was required for the formation of nitrotyrosine and nitrination of phagocytosed bacteria [7].

In contrast to these findings, the NO donor GEA 5171, given exogenously, has been shown to inhibit rather than stimulate neutrophil phagocytosis [106].
However, no studies with other NO donors have been carried out.

7. NO and neutrophil apoptosis

The role of endogenous NO in neutrophil apoptosis is less clear. The guanylate cyclase inhibitor LY 83583 increased the rate of spontaneous neutrophil apoptosis suggesting that cyclic GMP may limit neutrophil apoptosis [71]. However, it has not been shown that this cyclic GMP effect is subsequent to stimulation of guanylate cyclase by NO. Levels of neutrophil nitrite, a stable oxidation product of NO, increase as neutrophils undergo spontaneous apoptosis in culture [72]. However, since nitrite levels did not change between 6 and 16 h, but apoptosis increased 2-fold during this period, a causal relationship between NO and apoptosis could not be established. Furthermore, a reduction in nitrite was coincident with increased neutrophil apoptosis induced by anti-Fas or TNFα [72]. This observation would support the original contention that endogenous NO, if linked to guanylate cyclase, decreases apoptosis.

Exogenous nitric oxide released by high concentrations of agents such as SIN-1 and GEA 3162, as well as the NO adduct S-nitrosoglutathione, have been shown to enhance spontaneous neutrophil apoptosis in vitro [71,74,75]. This effect was particularly evident with shorter incubation times (4–6 h) and was confirmed using a variety of techniques designed to determine apoptosis rather than cell necrosis (annexin V binding, DNA fragmentation or morphological assessment of nuclear changes in apoptosis).

SIN-1 forms peroxynitrite (OONO−) and since the promotion of apoptosis observed with SIN-1 rather than GEA 3162 was dramatically inhibited by superoxide dismutase [71], this suggests that NO and peroxynitrite both promote neutrophil apoptosis. Indeed peroxynitrite (100–500 µM) was found to produce rapid apoptosis of neutrophils by 4 h with higher concentrations also inducing significant necrosis.

There is some controversy as to whether the effects of NO on neutrophil apoptosis are mediated by the subsequent generation of cyclic GMP. Fortenberry et al. [82] found the mechanism to be cyclic GMP-dependent, whereas Ward et al. [71] found NO promotion of apoptosis to be insensitive to the soluble guanylate cyclase inhibitor LY 83583, but that the process could be blocked by the caspase inhibitor ZVAD-fmk. The selectivity of these enzyme inhibitors is fundamental to the interpretation of these results and further work is required to elucidate the precise signaling pathways involved in both NO and peroxynitrite effects on neutrophil apoptosis. See also the articles by Kim et al. [107] and Kröncke et al. [108] in this issue for discussions of the mechanisms of NO-induced apoptosis.

8. Clinical significance of NO and neutrophils

In therapeutic terms, inhaled exogenous NO is being evaluated as a novel treatment for pulmonary arterial hypertension [76,77] in adult respiratory distress syndrome. In addition to its vasodilator action, NO has anti-inflammatory action by reducing neutrophil superoxide anion generation [78,79]. Similarly, inhaled NO prevents neutrophil-related lung injury in several animal models [80,81].

Since exposure of neutrophils to clinically relevant concentrations of NO has been found to promote neutrophil apoptosis in vitro [82], this mechanism might account for the subsequent reduction in neutrophil oxidative function [83]. Apoptosis has been shown to limit the toxic potential of the neutrophil [84]. However, this hypothesis has not, as yet, been tested ex vivo in humans.
Although there is considerable therapeutic potential for inhibition of neutrophil activation by NO, there is also the potential for detrimental effects of NO, particularly through peroxynitrite formation, resulting from the interaction of NO with neutrophil-derived superoxide anions [85,86]. Although results using exogenous NO or NO donors suggest that high levels of NO inhibit neutrophil activation, this hypothesis does not agree with results showing that induction of iNOS in neutrophils, and the subsequent production of large amounts of neutrophil NO, is linked to inflammatory conditions [87–90,109]. It seems difficult to imagine that exogenous and endogenously produced NO could have different effects given that NO is freely diffusable in cells and tissues.

Also of interest is the demonstration that, unlike in the rat, the NOS-like protein induced in human neutrophils in sepsis is different from the classical iNOS enzyme [91]. Although this induced protein displays considerable immunoreactivity with iNOS antibodies, the intense iNOS staining was not associated with increased neutrophil NOS activity. Furthermore, immunoreactivity was associated with a 100 kDa rather than 130 kDa protein. Clearly, the function of this protein needs to be characterised to help elucidate the role of iNOS in inflammation, and thus, the clinical potential of using NO donors as therapeutic agents.

Acknowledgements

I would like to thank Payong Wanikiat for performing the experiments on isolated neutrophils and with a rat model of ischaemia reperfusion injury, as part of her PhD thesis and for her thesis itself, which provided a starting point for the neutrophil and NO literature.

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[48] Radi R, Beckman JS, Bush KM, Freeman BA. Peroxyni-


