Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates

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Abstract: Reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), and cytokines are frequent companions at sites of acute inflammation. Previous work has established a clear link between the production of cytokines and the subsequent generation of ROI and RNI. However, more recent data indicates that ROI and RNI not only serve as end-stage effector molecules of pathogen destruction and tissue injury, but also as initiators of acute inflammation. Specifically, ROI and RNI will up-regulate cytokine gene expression since antioxidants inhibit interleukin 8 (IL-8) production and do not decrease production of other cytokines. Treatment with hydroxyl radical scavengers such as dimethyl sulfoxide (DMSO) will decrease the production of IL-8 in stimulated human whole blood, fibroblasts, type II epithelial cells, and hepatoma cells, but not other cytokines. Addition of exogenous ROI will increase IL-8 production in these same cells. Inhibition of nitric oxide synthase will decrease production of IL-8, whereas addition of nitric oxide (NO)-generating compounds will increase production of IL-8. The hydroxyl radical appears to be the final common pathway of cell activation for IL-8 synthesis, since DMSO will inhibit the NO-driven production of IL-8. Our data indicate that ROI and RNI can serve as intracellular second messengers to induce IL-8 gene expression. *J. Leukoc. Biol.* 59: 471–475; 1996.

Key Words: *interleukin-8 · interleukin-6 · superoxide · hydroxyl radical · antioxidants*

Recruitment and activation of neutrophils

The recruitment of neutrophils (PMNs) to sites of active inflammation remains a poorly defined process. Certain cytokines have been associated with neutrophil recruitment, such as tumor necrosis factor (TNF) or interleukin-1 (IL-1) [1], but careful experimentation has shown that they attract neutrophils through the induction of another newly translated protein mediator [2]. Interleukin-8 (IL-8) represents a newly described cytokine [3] and is a potent inducer of neutrophil chemotaxis. It is produced early in inflammation as a direct result of interaction of cells with such stimuli as endotoxin [4], and it will also be further up-regulated by the early, alarm cytokines such as TNF and IL-1 [5, 6]. Interestingly, a single dose of lipopolysaccharide (LPS) will serve to induce continuing production of IL-8 protein and persisting levels of IL-8 mRNA in vitro [5]. This finding stands in contrast to most other cytokines, where the mRNA rapidly disappears [7], and the protein levels plateau after a few hours [8]. There is now ample evidence that IL-8 has the ability to persist for several days during inflammatory states. Therefore, there is a theoretical basis and published data to support a role for IL-8 as the recruiter of neutrophils to sites of inflammation.

Reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), and tissue injury are also frequent companions at sites of acute inflammation (reviewed in refs. 9 and 10). The accepted hypothesis that links these observations may be stated as follows: PMNs are recruited to sites of acute inflammation; once at the site, they produce substantial quantities of ROIs [11, 12], which, together with released proteases [13], injure the tissue. However, ROI/RNI may serve a completely different function in acute inflammation, where they do not operate solely as end-stage effector molecules, but also as mediators regulating cytokine gene expression. ROIs have in fact been shown to be important inducers of both prokaryotic and eukaryotic gene expression by activation of various transcription factors including oxyR and nuclear factor-κB, respectively [14]. The predicted sequence is as follows: low intracellular concentrations of ROI and RNI serve to up-regulate expression of IL-8, which then induces neutrophil chemotaxis. This hypothesis does not in any way contradict previous work concerning ROI/RNIs and tissue damage [15–17], it merely expands their inflammatory role as initiators of the cascade that elicits neutrophils. Our data demonstrates an intimate link between the production of ROI, RNI, and the generation of neutrophil chemoattractants.

Abbreviations: ROI, reactive oxygen intermediates; RNI, reactive nitrogen intermediates; IL, interleukin; DMSO, dimethyl sulfoxide; NO, nitric oxide; PMN, polymorphonuclear neutrophils; TNF, tumor necrosis factor; LPS, lipopolysaccharide; SOD, superoxide dismutase; CGD, chronic granulomatous disease; L-NAME, (L-N=-nitroarginine methyl ester.

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BIOCHEMISTRY OF THE FORMATION OF ROI AND RNI

There have been several studies detailing the capacity of cytokines to induce ROI and RNI formation from a variety of cell types. Numerous cytokines have been demonstrated to enhance ROI and/or RNI production including TNF, IL-1, IL-6, IL-8, interferon-γ, and granulocyte-macrophage colony-stimulating factor. The metabolic intermediates and enzymes important in formation and degradation of ROIs have been defined [11, 18] and these pathways are shown schematically in **Figure 1**. The major ROI produced within the cell are the superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH). There are several compounds that may be used to dissect the various pathways involved in ROI formation, including the antioxidant hydroxyl radical scavengers [dimethyl sulfoxide (DMSO), dimethyl thiourea, thiourea, etc.]. Another antioxidant defense mechanism is to maintain high intracellular levels of glutathione, which will effectively scavenge the ROI.

RNIs, the most widely studied of which is nitric oxide (NO), are ultimately formed from the enzymatic generation of the highly reactive NO by the family of enzymes called NO synthases (NOS) [19]. There are three different isoforms of NOS identified to date. All three isoforms produce NO from the terminal guanidino group of L-arginine. Two of the isoforms are Ca^{2+}/calmodulin-dependent and are constitutively produced (ecNOS and ncNOS). In contrast, the third isoform (iNOS) is Ca^{2+}/calmodulin-independent and is inducible by treatment of cells with cytokines or microbial products [10]. NO can combine with ROIs to form other RNIs, such as nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and the hydroxyl radical [20, 21]. Under physiological conditions, NO readily complexes with protein-bound transition metal ions or thiol groups forming iron-nitrosyl proteins or S-nitrosoproteins, respectively. From a biological standpoint, these complexes have been implicated to be responsible for the involvement of NO in such diverse processes as neurotransmission, vasodilatation, platelet inhibition, host defense, and inflammation.

ROI AND THE INDUCTION OF CYTOKINES

Our laboratory has extensively studied the regulation of cytokine gene expression by ROI. We have shown that inhibition of ROI will decrease IL-8 production, and that augmentation of ROI will enhance IL-8 production [22, 23]. We have specifically been testing the hypothesis that low level intracellular formation of ROI will serve as second messengers for the induction of IL-8 gene expression.

Our first experiments evaluated human whole blood stimulated with 100 ng/ml of LPS. LPS is a potent stimulus that will induce the production of several cytokines . As can be observed in **Figure 2**, LPS stimulation for 24 h results in a substantial increase in IL-8 detectable in the plasma. Unstimulated samples have 0.6 ± 0.2 ng/ml of

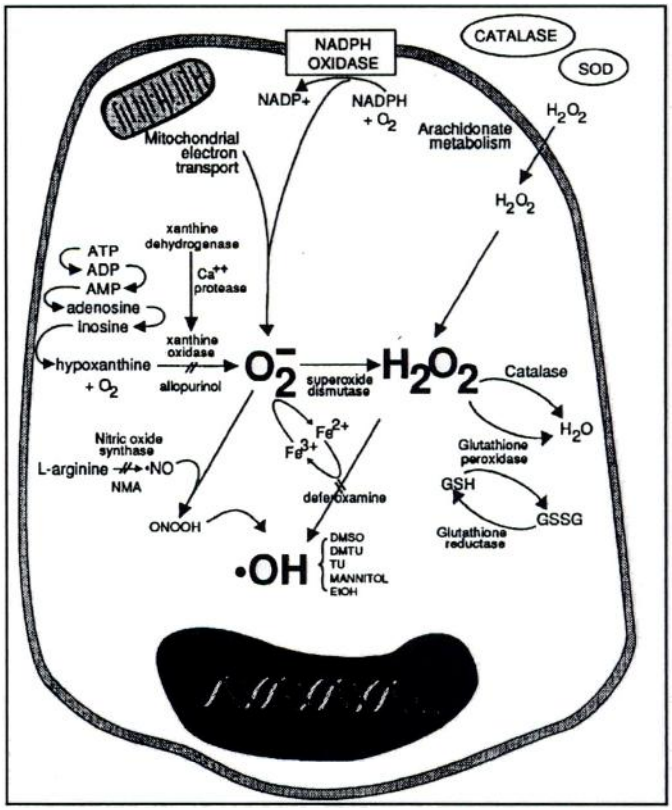

Fig. 1. Metabolic pathways of ROI and RNI. The major pathways involved in the generation of ROI and RNI, as well as the inhibitors that will block their production. (Reproduced with permission from ref. 22).

IL-8, and LPS stimulation increases this amount to 4.0 ± 1.0 ng/ml (mean ± SE for 3 individuals). Addition of 1% DMSO immediately prior to the LPS stimulation caused a significant reduction in the amount of IL-8 (1.8 ± 1.0 ng/ml, $P < 0.05$ compared with LPS). To demonstrate that the inhibition of IL-8 was specific and not merely due to loss of cell viability, IL-6 was measured in these same samples. As can be observed, there was no reduction in the amount of IL-6 by DMSO. These results have been extremely consistent in our laboratory and have been observed reproducibly in dozens of volunteers over the past years. In human whole blood, IL-8 production is inhibited by oxygen radical scavengers, whereas that of IL-6 and other cytokines is not. We sought to determine whether the inhibition of IL-8 occurred at the transcriptional or translational level by performing Northern blot analysis. LPS results in a substantial increase in mRNA coding for IL-8, whereas DMSO causes a reduction in mRNA expression (data not shown). There are several other stimuli that will serve to induce the formation of IL-8, including phytohemagglutinin, immune complexes, and recombinant cytokines such as TNF or IL-1 [23]. Regardless of the stimulus that is used in the whole blood, DMSO will decrease the production of IL-8, and this inhibition occurs at the level of the mRNA.

The biochemistry of ROI formation is complex and many different reactive intermediates may be responsible for the up-regulation of IL-8. We investigated these pathways by

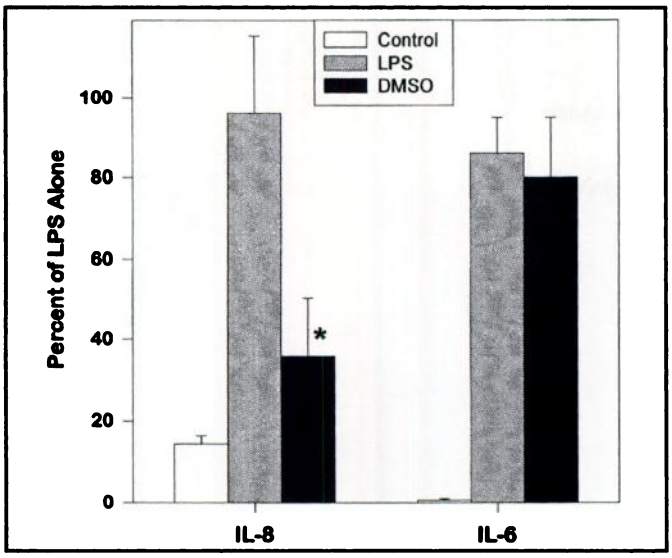

Fig. 2. Antioxidant inhibition of IL-8 production. Human whole blood was stimulated with 100 ng/ml of LPS in the presence or absence of 1% DMSO, and cytokines in the plasma measured 24 h later. DMSO caused a significant reduction in the amount of IL-8, with no reduction in the amount of IL-6. Values are means ± SE. $*P < 0.05$ compared to LPS.

using several different inhibitors in LPS-stimulated whole blood. All of the hydroxyl radical scavengers that were tested effectively decreased the production of IL-8. The order of potency of inhibition, listed from most potent to least potent was (concentration in parenthesis): dimethyl thiourea (75 mM) > ethanol (86 mM) > DMSO (140 mM) > thiourea (75 mM) > mannitol (50 mM). The addition of catalase or superoxide dismutase (SOD) did not block the production of IL-8. These results may appear paradoxical, since the hydroxyl radical is formed through superoxide or hydrogen peroxide. However, our hypothesis states that it is intracellular levels of ROI that drive the reaction. The large catalase and SOD enzymes will not penetrate the cell to achieve sufficiently high concentrations to alter intracellular levels of ROI unless steps are taken to modify the enzymes to improve their bioavailability [24]. Deferoxamine will chelate iron and prevent the Fenton reaction ($H_2O_2 \rightarrow \cdot OH$) from occurring, however, addition of deferoxamine did not reduce IL-8. This may well be due to the whole blood system that we use, which has very high iron binding capacity.

We further explored the source of the ROIs in LPS-stimulated whole blood by using other inhibitors. Allopurinol and oxypurinol will inhibit xanthine oxidase and prevent the formation of superoxide. Neither of these inhibitors effectively decreased IL-8 production. Superoxide is also formed through the action of NADPH oxidase, and this enzyme is deficient in patients with chronic granulomatous disease (CGD) [25]. When whole blood from patients with CGD is stimulated with LPS, phytohemagglutinin, immune complexes, or IL-1, there is an up-regulation of IL-8, which is inhibitable by 1% DMSO [22]. This indicates that the ROI are not formed through the action of NADPH oxidase. All of these data would point to the mitochondria as the source of the superoxide, which is consistent with publications indicating that mitochondria may serve as a source of ROI [26, 27].

All of the data presented until this time have dealt with scavenging of ROI and decreasing IL-8. We also evaluated whether addition of exogenous ROI would increase the production of IL-8. H_2O_2 was added directly to human whole blood where it caused a substantial increase in the amount of IL-8, and this increase was inhibitable by DMSO [23]. These results again point to the importance of ROI as regulators of cytokine gene expression.

Our studies were extended to isolated, cultured cells to determine whether other cell types also exhibited regulation of IL-8 by ROI. Isolated human fibroblasts were incubated in the presence of glucose oxidase (5 mU/ml) and the supernatants collected at different time points (**Fig. 3**). Glucose oxidase caused a substantial increase in the IL-8 concentration in the supernatant, which could be inhibited by the addition of catalase. In this setting, the glucose oxidase is generating H_2O_2 in the extracellular media, which will diffuse into the cell to up-regulate IL-8. Because the ROI is generated outside of the cell, catalase has the opportunity to inactivate the H_2O_2 before it moves into the cell. This is in contrast to the LPS-stimulated whole blood, where catalase is not effective because the ROI are generated within the cell. Other cell lines were tested for their ability to produce IL-8 in response to exogenous ROI. Fibroblasts, hepatoma cells (HEP-G2) and type II alveolar epithelial cells (A549) all secrete IL-8 into the supernatant when stimulated with 1% H_2O_2, and this induction could be inhibited by the addition of catalase [22].

Other investigators have examined the ability of ROI to serve as intracellular second messengers [28]. A recent publication has supported our concept concerning the role

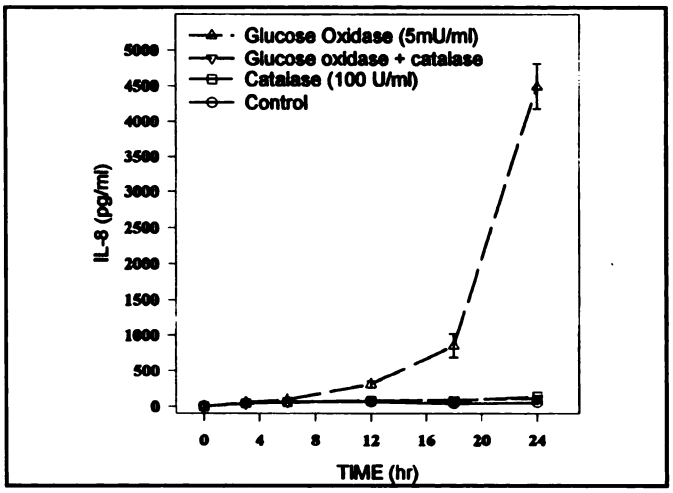

Fig. 3. Kinetics of glucose oxidase induction of IL-8 in cultured fibroblasts. Glucose oxidase added to the media of cultured human fibroblasts induces the synthesis of IL-8. This could be completely prevented by the addition of catalase, which will metabolize H_2O_2. Values are means ± SE for triplicate wells. No stimulus, catalase alone, and catalase plus glucose oxidase failed to induce up-regulation of IL-8. (Adapted, with permission, from ref. 22).

of ROI in the induction of chemokine expression with data showing that antioxidants will also depress production of monocyte chemotactic protein [29]. In the monocyte cell line U937, antioxidants will prevent the up-regulation of TNF [30]. In contrast, our studies did not show any significant inhibition of TNF using stimulated whole blood. The differences probably relate to the experimental conditions, since the whole blood contains substantial oxidant scavenging capacity. In this regard, experiments in our laboratory have shown that the production of TNF by isolated human mononuclear cells may be inhibited by 1% DMSO (unpublished data). A report has shown that the stress induced by exposure to a heavy metal, cadmium, will induce IL-8 in isolated human mononuclear cells [31]. Our work with fibroblasts failed to show that cadmium would induce IL-8 [22]. Additional studies in vivo have shown that steps to maintain the intracellular levels of glutathione will inhibit TNF production and decrease mortality [32]. In this setting, there is a severe stress (i.e., lethal LPS) and the intracellular stores of glutathione are not sufficient to prevent substantial accumulation of ROIs.

RNI INDUCTION OF CYTOKINES

Our studies demonstrating the strong regulatory effect of ·OH on IL-8 production led us to explore the possibility that RNIs may also exert similar effects on cytokine production. We explored this hypothesis because NO has been shown to be involved in intracellular signaling as well as ·OH formation (Fig. 1) [19]. These experiments were designed to parallel those used in examining the regulation of cytokine gene expression by ROIs. The availability of NO synthase inhibitors and NO-generating compounds have made it possible to evaluate the effect of NO in regulating cytokine gene expression. Thus far, our studies have provided ample evidence substantiating an immunoregulatory role for NO in IL-8 production in the human whole blood system and in the human endothelial cell line, ECV304.

Figure 4 depicts a representative set of data from one donor showing the LPS (1 μg/ml) -induced increase in both IL-8 (18.8 ng/ml) and IL-6 (87.8 ng/ml). Addition of 5 mM L-N≈-nitroarginine methyl ester (L-NAME), a NO synthase inhibitor, to blood 2 h before LPS stimulation resulted in the substantial decrease in IL-8 but not IL-6 protein levels. This inhibitory effect of L-NAME was dose-dependent and also occurred at the mRNA level. The observation that NO synthase inhibitors have the potential to inhibit IL-8 production in human whole blood has been extended to the human endothelial cell line, ECV304. TNF is a potent inducer of IL-8 in endothelial cells [33]. Stimulation of ECV304 cells with 50 ng/ml of TNF induced significant levels of IL-8, which was inhibited in a dose-dependent manner by the addition of L-NAME [34].

The next series of experiments was used to evaluate whether exogenously added NO could stimulate cytokine production in the whole blood model or the ECV304 cells.

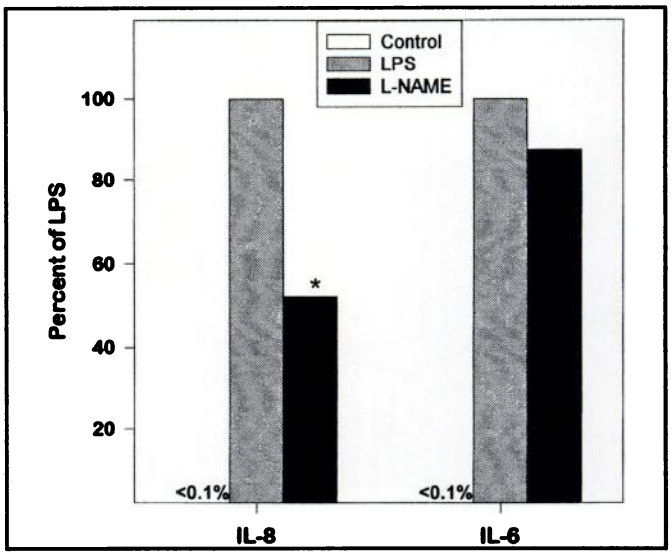

Fig. 4. Inhibition of NO synthase decreases IL-8 production. Human whole blood was stimulated with 1 μg/ml of LPS in the presence or absence of 5 mM L-NAME, and cytokines in the plasma measured 24 h later. Blockade of NO synthase resulted in a reduction in the amount of IL-8, with no reduction in the amount of IL-6. Data are from a single donor but are representative of >15 individual donors. When the groups are compared, 5 mM L-NAME causes a significant inhibition of IL-8 production. *$P < 0.05$ compared to LPS.

Studies were conducted using ethanamine-2,2′-(hydroxynitrosohydrazono)bis (DETA NONOate, Cayman Chemical, Ann Arbor, MI), a spontaneous NO generator. DETA NONOate added to either whole blood or the ECV304 cells resulted in a dose-dependent increase in IL-8. Addition of 10 mM DETA NONOate to whole blood, or 2 mM to ECV304 cells, was able to induce significant levels of IL-8 after 24 h incubation. IL-8 levels continually increased up to 72 h after the addition of 10 mM DETA NONOate to the whole blood. No measurable IL-6 was detected in the same samples.

As discussed above, oxidant stress, particularly that caused by ·OH, appears to represent one of the signals that causes up-regulation of IL-8 gene transcription. Interestingly, one of the intracellular pathways that leads to ·OH formation involves the formation of peroxynitrite from the rapid reaction of NO with $O2^-$ (Fig. 1). With this in mind, the results from a representative experiment displayed in **Table 1** show the effect of L-NAME and the ·OH scavenger, DMSO, on DETA NONOate or H_2O_2-induced IL-8 production in whole blood. Consistent with our previous reports, 0.15% H_2O_2 induced IL-8 production, which was significantly reduced by 1% DMSO [22]. Since L-NAME is a substrate competitor for L-arginine and does not have any known direct scavenging effect on radicals, it is not surprising that IL-8 production by either DETA NONOate or H_2O_2 was not inhibited. However, addition of DMSO to DETA NONOate-stimulated blood dramatically suppressed IL-8 production compared with the sample treated with the stimulant alone. These results strongly indicate a potentially important link between ·OH and NO induction

TABLE 1. Effect of L-NAME and DMSO on ROI or RNI- Stimulated IL-8 Production.

Stimulus (concentration)	IL-8 (ng/ml) No treatment	+ L-NAME	+ DMSO
Control	1.5	2.5	ND
DETA NONOate (10 mM)	10.3	10.5	1.8
H_2O_2 (0.15%)	4.7	4.9	0.9

Human whole blood was stimulated as indicated and IL-8 measured 24 h later. ND, not done.

of IL-8 production in human whole blood, since the hydroxyl radical may be mediating the action of NO.

CONCLUSIONS

ROI and RNI are formed within the cytoplasm following stimulation of cells. These intracellular ROI and RNI may serve as second messengers to up-regulate expression of cytokines, and IL-8 is particularly sensitive to these changes. Blockade of NO synthase or scavenging of hydroxyl radicals will decrease the formation of IL-8 and addition of exogenous RNI or ROI will increase gene expression of IL-8. Our data point to a new role for these intracellular reactive species, as regulators of the immune response by augmenting cytokine gene expression. There is an important causal link between the formation of NO and the generation of hydroxyl radicals leading to the induction of cytokine gene expression.

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