Regulation of Interleukin 8 Gene Expression by Oxidant Stress*

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Interleukin 8 (IL-8) is a recently described cytokine that functions as a potent neutrophil chemoattractant and activator. We sought to examine the link between the generation of reactive oxygen intermediates (ROI) and the regulation of IL-8 gene expression to specifically test the hypothesis that ROI would induce production of IL-8 mRNA and protein. In lipopolysaccharide-stimulated human whole blood, the OH radical scavenger dimethyl sulfoxide (Me₂SO) dramatically inhibited (~90%) IL-8 production, but had minimal effects on the production of tumor necrosis factor, interleukin 1β (IL-1), and IL-6. To determine whether NADPH-oxidase-generated free radicals were critical in the regulation of IL-8, studies were performed using blood from patients with chronic granulomatous disease. In both normal individuals and patients with chronic granulomatous disease, production of IL-8 could be initiated with lipopolysaccharide, phytohemagglutinin, or aggregated immune complexes, and this production could be inhibited by Me₂SO (1% v/v).

To examine if oxidant stress represents a ubiquitous manner in Hep-G2 cells, A549 pulmonary type I1 epithelial cells, and human skin fibroblasts; this induction could be prevented by addition of catalase. The production of IL-8 appeared to be specific to an oxidant stress since exposure of the cells to heat shock or chemical stress did not induce expression of IL-8. These studies demonstrate that oxidant stress is an important regulator of IL-8 gene expression and support the hypothesis that low levels of ROI may serve to initiate IL-8 production which then serves to recruit neutrophils to sites of inflammation.

Numerous studies have implicated both neutrophils and reactive oxygen intermediates as important mediators of the tissue damage accompanying ischemia/reperfusion (1, 2). These same causative agents have also been implicated in the pathophysiology of other disease states including arthritis and adult respiratory distress syndrome (3). The toxic effects of neutrophils can be attributed not only to the degranulatory release of various proteases, but also to the production of reactive oxygen intermediates (ROI)1 by NADPH-oxidase during the respiratory burst. Superoxide anion (O₂⁻), which itself has not been implicated as an extensive mediator of cell toxicity, can be further reduced to hydrogen peroxide (H₂O₂) and hydroxyl radical (OH·), molecules which have been shown to be very damaging to surrounding tissue (3). Substantial evidence, however, also indicates that tissue cells of nonimmune origin can also release ROI. Perhaps the most significant source of non-leukocyte generated ROI during ischemia/reperfusion is the production of O₂⁻ by xanthine oxidase (1, 2). The cellular alterations accompanying hypoxia promote the conversion of xanthine dehydrogenase to xanthine oxidase as well as the formation of substrate for xanthine oxidase from metabolites of ATP. Many other potential mechanisms of ROI generation by nonimmune cells, however, also exist, including mitochondrial electron transport, peroxisomal reactions, and metabolism of arachidonate (4).

A potential connection between the generation of ROI in tissues following ischemia/reperfusion and the subsequent infiltration of neutrophils which release more ROI and other damaging agents was established by the observation of Petrone et al. (5) that treatment of plasma with O₂⁻ resulted in the generation of a neutrophil chemoattractant. Other investigators have demonstrated the generation of neutrophil chemoattractant activity from the complement component C5 upon treatment of the protein with H₂O₂ (6) and from arachidonic acid following incubation with xanthine oxidase (7). Although these chemotactic factors have not been firmly identified, they may represent C5a and leukotriene B₄. These are well characterized chemoattractants that are generated from pre-existing factors and do not require de novo protein synthesis.

Recently (8), we have demonstrated that ROI appear to have important regulatory effects on the production of interleukin 8 (IL-8), a recently described cytokine that functions as a potent neutrophil chemoattractant and activator (9). In contrast to other classic neutrophil chemoattractants such as leukotriene B₄, C₅a, platelet activating factor, and the bacterial peptide fMLP, IL-8 is an extremely stable protein that is resistant to proteolysis and denaturation and has a pro-

1 The abbreviations used are: ROI, reactive oxygen intermediates; IL, interleukin; Me₂SO, dimethyl sulfoxide; LPS, lipopolysaccharide; TNF, tumor necrosis factor; PHA, phytohemagglutinin; IC, immune complexes; CGD, chronic granulomatous disease; WBC, white blood cells; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
longed biologic activity in vivo. In addition, we have observed a protracted expression of IL-8 messenger RNA (mRNA) in the continued presence of a stimulating agent in studies using human whole blood (10). The stability of the IL-8 protein and the agglutination of human polymorphonuclear neutrophils indicates that the biologic impact of IL-8 may be greater than that of other neutrophil chemotaxotants. Furthermore, since IL-8 is produced de novo in the presence of an inflammatory stimulus and not from pre-existing or latent factors, modulation of IL-8 production may provide a more promising pharmacologic target than other chemotaxotants.

In previous studies using whole blood as a model of cytokine production, we have demonstrated that the OH scavenging agent dimethyl sulfoxide (Me$_2$SO) dramatically inhibited IL-8 production in blood stimulated with lipopolysaccharide (LPS) or other agents such as tumor necrosis factor (TNF), interleukin 1β (IL-1β), phytohemagglutinin (PHA), and aggregated immune complexes (IC) (8). In addition, other OH scavenging agents such as dimethyl thioare and thioare had a similar suppressive effect on LPS-stimulated IL-8 production (8). In the present study, we confirm and extend these findings. First, based on studies using blood from patients with chronic granulomatous disease (CGD), NADPH oxidase does not appear to be required as a source of ROI. Secondly, studies using cultured cell lines lacking NADPH-oxidase as a source of ROI show a pattern of responsiveness similar to that observed in normal whole blood. Specifically, Me$_2$SO suppressed IL-8 production in TNF-stimulated Hep-G2 cells, and oxidant stress stimulated IL-8 release in Hep-G2 cells, human skin fibroblasts, and A549 pulmonary type II epithelial cells. These studies provide further evidence implicating ROI generated independently of NADPH-oxidase as important regulators of IL-8 gene expression.

**Materials and Methods**

Reagents—Me$_2$SO, H$_2$O$_2$, thioare, mannitol, ethanol, catalase (from bovine liver), xanthine oxidase (grade I from buttermilk), xanthine, glucose oxidase (type V-S from Aspergillus niger), PHA, LPS (Escherichia coli 0111:B4), sodium arsenite, cadmium sulfate, and sodium azide were obtained from Sigma. Dimethyl thioare was purchased from Aldrich. Endotoxin-free aggregated IC were prepared as described (11) and were generously provided by Brian Wharram. IL-1β was the generous gift of the Upjohn Co.

Whole Blood—Experiments were performed essentially as described (10). Briefly, blood from normal male donors, or from male donors with X-linked CGD was drawn into heparinized syringes (20 units heparin/ml). The CGD patients' neutrophils were previously found to be incapable of generating O$_2^-$ (12). The blood was then placed in 1-ml aliquots into sterile Eppendorf tubes, and the various stimulating agents or ROI scavengers were added as required. The capped tubes were placed on a rotator in a 37°C tissue culture incubator for 21-24 h. The samples were then harvested by centrifuging the tubes at 600 × g for 5 min at 4°C. The plasma was removed, diluted 1:5 with RPMI 1640 (Life Technologies, Inc.) supplemented with 25 mM HEPES and 1% fetal calf serum. The buffy coat was then removed and contaminating red blood cells were lysed with 0.84% NH$_4$Cl. The white blood cell (WBC) pellets were then either used to determine cellular viability or to assay IL-8 mRNA levels by Northern blot analysis.

Cell Culture—A549 human type II pulmonary epithelial cells and Hep-G2 human hepatoma cells were obtained from American Type Culture Collection (Rockville, MD). Normal infant foreskin fibroblasts from cultures established at the University of Michigan Hospital were generously provided by Dr. Sov Karmiol. Stock cultures of cells were grown in a humidified atmosphere containing 5% CO$_2$ and split twice weekly by trypsinization. Fibroblasts and A549 cells were grown in Dulbecco's modified Eagle's medium and Hep-G2 cells in RPMI (both from Life Technologies, Inc.). All media was supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine, 25 mM HEPES (standard additions) and 10% fetal calf serum. Fibroblasts were used between 12-18 population doublings. For experiments, cells were plated in 6-well culture dishes at a concentration of 3-6 × 10$^4$ cells/well (fibroblasts and A549 cells) or 18 × 10$^4$ cells/well (Hep-G2s) and grown for 3-4 days until nearly confluent. At the time of the experiment, cells were rinsed with phosphate-buffered saline and overlaided with 1 ml of serum-free media with standard additions. After a 1-2-h period of equilibration at 37°C, triplicate wells of cells were dosed with the various stimulants and ROI scavengers. At the indicated time points, the cell supernatants were centrifuged at 1500 × g for 5 min to clear any cellular debris. The cell-free supernatants were transferred to clean tubes and stored at -20°C for later analysis of cytokine levels. The cells were then processed as described below to assess viability or mRNA expression levels.

**Cell Viability—**For whole blood studies, WBC viability was assessed using MTT-tetrazolium (Sigma) as described (13). For the cell culture studies, the cell supernatants were replaced by 1 ml of fresh serum-free media with standard additions. MTT-tetrazolium (50 μM, 5 mg/ml solution) was then added, and the cells were incubated at 37°C for 1 h for Hep-G2 cells and for 1-3 h for other cell lines. A 750-μl aliquot of the supernatant was removed and replaced with 0.04 N HCl/isopropyl alcohol. Once the MTT crystals were solubilized (approximately 1 h), triplicate aliquots (200 μl) of the supernatant were added into 96-well plates. The absorbance was then read at 550 nm using a Bio-Tek microplate reader (Bio-Tek Instruments, Inc., Winoski, VT). The triplicate absorbance values were averaged, and the data for the various treatment groups was compiled.

**Northern Blot Analysis—**The WBC pellet from 1 ml of blood and the cells in the 30-mm tissue culture dishes were dissolved in 0.5 ml of an RNA extraction buffer containing guanidine isothiocyanate, 0.5% Na-aurorsarcosine, and 0.1 M 2-mercaptoethanol and were stored at -70°C for later analysis. Total RNA was extracted, and Northern blot analysis was performed using previously described methods (10). The 5'5'-end labeled probes consisted of an IL-8 30-mer 5'GTT-GGC-GCA-GTO-TCC-CTC-AAT-CAA-3' (14) and a β-actin 42-mer 5'GTC-TTC-GGTATT-GAA-GGT-CCT-AAA-CAT-GAT-CGT-GTT-CTT-3' (15). Blots were first probed for IL-8 and then stripped and reprobed for β-actin. The relative intensities of the bands were quantitated in each lane based on the intensity of the actin bands, and the intensity of each lane was then expressed as a percentage of the intensity of the darkest band. The performance of the image analysis system was validated by serially diluting a sample containing mRNA coding for IL-8 and subjecting the material to Northern blot analysis. The IL-8 probe was probed for IL-8 and a linear increase in signal was observed with increasing mRNA (r$^2 = 0.99$).

**Cytokine Assays—**IL-8 and TNF protein concentrations were determined using specific ELISAs that were developed in our laboratory. Native and biotinylated forms of a polyclonal rabbit anti-human IL-8 and a rabbit anti-human TNF antibody were used at the primary and secondary antibody titers, respectively, as described (10). The IL-8 ELISA was performed essentially as described (10) and does not detect the presence of other cytokines such as TNF, IL-6, and IL-1β, or other members of the IL-8 gene family such as neutrophil-activating peptide 2, platelet factor 4, connective tissue-activating peptide III, or melanoma growth-stimulating activity. The IL-8 ELISA consistently detects IL-8 concentrations <30 pg/ml. The TNF ELISA was performed in essentially the same manner as the IL-8 ELISA, using primary and secondary antibody concentrations of 1 and 3 μg/ml, respectively. A lower limit of sensitivity of approximately 230 pg/ml was typically obtained. IL-6 levels were measured as described (16). IL-1β-H6 and biotinylated IL1β-H6 were the mouse monoclonal antibodies used as the capture and secondary antibodies, respectively. IL-6 concentrations were assessed by bioassay using the B-9 cell line (10, 17).

**Statistical Analysis—**The significance of the differences between groups was assessed by Student's t-test for single comparisons and by Student-Newman-Keuls test for multiple comparisons.

**Results**

Me$_2$SO Suppresses IL-8 Production in Human Whole Blood—Experiments were performed in which whole blood was stimulated for 24 h with concentrations of LPS ranging from 1 ng/ml to 10 μg/ml. Measurement of the plasma cytokine levels demonstrated that LPS caused a dose-dependent...
stimulation of IL-8, TNF, IL-1β, and IL-6 production (Fig. 1), with plasma levels of these cytokines increasing in an essentially log-linear manner in response to increasing concentrations of LPS.

LPS is known to elicit ROI production by leukocytes (18). In an effort to determine whether these ROI affect the extent of cytokine release in LPS-stimulated blood, identical samples were incubated in parallel with the addition of the OH· scavenging agent Me2SO (1% v/v). The presence of Me2SO was found to cause a striking suppression in the plasma levels of IL-8. As compared to blood stimulated with LPS alone, Me2SO treatment significantly reduced plasma IL-8 concentrations at every LPS concentration, with an average level of suppression of approximately 90%. In the presence of 1 μg/ml LPS, Me2SO treatment reduced plasma IL-8 concentrations from 72.2 ± 25.6 ng/ml to 7.3 ± 2.7 ng/ml (mean ± S.E., n = 10), an 87.8 ± 3.4% decrease. In contrast, Me2SO had no effect on the LPS-stimulated production of TNF (Fig. 1B) or IL-6 (Fig. 1D), with the dose-response curves in the presence and absence of Me2SO being essentially superimposable. The effect of Me2SO on IL-1β release was more complex. In the presence of low levels of LPS (1 and 10 ng/ml), 1% Me2SO suppressed plasma IL-1β levels. At the higher LPS concentrations, an augmentation of IL-1β release was observed upon Me2SO treatment, with this reaching statistical significance only in the presence of 10 μg/ml LPS (45.8 ± 5.2 ng/ml versus 104.0 ± 16.0 ng/ml (mean ± S.E., n = 5), for LPS alone and LPS + 1% Me2SO, respectively; p < 0.05, Student’s t test).

Me2SO was found to effectively suppress IL-8 release not only in response to LPS, but also in response to other stimulants such as PHA and IC. Fig. 2 shows the compiled results from two experiments using five blood donors each. Blood was stimulated with LPS (1 μg/ml), PHA (10 μg/ml), or IC (250 μg/ml) in the presence of increasing concentrations of Me2SO, and plasma IL-8 levels were measured at a 24-h time point.

![Fig. 2.](image_url)

**Fig. 2.** Me2SO (DMSO) dose-dependently inhibits IL-8 production in whole blood stimulated by LPS, PHA, and IC. Blood from different donors was stimulated with LPS, PHA, or IC in the presence of increasing concentrations of Me2SO. Plasma samples were harvested at a 24-h time point and analyzed for IL-8 by ELISA. The compiled results from two different studies are shown, and the data are expressed as a percentage of the IL-8 produced in the presence of the stimulus alone (mean ± S.E., n = 10). The different groups for each stimulus were compared by Student-Newman-Keuls test (*p < 0.05 versus the stimulus alone).

In an effort to confirm our hypothesis that ROI are important in the regulation of IL-8 gene expression, whole blood was stimulated with H2O2 (0.015%) as a means of directly exposing the leukocytes to an oxidant stress. Importantly, H2O2 elicited the production of significant levels of IL-8 (Table I), and the addition of Me2SO dose dependently inhibited this production. Significant suppression was observed at a Me2SO concentration of 1% (Fig. 3).
Regulation of IL-8 Gene Expression

**Table I**

| Stimulus | No MeSO | 1% MeSO | No MeSO | 1% MeSO |
|----------|---------|---------|---------|---------|
| Control  | 0.489 ± 0.068 | 0.559 ± 0.070 | 143.5 ± 8.6 | 1,097.8 ± 247.0 |
| LPS (1 μg/ml) | 0.729 ± 0.070 | 0.559 ± 0.070 | 10,760 ± 1,236 | 1,097.8 ± 247.0 |
| PHA (10 μg/ml) | 0.553 ± 0.110 | 0.533 ± 0.047 | 24,991 ± 5,684 | 257.2 ± 66.4 |
| IC (250 μg/ml) | 0.411 ± 0.076 | 0.450 ± 0.055 | 36,542 ± 5,652 | 692.2 ± 123.9 |
| H2O2 (0.15%) | 0.240 ± 0.041 | 0.346 ± 0.086 | 3,585 ± 1,335 | 247.2 ± 23.9 |

*As assessed by reduction of MTT and measurement of absorbance at 550 nm.

Cell viability studies demonstrated that the MeSO-mediated suppression of IL-8 production in stimulated whole blood was not due to cellular toxicity. The viability of WBC isolated from blood samples was assessed by the ability of the cells to metabolize MTT-tetrazolium to a colored end product, a reaction occurring only in live cells (19). As shown in Table I, MeSO at a concentration of 1% did not significantly affect cell viability in the presence of any of the stimulating agents. Although WBC viability was somewhat reduced in the blood samples incubated with H2O2, this difference was not statistically significant as compared to control cells. The actual concentrations of IL-8 produced in response to the various stimuli are also shown in Table I. PHA and IC were stronger inducers of IL-8 production than LPS, which was in turn stronger than H2O2. However, MeSO was able to profoundly suppress IL-8 release into the plasma in all instances.

To determine whether MeSO exerted its inhibitory effects at the level of mRNA, the expression of IL-8 mRNA was examined in the presence and absence of 1% MeSO, with WBC being harvested for RNA extraction at a 24-h time point. The compiled results from the Northern blots of five different donors are shown in Fig. 4A. The results were corrected to represent equivalent RNA loading in each lane based on the intensity of the β-actin bands and are expressed as a percentage of the band of maximal intensity on each blot. Stimulation of blood with LPS (1 μg/ml), PHA (10 μg/ml), IC (250 μg/ml), or H2O2 (0.15%) caused a significant elevation in IL-8 mRNA levels as compared to cells incubated under control conditions (#p < 0.05, Student-Newman-Keuls test). In addition, treatment with 1% MeSO significantly reduced the levels of IL-8 mRNA in every instance (*p < 0.05; **p < 0.001, Student’s t test). Fig. 4B depicts a representative blot that was sequentially probed for IL-8 and β-actin. The higher molecular weight band is nonspecific hybridization to the 28 S rRNA.

**Fig. 3.** MeSO (DMSO) dose-dependently inhibits IL-8 production in whole blood stimulated by H2O2. Blood from different donors was stimulated with 0.15% H2O2 in the presence of increasing concentrations of MeSO. Plasma samples were harvested at the level of mRNA, the expression of IL-8 mRNA was examined in the presence and absence of 1% MeSO, with WBC isolated from blood samples was assessed by the ability of the cells to metabolize MTT-tetrazolium to a colored end product, a reaction occurring only in live cells (19). As shown in Table I, MeSO at a concentration of 1% did not significantly affect cell viability in the presence of any of the stimulating agents. Although WBC viability was somewhat reduced in the blood samples incubated with H2O2, this difference was not statistically significant as compared to control cells. The actual concentrations of IL-8 produced in response to the various stimuli are also shown in Table I. PHA and IC were stronger inducers of IL-8 production than LPS, which was in turn stronger than H2O2. However, MeSO was able to profoundly suppress IL-8 release into the plasma in all instances.

**Fig. 4.** MeSO (DMSO) inhibits IL-8 mRNA expression. Whole blood from five different donors was stimulated with LPS (1 μg/ml), PHA (10 μg/ml), IC (250 μg/ml), and H2O2 (0.15%) in the presence and absence of 1% MeSO. WBC were isolated at a 24-h time point and subjected to Northern blot analysis. A, the intensity of the IL-8 bands of blots from five different donors were adjusted based on the intensity of the β-actin bands to give equivalent RNA loading in each lane. The compiled results (n = 5) represent mean ± S.E. All of the stimuli induced substantial amounts of IL-8 mRNA. *p < 0.05 versus control, Student-Newman-Keuls test. The induction of IL-8 mRNA could be inhibited by treatment with MeSO *p < 0.05 versus stimulus alone, Student’s t test. B, autoradiograms of a representative blot sequentially probed for IL-8 and β-actin. The higher molecular weight band is nonspecific hybridization to the 28 S rRNA.
be clearly visualized. The regulation of IL-8 is probably occurring at more than 1 level, since stimuli which produce similar levels of RNA (LPS, PHA, IC), differ more than 3-fold in the amount of IL-8 protein which is produced (Table I compared to Fig. 4).

IL-8 Regulation in CGD Patients—The ability of Me\textsubscript{2}SO, an effective OH\textsuperscript{•} scavenger, to suppress IL-8 production suggested the involvement of ROI in eliciting IL-8 expression. This possibility was strongly supported by the fact that addition of \textsubscript{H}O\textsubscript{2} to the blood elicited IL-8 production. Proinflammatory stimuli such as LPS, PHA, and IC are known to activate monocytes and neutrophils to enzymatically generate ROI via the NADPH-oxidase on the cell membrane. To test whether this source of ROI is important in inducing IL-8 expression, experiments were performed in which blood from CGD patients was stimulated with a variety of agents in the presence and absence of 1% Me\textsubscript{2}SO. Fig. 5 shows the plasma IL-8 levels measured in experiments using blood from two different CGD patients. Stimulation of the CGD blood with LPS (1 \mu g/ml), PHA (10 \mu g/ml), IC (250 \mu g/ml), IL-1\beta (100 ng/ml), and \textsubscript{H}O\textsubscript{2} (0.15%) resulted in substantial IL-8 production, particularly in the case of patient B. Treatment of these stimulated blood samples with 1% Me\textsubscript{2}SO caused substantial suppression of the plasma IL-8 concentrations. The level of suppression ranged from 50 to 92% for patient A and from 77 to 99% for patient B.

IL-8 mRNA levels were also assessed in these experiments. Fig. 6 shows the results of the densitometric analysis of Northern blots which were sequentially probed for IL-8 and \beta-actin. A pattern of responsiveness similar to that shown in Fig. 3 was observed, with Me\textsubscript{2}SO causing a substantial suppression in the level of IL-8 mRNA expression. These results demonstrate that CGD blood responded to stimuli in the absence and presence of Me\textsubscript{2}SO in a manner essentially identical to blood from normal donors.

To further define the reactive oxygen intermediates which may be driving the production of IL-8, other antioxidants were studied. Blood from a patient with CGD was mixed with 1 \mu g/ml of LPS in the presence of Me\textsubscript{2}SO, dimethyl thiourea, thiourea, mannitol, or ethanol (Table II). All of these reagents effectively reduced the amount of plasma IL-8, in a manner very similar to our previous report using normal volunteers (8).

ROI Regulation of IL-8 Production in Cultured Cells—A wide variety of cell types of nonimmune origin have been shown to produce ROI (20-23), and several potential pathways of ROI generation may be involved (4, Fig. 7). We hypothesized that if ROI are crucial regulators of IL-8 expression, then the pattern of IL-8 regulation observed in whole blood should also be evident in many types of cultured cells. Additionally, if ROI cause up-regulation of IL-8 production in these cells endogenous to normal organs, then the potential exists that following an oxidant stress, the resident, nonimmune cells could generate a chemotactic factor to recruit neutrophils to the site of injury. To address this issue, experiments were performed using three different cell lines: Hep-G2 cells, fibroblasts, and A549 cells. All of these cells have been shown by other investigators to produce IL-8 in response to TNF and IL-1\beta (24-27).

To test the ability of Me\textsubscript{2}SO to inhibit cytokine-stimulated IL-8 production, Hep-G2 cells were stimulated with 10 ng/ml TNF alone or in combination with increasing concentrations of Me\textsubscript{2}SO (Fig. 8). Cell-free supernatants were harvested at a 24-h time point. Compared to the IL-8 levels measured in the presence of TNF alone, Me\textsubscript{2}SO caused a dose-dependent reduction in IL-8 release. A significant suppression (\textsubscript{p} < 0.05, Student-Newman-Keuls test) in IL-8 levels was observed at the three highest Me\textsubscript{2}SO concentrations (0.25–1%), with a 73.5 ± 0.9% suppression measured in the presence of 1% Me\textsubscript{2}SO. Moreover, cell viability was also assessed using MTTtetrazolium and was not found to be significantly altered by Me\textsubscript{2}SO concentrations of ±1%.

Studies were also performed in an effort to stimulate IL-8 production by exposing cells to oxidant stress. Fig. 9 shows the results of representative experiments in which fibroblasts, Hep-G2 cells, and A549 cells were incubated for 24 h in the presence of \textsubscript{H}O\textsubscript{2} (0.25–2 mM). A dose-dependent induction of IL-8 protein expression was detected in the cell supernatants, with significant elevations in IL-8 concentrations being detected at \textsubscript{H}O\textsubscript{2} concentrations of 0.5 mM and greater in all instances (\textsubscript{p} < 0.05; \textsuperscript{**p} < 0.01 as compared to the same \textsubscript{H}O\textsubscript{2} concentration in the presence of catalase, Student's t test). In the fibroblasts and the A549 cells, some fall-off in the level of IL-8 produced was observed at the higher \textsubscript{H}O\textsubscript{2} concentrations; this is likely to be due to a decrease in cell viability (see below). Concomitant addition of catalase (100 units/ml) at the time of stimulation with \textsubscript{H}O\textsubscript{2} was able to completely prevent IL-8 production, indicating that this response was, in fact, specifically due to the presence of \textsubscript{H}O\textsubscript{2} (Fig. 9).

A similar induction of IL-8 was observed in fibroblasts exposed to the \textsubscript{H}O\textsubscript{2}-generating enzyme glucose oxidase. Fig. 10 shows the results of an experiment in which fibroblasts received various treatments, and cell supernatants were harvested at 3, 6, 12, 18, and 24 h. The treatment groups included controls (no addition), catalase alone (100 units/ml), glucose oxidase (5 milliunits/ml), and glucose oxidase plus catalase. While basal levels of IL-8 were detected in the supernatants of control cells and cells treated with catalase alone, the cell supernatant IL-8 levels increased in a log-linear manner in

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**Fig. 5. Stimulation of IL-8 production in blood from CGD patients is suppressed by Me\textsubscript{2}SO (DMSO).** Blood from two patients with clinically diagnosed CGD was stimulated with LPS (1 \mu g/ml), IC (250 \mu g/ml), PHA (10 \mu g/ml), IL-1\beta (100 ng/ml), and \textsubscript{H}O\textsubscript{2} (0.15%) in the presence and absence of Me\textsubscript{2}SO (1% v/v). Plasma was harvested at a 24-h time point and analyzed for IL-8 by ELISA.
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Fig. 6. Stimulation of IL-8 mRNA expression in blood from CGD patients is suppressed by Me2SO (DMSO). Blood from two patients with clinically diagnosed CGD was stimulated as described in the legend to Fig. 5 in the presence and absence of Me2SO (1% v/v). WBC were isolated at a 24-h time point and subjected to Northern blot analysis. The IL-8 mRNA results were corrected based on the intensity of the β-actin bands of the same blot to give equivalent RNA loading in each lane and are expressed as a percentage of the band of maximal intensity.

Table II
Effects of different antioxidants on IL-8 production in LPS-stimulated blood from a patient with chronic granulomatous disease
Whole blood was stimulated with 1 µg/ml of LPS.

| Antioxidant     | Conc.  | IL-8, pg/ml |
|-----------------|--------|-------------|
| None            |        | 14,111      |
| Me2SO           | 1%     | 1,188       |
| Dimethyl thioare | 7.5 mM | <50         |
| Thiourea        | 75 mM  | 1343        |
| Mannitol        | 150 mM | 9,257       |
| Ethanol         | 0.5%   | 1,050       |
| No LPS          |        | 568         |

Fig. 7. Potential intracellular pathways of ROI generation.
The abbreviations used are: SOD, superoxide dismutase; DMSO, dimethyl sulfoxide; DMTU, dimethyl thioare; TU, thiourea; NMA, N-methyl-L-arginine.

Fig. 8. Me2SO (DMSO) suppresses TNF-stimulated IL-8 production in Hep-G2 cells. Hep-G2 cells were stimulated with 10 ng/ml TNF in the presence of increasing concentrations of Me2SO. Cell supernatants were harvested at a 24-h time point, and IL-8 levels were measured by ELISA (closed circles). Cellular viability was also assessed by the ability of the cells to reduce MTT and is expressed as absorbance units at 550 nm (open diamonds). The results represent mean ± S.E. (n = 3). *p < 0.05 versus TNF alone, Student-Newman-Keuls test.

Because the production of IL-8 in whole blood and various...
which fibroblasts and Hep-G2 cells were stimulated for color development of control cells incubated with MTT-tetrazolium, 1 mM H₂O₂ reduced fibroblast viability by approximately 50%. Moreover, catalase conferred full protection from the H₂O₂-mediated toxicity. Although these observations serve to emphasize the different susceptibilities of various cell lines to oxidant stress, they also suggest the possibility that the induction of IL-8 expression by H₂O₂ and glucose oxidase in fibroblasts may be a nonspecific response of the cell to a cytotoxic environmental stress. Studies were therefore performed in which fibroblasts were exposed to various types of cellular stress including heat shock and chemical stress as well as oxidant stress. The results of such an experiment are shown in Table IV. The cells were exposed to heat shock by incubating them at 42 °C for 1, 2, or 3 h with the remainder of the 24-h incubation being conducted at 37 °C. These conditions were not found to elicit IL-8 production. Likewise, incubation of fibroblasts for 24 h with various types of chemical stress (such as cadmium sulfate, sodium arsenite, ethanol, ammonium chloride, and sodium azide) also did not induce IL-8 release. Oxidant stress, however, did stimulate IL-8 production. Although a minimal response to H₂O₂ was observed in this particular experiment, glucose oxidase and xanthine oxidase, proteins which enzymatically generate ROI, stimulated substantial levels of IL-8 release (Table IV).

DISCUSSION

Low levels of ROI are produced as a normal part of cellular metabolism, and cells contain several enzymes, such as cata-

![Figure 9](image_url)

**Fig. 9.** H₂O₂ dose-dependently stimulates IL-8 production in cultured cells. Fibroblasts, Hep-G2 cells, and A549 cells were stimulated with increasing concentrations of H₂O₂ in the presence and absence of catalase (100 units/ml). Cell supernatants were harvested at a 24-h time point, and IL-8 levels were assessed by ELISA. The results represent mean ± S.E. (n = 3). *p < 0.05; **p < 0.01 versus the same H₂O₂ concentration in the presence of catalase, Student’s t test. HSF, human skin fibroblasts.

![Figure 10](image_url)

**Fig. 10.** Glucose oxidase (GOX) stimulates IL-8 protein and mRNA expression in fibroblasts. Fibroblasts were treated with 5 milliunits/ml glucose oxidase, 100 units/ml catalase, or a combination of glucose oxidase and catalase. Control cells received no addition. A, cell supernatants were harvested at the indicated time points and analyzed for IL-8 protein by ELISA. The results represent mean ± S.E. (n = 3). *p < 0.05 versus other treatments at the same time point, Student-Newman-Keuls test. B, total cellular RNA was extracted at the indicated time points and analyzed by Northern blot. The results (mean ± S.E., n = 3) represent IL-8 mRNA levels corrected for the intensity of the β-actin bands to give equivalent RNA loading in each lane and are expressed as a percentage of the maximal IL-8 levels for each blot.
lase, glutathione peroxidase, and superoxide dismutase, whose function is to detoxify these radicals (4). Higher levels of ROI are generated upon the activation of xanthine oxidase or during the respiratory burst of phagocytic cells, and these greater concentrations have been associated with tissue damage (1, 3). A new role for free radicals has been proposed with the discovery that nitric oxide (NO), a nitrogen-centered radical, is important in intracellular signaling (28). It satisfies many of the criterion for a second messenger in that it is

ubiquitously present, it can be rapidly produced in a regulated manner, and can be readily inactivated. Recently, Schreck and Bauerle (29) have theorized that certain oxygen-centered radicals may play a similar role as intracellular messengers. The results of the present study indicate that ROI are important in regulating IL-8 gene expression, confirming and extending the findings of a preliminary report (8). The OH radical scavenger Me2SO specifically suppressed IL-8 production in LPS-stimulated human whole blood. Me2SO similarly suppressed IL-8 production upon stimulation with PHA or IC. Moreover, addition of H2O2, a direct oxidant stress, to blood stimulated IL-8 release, while concomitant addition of Me2SO inhibited IL-8 release.

The NADPH-oxidase of phagocytic cells likely constitutes the most significant source of ROI in stimulated whole blood. To determine whether this enzyme is the sole participant in the induction of IL-8 expression by ROI, experiments were performed using blood from patients with CGD, an inherited condition in which components of the NADPH-oxidase of phagocytic cells are either absent or nonfunctional (30). CGD patients thus often have recurring infections due to the inability of neutrophils to adequately clear microorganisms without the aid of NADPH-oxidase generated ROI. Experiments using blood from CGD patients demonstrated that the production of IL-8 was regulated in a manner similar to that observed for normal blood donors: a variety of stimulating agents elicited production of IL-8, and this production was substantially inhibited at both the level of protein and mRNA by the OH scavenger Me2SO. Furthermore, other anti-oxidants also suppressed LPS-induced IL-8 production. To propose that IL-8 production is regulated by endogenously produced ROI even in CGD patients is in disagreement with current dogma emphasizing the inability of the cells of CGD patients to produce any ROI whatsoever. However, several non-NADPH-oxidase-dependent sources of ROI production also exist, including radicals generated during mitochondrial electron transport and arachidonate metabolism (4). Moreover, many different cell types, including endothelial cells (18, 19), fibroblasts (20), and chondrocytes (21), have been shown to produce measurable levels of ROI in response to cytokine stimulation in spite of the fact that they lack a classic NADPH-oxidase system for generating ROI.

Additional studies were therefore performed in order to document the ability of ROI to regulate IL-8 production in cultured cells. Although Me2SO, even at low concentrations (0.25%), was found to cause toxicity in fibroblasts (data not shown), this OH scavenger was observed to dose dependently inhibit TNF-stimulated IL-8 production in Hep-G2 cells with no effect on cell viability. A similar suppressive effect of Me2SO has been observed for TNF- and LPS-stimulated IL-8 production in a transformed endothelial cell line (data not shown). These findings suggest that endogenously produced ROI are involved in up-regulating IL-8 gene expression in cells of nonimmune origin as well as in leukocytes.

### Table III

| Viability of fibroblasts and Hep-G2 cells after a 6- and 24-h incubation with H2O2 alone or combined with catalase | Fibroblasts | HEP-G2 |
|---------------------------------------------------------------|------------|--------|
|                                                               | 6 h        | 24 h   |
|                                                               | 6 h        | 24 h   |
| Control                                                      | 100 ± 3.4* | 100 ± 3.4 | 100.0 ± 1.4 | 100.0 ± 0.5 |
| Catalase                                                    | 88.3 ± 1.0 | 107.1 ± 3.2 | 98.7 ± 1.2 | 99.2 ± 0.1 |
| H2O2                                                        | 52.1 ± 0.9 | 50.8 ± 2.1 | 92.7 ± 1.5 | 83.2 ± 1.6 |
| H2O2 + catalase                                            | 85.9 ± 1.0 | 73.1 ± 0.6 | 96.0 ± 1.8 | 104.5 ± 0.3 |

*Results are expressed as a percentage of the viability of the control group at each time point for each cell line (mean ± S.E.).

[^1]: Fibroblasts and Hep-G2 cells were incubated with 1 mM H2O2 and 2 mM H2O2, respectively.
Regulation of IL-8 Gene Expression

| Table IV | Effect of heat shock, chemical stress, and oxidant stress on IL-8 production in human fibroblasts |
|----------|-----------------------------------------------------------------------------------------------|
| Stimulus | IL-8 (pg/ml) |
| Control  | <24.2* |
| 1 h 42 °C | <24.2 |
| 2 h 42 °C | <24.2 |
| 3 h 42 °C | <24.2 |
| 50 μM cadmium sulfate | <24.2 |
| 50 μM sodium arsenite | <24.2 |
| 0.5% ethanol | <24.2 |
| 1.5 mM ammonium chloride | <24.2 |
| 1.5 mM sodium azide | <24.2 |
| 1 mM H₂O₂ | 110.2 + 9.8 |
| 5 millimol/ml glucose oxidase | 777.5 ± 68.1 |
| 12.5 millimol/ml xanthine oxidase | 1721.1 ± 124.5 |

*The lower limit of the assay was 24.2 pg/ml. Detectable IL-8 levels represent mean ± S.E. (n = 3).

While exposure of cells to an oxidant stress such as H₂O₂ or glucose oxidase stimulated the production of IL-8, such treatments were also significantly toxic to the cells. Since oxidant stress causes the production of heat shock proteins (31), other treatments which also induce these proteins (31) were evaluated with regard to their ability to induce IL-8. Heat shock at 42 °C and various chemical inducers of heat shock proteins were found to be ineffective inducers of IL-8. The production of IL-8 therefore appears to be a specific response to oxidant stress. A fundamental difference, however, appears to exist in the induction of IL-8 by endogenously produced ROI and by exogenously added ROI. The low levels of endogenously produced ROI induce IL-8 without cellular toxicity, whereas toxic or near toxic levels of exogenously added H₂O₂ are necessary to stimulate IL-8 production. This perhaps suggests that the intracellular localization of the ROIs is important: if endogenously produced ROI are generated in close proximity to their intracellular target, only low levels may be necessary, while high levels of H₂O₂ may be required to allow sufficient levels of ROI to diffuse to the target site through a detoxifying array of intracellular enzymes.

Regulation of gene expression by oxidant stress is not unique to IL-8 as the transcription of several other genes is also apparently regulated by cellular redox status. For example, the transcription of HIV-1 in TNF- or PMA-stimulated cells has been shown to be suppressed by N-acetyl cysteine, which reacts with ROI and replenishes intracellular glutathione levels (32, 33). In addition, TNF production in PMA-stimulated U373 cells was significantly blocked by the antioxidant butylated hydroxyanisole (34). Alterations in ambient oxygen tension also affect cytokine gene expression. Hypoxia (35) and reoxygenation of hypoxic cells (36) induces IL-1 production in mononuclear cells, and hypoxia and hyperoxia, alone or in sequence, have been shown to induce IL-8 (37). A unifying mechanism underlying these studies may involve regulation of the activation state of nuclear factor-κB (NF-κB) by the redox status of the cell. NF-κB is a DNA-binding protein involved in the transcriptional activation of a variety of host defense genes (38), such as TNF (34) and IL-8 (39), as well as viruses such as HIV-1 (32, 33). Studies have shown that oxidant stress, such as H₂O₂, can activate NF-κB to its DNA binding form (40), while various antioxidants can inhibit NF-κB activation in cells stimulated with cytokines or agents such as LPS or PMA (32, 33, 41). Whether a mechanism of involving modulation of NF-κB DNA binding is involved in the MeSO-mediated suppression of IL-8 production is presently unclear.

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