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### Research Article

Ischemia-reperfusion and hyperoxia-induced pulmonary injury are associated with the presence of activated neutrophils (PMN) and cellular injury. Although the signals orchestrating the directed migration of these PMN during the pathogenesis of these disease states remain to be fully elucidated, it appears they may be dependent upon the production of certain neutrophil activating/chemotactic factors such as C5a, leukotriene B4, platelet-activating factor, and IL-8. The production of the latter chemotaxin by mononuclear phagocytes is especially intriguing as these cells can mediate inflammatory cell migration by either directly generating IL-8, or by inducing its production from surrounding nonimmune cells. In light of these observations, we propose that ischemia-reperfusion and oxidant stress, in vivo, may be simulated by anoxia-hyperoxia induced stress in vitro, and that this stress may act as a stimulus for the production of IL-8. We now show that isolated human blood monocytes respond to such an oxygen stress with augmented production of IL-8. In initial studies, monocytes demonstrated an increase in the production of IL-8 under anoxic preconditioning. Subsequently, monocytes were cultured under one of the following conditions for 24 h: (a) room air/5% CO<sub>2</sub>; (b) 95% N<sub>2</sub>/5% CO<sub>2</sub> for 6 h, followed by room air/5% CO<sub>2</sub> for 18 h; (c) 95% N<sub>2</sub>/5% CO<sub>2</sub> for 6 h, followed by 95% O<sub>2</sub>/5% CO<sub>2</sub> for 18 h; (d) room air/5% CO<sub>2</sub> for 6 [...]

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# Anoxia-Hyperoxia Induces Monocyte-derived Interleukin-8

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## Abstract

Ischemia-reperfusion and hyperoxia-induced pulmonary injury are associated with the presence of activated neutrophils (PMN) and cellular injury. Although the signals orchestrating the directed migration of these PMN during the pathogenesis of these disease states remain to be fully elucidated, it appears they may be dependent upon the production of certain neutrophil activating/chemotactic factors such as C5a, leukotriene B<sub>4</sub>, platelet-activating factor, and IL-8. The production of the latter chemotaxin by mononuclear phagocytes is especially intriguing as these cells can mediate inflammatory cell migration by either directly generating IL-8, or by inducing its production from surrounding nonimmune cells. In light of these observations, we propose that ischemia-reperfusion and oxidant stress, *in vivo*, may be simulated by anoxia-hyperoxia induced stress *in vitro*, and that this stress may act as a stimulus for the production of IL-8. We now show that isolated human blood monocytes respond to such an oxygen stress with augmented production of IL-8. In initial studies, monocytes demonstrated an increase in the production of IL-8 under anoxic preconditioning. Subsequently, monocytes were cultured under one of the following conditions for 24 h: (a) room air/5% CO<sub>2</sub>; (b) 95% N<sub>2</sub>/5% CO<sub>2</sub> for 6 h, followed by room air/5% CO<sub>2</sub> for 18 h; (c) 95% N<sub>2</sub>/5% CO<sub>2</sub> for 6 h, followed by 95% O<sub>2</sub>/5% CO<sub>2</sub> for 18 h; (d) room air/5% CO<sub>2</sub> for 6 h, followed by 95% O<sub>2</sub>/5% CO<sub>2</sub> for 18 h; or (e) 95% O<sub>2</sub>/5% CO<sub>2</sub>. Supernatants were isolated and analyzed for IL-8 antigen by specific IL-8 ELISA, demonstrating the production of monocyte-derived IL-8: 5.9±0.9, 11.4±1.7, 21.1±2.3, 14.6±2.4, and 26.3±4.7, ng/ml by designated conditions a, b, c, d, and e listed above, respectively. This variance in IL-8 production reflects altered rates of transcription as shown by Northern blot analysis and nuclear run-off assay. Furthermore, when monocytes were concomitantly treated with LPS (100 ng/ml) under *in vitro* hyperoxic conditions, both IL-8 steady-state mRNA and antigenic activity were two- to threefold greater than under room air conditions. The association of anoxic preconditioning and oxygen stress with augmented production of monocyte-derived IL-8 support the potential role for ischemia-reperfusion and hyperoxia-induced IL-8 production *in vivo*, providing a possible mechanism for PMN migration/activation in disease states characterized by

altered tissue oxygenation. (*J. Clin. Invest.* 1992. 90:791-798.) Key words: cytokine • ischemia-reperfusion • inflammation • neutrophil • neutrophil chemotactic factor

## Introduction

PMN accumulation and invasion upon reperfusion of a previously ischemic region is a well described phenomenon seen in a heterogeneous variety of tissues (1-4). When neutrophil infiltration extends beyond the borders of ischemia, as frequently occurs after reperfusion, the release of activated PMN contents may also cause indiscriminate destruction of still viable tissue, resulting in extension of the area of injury. For example, in both myocardium and gut, ischemia followed by reperfusion is associated with vigorous PMN infiltration, and varying degrees of subsequent neutrophil-dependent tissue damage (5, 6). PMN invasion with subsequent cellular injury is therefore a hallmark of ischemia-reperfusion events in a number of clinically relevant settings. The emigration of neutrophils, from within the vascular compartment to the site of eventual inflammation within reperfused tissue, is a complex and dynamic process; the neutrophil must first reversibly adhere to the vascular endothelium, followed by transendothelial diapedesis, and chemotactically directed migration to the eventual inflammatory locus. Although nonviable and dying cells can release a variety of short-lived mediators which possess neutrophil chemotactic activity, this may not fully account for the significant influx of neutrophils into salvageable zones of ischemia, and would be unlikely to account for neutrophil invasion of bordering nonischemic tissue. This raises the question: do cells in a region of oxidant stress, which do not incur terminal injury, actively generate a neutrophil chemoattractant that will account for neutrophil infiltration once reperfusion occurs? Although the spectrum of the neutrophil activities above is well described, the signals orchestrating these activities in the setting of reperfusion remain to be completely elucidated, but likely rely upon the production of one or more previously identified neutrophil activating/chemotactic factors, such as IL-8.

IL-8 is a recently described cytokine that has both neutrophil activation and chemotactic properties (7-15). Mononuclear phagocytes such as monocytes and macrophages appear to be the predominant cellular sources of IL-8, but this cytokine can also be produced by several nonimmune cells in response to the monocyte/macrophage-derived cytokines tumor necrosis factor (TNF),<sup>1</sup> and IL-1 (13-16). Thus, activated mononuclear phagocytes can mediate inflammatory cell migration by either directly generating IL-8, or by inducing its production from surrounding nonimmune cells. IL-8 interacts with its own specific neutrophil receptor to induce motility, directional migration, exocytosis of storage enzymes, and expression of the leukocyte integrin CD11b/CD18 complex (7-

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1. Abbreviation used in this paper: TNF, tumor necrosis factor.

10). Its biological activity is maintained in the presence of significant changes in pH, and is relatively resistant to proteolysis and denaturation as compared to other known chemotactic factors (7–11). The stability of IL-8 suggests that the production of this cytokine at in vivo sites of acute inflammation may have prolonged biological activity for the recruitment of neutrophils, making it a pivotal cytokine in acute neutrophil-mediated disease states.

In this study we proposed that certain aspects of ischemia-reperfusion injury may be simulated in vitro by anoxia-hyperoxia-induced stress upon monocytes, or other tissue-based, mononuclear immune cells, and that this stress can act as a stimulus for the production of IL-8, with resultant in vivo neutrophil influx. In testing this hypothesis, we were able to demonstrate that anoxia pretreatment of monocytes definitely primes these cells for the augmented production of IL-8, regardless of their subsequent level of oxidant stress. Similarly, under conditions of oxidant stress (hyperoxia) monocytes displayed augmented production of IL-8. We also demonstrated that anoxia-hyperoxia acts synergistically with LPS for the induction of monocyte-derived IL-8. Lastly, we demonstrated that the differences seen in IL-8 production reflected changes occurring at the transcriptional level. The association between oxygen stress and augmented production of IL-8 in these studies clearly support the role of IL-8 as a potential mediator in the migration and activation of neutrophils seen in ischemia-reperfusion injury and hyperoxia-induced tissue injury.

## Methods

**Reagent preparation.** Anti-human IL-8 antibodies were produced by immunization of rabbits with recombinant human IL-8 administered in multiple intradermal sites with complete Freund's adjuvant and boosted with recombinant IL-8 in incomplete Freund's adjuvant. IL-8 antibodies, in concentrations used in the ELISA, reacted with recombinant IL-8 in Western analysis, and failed to cross-react with connective tissue activating protein III, neutrophil activating peptide-2, beta-thromboglobulin, GRO/MGSA, or platelet factor 4. Stock LPS (*Escherichia coli* 0111:B4; Sigma Chemical Co., St. Louis, MO) was prepared at a concentration of 200 µg/ml in sterile RPMI 1640 (Whitaker Biomedical Products, Walkersville, MD), 1 mM glutamine, 25 mM Hepes, 100 U/ml penicillin, 100 ng/ml streptomycin (Hazelton Research Products, Inc., Denver, PA) (complete media).

**Cell isolation procedures.** Blood from normal, healthy volunteers was obtained in heparinized syringes, and monocytes were isolated by Ficoll-Hypaque density gradient centrifugation. Mononuclear cells were washed three times in complete media, and total cell counts were performed using a hemocytometer. The mononuclear cells were suspended in complete media at a concentration of  $3 \times 10^6$  cells/ml, and immediately plated as 1-ml aliquots, into 35-mm plastic culture plates (Costar Corp., Cambridge, MA). Cells were then incubated for 1 h at 37°C in humidified 95% air/5% CO<sub>2</sub>, to allow monocytes to adhere to the culture dishes. Nonadherent cells, and media, were then removed from the dishes, and adherent cells were rinsed twice with complete media warmed to 37°C. The cultures were then overlaid with 1 ml of complete media, and incubated according to the protocol below.

**Incubation of monocytes.** Since our laboratory and others have demonstrated monocyte activation and IL-8 mRNA induction with adherence to plastic and biological surfaces alone (17, 18), adherence-purified monocyte cultures were uniformly incubated to minimize differing durations of contact with plastic as a confounding variable. After adherence purification, the monocyte culture plates were placed in modular incubation chambers (Forma Scientific, Inc., Marietta, OH), which were then sealed and flushed with the appropriate gas mixture

for 5 min. The sealed modular chambers containing the monocyte cultures were humidified, and incubated at 37°C for 24 h. To establish the kinetics of anoxia-induced IL-8 expression by monocytes, we performed an initial set of experiments in which monocytes from each of 10 subjects were exposed to varying durations (0.5, 1, 1.5, 3, 6, 9, and 12 h) of anoxic conditions, before 12 h of exposure to hyperoxia. These conditions were achieved by flooding the sealed modular chambers with gas mixtures of either: (a) 95% nitrogen/5% CO<sub>2</sub>, to simulate anoxic conditions; or (b) 95% oxygen/5% CO<sub>2</sub>, to simulate hyperoxic conditions. Oxygen tension within the chambers was confirmed both before and after each experiment by use of an oxygen analyzer (Beckman Instruments, Fullerton, CA). Before their anoxic "priming", monocyte cultures were incubated for varying durations at room air (normoxic) conditions, so that a constant 24 h total incubation time was achieved. The monocyte supernatants were then isolated, and analyzed for IL-8 antigen by specific IL-8 ELISA.

Once the kinetics of anoxia-induced IL-8 generation were established, monocytes were isolated and plated as above and incubated at one of five possible sets of conditions: (a) 24 h at room air; (b) 6 h of anoxia, followed by 18 h at room air; (c) 6 h of anoxia, followed by 18 h of hyperoxia; (d) 6 h at room air, followed by 18 h of hyperoxia; or (e) 24 h of hyperoxia. These conditions were chosen to provide a 24-h normoxic control group, a 24-h hyperoxic control group, and combinations in which oxygen tension increased sequentially. For each of the five sets of conditions, cell viability of > 95% was confirmed by trypan blue exclusion, and pH of the media was unchanged. The monocyte supernatants were then isolated and analyzed for IL-8 antigen as above, while cell pellets were extracted for total RNA, or used in nuclear run-off analysis.

Lastly, we studied the effects of oxidant stress and concomitant exposure to an agonist such as LPS. Monocyte cultures were stimulated at time 0 with 100 ng/ml of LPS, a dose that represented the half-maximal stimulus for production of monocyte-derived IL-8 (19), and then exposed to one of the five conditions described above. The resulting monocyte supernatants were then analyzed for IL-8 antigen as above. In other experiments total RNA was extracted from cell pellets that had been generated in a time-dependent fashion.

**Interleukin-8 ELISA.** Monocyte-derived antigenic IL-8 was quantitated using a modification of a double ligand method as previously described (13). Briefly, flat-bottomed 96-well microtiter plates (Immuno-Plate I 96-F; Nunc, Roskilde, Denmark) were coated with 50 µl/well of rabbit anti-IL-8 antibody (1 ng/µl in 0.6 M NaCl, 0.26 M H<sub>3</sub>BO<sub>4</sub>, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C, washed with PBS, pH 7.5, 0.05% Tween-20 (wash buffer), and nonspecific binding sites blocked with 2% BSA in PBS. Plates were rinsed three times with wash buffer, and diluted (neat, 1:5, and 1:10) monocyte-derived conditioned media (50 µl) in duplicate was added, followed by incubation for 1 h at 37°C. Plates were washed three times with wash buffer, 50 µl/well of biotinylated rabbit anti-IL-8 (3.5 ng/µl in PBS, pH 7.5, 0.05% Tween-20, and 2% FCS) was added, and plates incubated for 30 min at 37°C. Plates were again washed three times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) was added, and the plates incubated for 30 min at 37°C. Plates were washed three times and chromogen substrate (Bio-Rad Laboratories) added. The plates were incubated at room temperature to the desired extinction, and the reaction terminated with 50 µl/well of 3 M H<sub>2</sub>SO<sub>4</sub> solution. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant IL-8; 1,000 ng to 1 pg/ml (50 µl/well). This ELISA method consistently detected IL-8 concentrations in a linear fashion > 10 pg/ml. TNF and IL-1β ELISAs were performed in a similar fashion using specific anti-TNF and anti-IL-1β antibodies, respectively.

**Western blot analysis.** Monocyte supernatant samples, 20 ng recombinant human IL-8 (72-amino acid species) (PeproTech, Rocky Hill, NJ), and molecular weight standards were loaded in 50-µl aliquots and separated by SDS-PAGE according to the method of Laemmli (20) (16% polyacrylamide containing 0.8% bisacrylamide), and then transferred electrophoretically to nitrocellulose by 14-min electrical transfer

at 24 V in 12 mM Tris, 96 mM glycine, in 20% methanol. Nitrocellulose sheets were blotted overnight at 4°C with TBS (20 mM Tris-Cl pH 8.2, 140 mM NaCl) with 5% nonfat dry milk. Blots were incubated with rabbit anti-human IL-8 at 1:500 concentration in TTBS (0.05% Tween in Tris-buffered saline) for 2 h at 4°C. The blots were washed three times with TTBS, then incubated with biotinylated goat anti-rabbit IgG at 1:1,000 dilution (Vector Laboratories, Inc., Burlingame, CA) for 1 h. After washing three times with TTBS, blots were treated 1 h with streptavidin-peroxidase at 1:5,000 dilution (Vector Laboratories) followed by three washings in TBS and reaction with 0.5 mg/ml 4-chloro-1-naphthol (Sigma Chemical Co.) in TBS with 0.05% hydrogen peroxide. Prestained rainbow molecular weight markers (Amersham Corp., Arlington Heights, IL) were used as a reference.

**Northern blot analysis.** Total cellular RNA from monocytes was isolated as previously described (21). Briefly, monocyte monolayers were scraped into a solution containing 25 mM Tris, pH 8.0, 4.2 M guanidine isothiocyanate, 0.5% *N*-lauroylsarcosine, and 0.1 M 2-mercaptoethanol. After homogenization, the above suspension was added to a solution containing an equal volume of 100 mM Tris, pH 8.0, 10 mM EDTA, and 1.0% SDS. The mixture was then extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was alcohol precipitated and the pellet dissolved in diethyl pyrocarbonate H<sub>2</sub>O. Total RNA was separated by Northern blot analysis using denaturing formaldehyde, with 1% agarose gels, transblotted to nitrocellulose, baked, prehybridized, and hybridized with a <sup>32</sup>P-5' end-labeled oligonucleotide probe. A 30-mer oligonucleotide probe was synthesized using the published cDNA sequence for human-derived IL-8 (11). The probe was complementary to nucleotides 262–291 and had the sequence 5'-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3'. Blots were washed, and autoradiographs were quantitated using laser densitometry (Ultrascan XS; LKB Instruments Inc., Houston, TX). Equal loading of all wells was assessed by monitoring 28s and 18s ribosomal RNA.

**Nuclear transcriptional analysis.** A nuclear run-off transcription assay was performed using a modification of the technique described previously (22). Briefly, adherent monocyte monolayers were washed twice with PBS, then scraped into buffer containing 10 mM Tris pH 7.4, 3 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub>. After centrifugation, the pellet was treated with 1 ml DP-40 lysis buffer followed by dounce homogenization. The nuclei were then recovered by centrifugation and the pellet from ~ 5 × 10<sup>7</sup> nuclei, in glycerol storage buffer, was suspended in 200 μl of 2× reaction buffer containing 10 mM Tris pH 8.0, 5 mM MgCl<sub>2</sub>, 300 mM KCl, 5 mM DTT, 10 μl of 100 mM XTP (A, G, C), and 10 μl <sup>32</sup>P-UTP (3,200 Ci/mmol). After 30 min at 30°C, 25 μl of RNase-free DNase (1 mg/ml) was added. After 5 min at 30°C, 200 μl of 10 mM Tris pH 7.4 containing 1% SDS and 5 mM EDTA, and 10 μl proteinase K (10 mg/ml; Bethesda Research Laboratories, Gaithersburg, MD) was added. The mixture was then incubated at 42°C for 30 min, extracted with phenol/chloroform, and the RNA precipitated to HA.45 μm filters (Millipore Corp., Bedford, MA) with 10% TCA/60 mM sodium pyrophosphate. The RNA was eluted off the filters with buffer containing 1% SDS, 10 mM Tris (pH 7.5), and 5 mM EDTA, then extracted again with phenol/chloroform/isoamyl alcohol, then reprecipitated with 3 M sodium acetate and ethanol. Precipitated RNA was resuspended in *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid/NaCl solution and equivalent counts per minute per milliliter hybridized with slot blots with plasmids containing either the IL-8 or β-actin inserts. The plasmids containing the IL-8 or human β-actin inserts were grown in *E. coli* and denatured by adding 1/10 vol of 3 M NaOH and incubating for 1 h at 65°C. The plasmids were placed on ice and neutralized by adding an equal vol of 2 M NH<sub>4</sub> acetate, pH 7.0. 5 μg of DNA was placed onto nitrocellulose via a slot blot apparatus (Schleicher and Schuell, Keene, NH). The filters were dried under vacuum for 2 h at 80°C and then prehybridized at 60°C in 10 mM TES (Sigma Chemical Co.) containing 0.2% SDS, 10 mM EDTA, 250 μg/ml *E. coli* RNA, 0.3 M NaCl, and 1× Denhardt's overnight. The <sup>32</sup>P-labeled mRNA (10 × 10<sup>6</sup> cpm/ml) generated was suspended in the

above buffer and incubated at 60°C with the immobilized DNA for 48 h. Filters were washed twice for 1 h at 65°C in 2× standard saline citrate (SSC), treated with 10 ng/ml RNase A (Sigma Chemical Co.) for 30 min at 37°C, washed with 2× SSC, then exposed to Kodak XAR-2 film at –70°C with intensifying screens. Quantitative results were obtained by laser densitometry, and standardized to β-actin.

**Statistical analysis.** Experiments were performed in triplicate for each time point. Each subject supplied monocytes for both control and experimental conditions in the individual experiments. Results are presented as means ± SEM. Data were analyzed by MacIntosh II computer using Statview II statistical software package (Abacus Concepts, Inc., Berkeley, CA). Groups of data were evaluated by analysis of variance. Data that appeared statistically significant were compared by Student's *t* test for comparing the means of multiple groups, and were considered significant if *P* values were < 0.05.

## Results

**Anoxia pretreatment induces monocyte-derived IL-8.** Our initial experiments were performed to establish whether anoxic preconditioning of monocytes in culture would augment the constitutive expression of IL-8 by these cells when challenged with a subsequent oxidant stress, and the duration of anoxic exposure necessary to maximize this effect. The data presented in Fig. 1 show the results of varying durations of anoxic preconditioning, followed by 12 h of hyperoxic incubation, for the production of monocyte-derived antigenic IL-8. The results were standardized to the control group (12 h room air/12 h hyperoxia) and expressed as percent of the control. This yielded values of 321 ± 40%, 283 ± 28%, 252 ± 14%, 188 ± 14%, 157 ± 17%, and 131 ± 17%, for 12, 9, 6, 3, 1.5, and 0.5 h, respectively, thus demonstrating a time-dependent relationship between duration of anoxic conditioning and subsequent hyperoxia-induced IL-8 expression. 12, 9, and 6 h of anoxic preconditioning were similar for their effects, resulting in significantly greater antigenic IL-8 production as compared to 3, 1.5, 0.5, and 0 h of anoxic preconditioning. While no significant difference in IL-8 production was seen among 12, 9, and 6 h of anoxic conditioning, 90% cell viability was not consistently

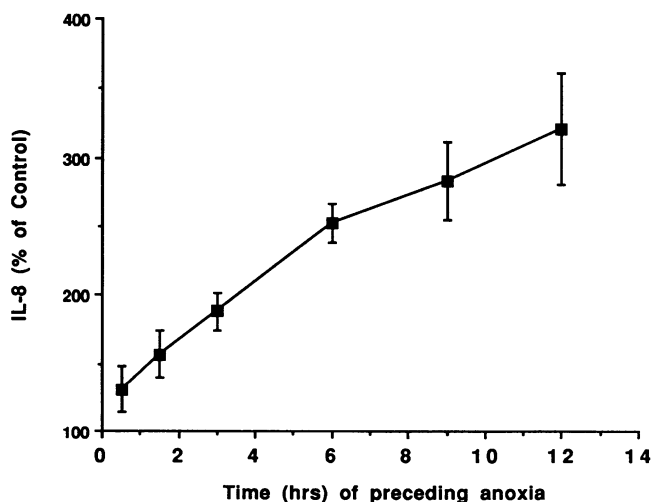


Figure 1. Time course of monocyte exposure to anoxia (95% N<sub>2</sub>/5% CO<sub>2</sub>) followed by exposure to hyperoxia (95% O<sub>2</sub>/5% CO<sub>2</sub>). Interleukin-8 is expressed as a percentage of control (exposure to 12 h room air/5% CO<sub>2</sub> and 12 h hyperoxia).

achieved after 12 h of anoxic exposure. To minimize altered cell viability as a potential variable, we limited duration of anoxic exposure to 6 h, and all subsequent experiments involving exposure of cells to combinations of conditions therefore used 6 h as the period of initial preconditioning.

*Sequential anoxic/hyperoxic stress augments IL-8 production.* Having established 6 h as a representative duration for our initial anoxic conditions, we exposed our monocyte cultures to one of the five following sets of conditions: (a) 24 h at room air; (b) 6 h of hypoxia followed by 18 h at room air; (c) 6 h of hypoxia followed by 18 h of hyperoxia; (d) 6 h at room air, followed by 18 h of hyperoxia; or (e) 24 h of hyperoxia, and then isolated supernatants for determination of antigenic IL-8 activity. Supernatants were also analyzed for antigenic IL-1 $\beta$  and TNF activity. Levels of antigenic IL-1 $\beta$  were  $2.5 \pm 0.9$  ng/ml,  $2.2 \pm 0.9$  ng/ml,  $2.5 \pm 2.0$  ng/ml,  $2.2 \pm 1.8$  ng/ml, and  $2.7 \pm 0.4$  ng/ml for conditions a, b, c, d, and e, respectively, while TNF levels were  $< 1$  ng/ml for all groups; in comparing all sets of conditions, no significant differences were found for either of these cytokines. Levels of antigenic IL-8 were  $5.9 \pm 0.9$  ng/ml,  $11.4 \pm 1.7$  ng/ml,  $21.1 \pm 2.3$  ng/ml,  $14.6 \pm 2.4$  ng/ml, and  $26.3 \pm 4.7$  ng/ml, for conditions a, b, c, d, and e, respectively (Fig. 2). Of note is that IL-8 production by the 24-h room air control group differed significantly from all other groups, with  $P$  values  $< 0.01$ . Antigenic IL-8 production by cell cultures exposed to the 6 h anoxia/18 h hyperoxia conditions differed significantly from both the anoxia/room air and room air/hyperoxia groups; however, no difference was shown between the anoxia/hyperoxia group and the 24-h hyperoxia control group. Lastly, there was a significant difference in antigenic IL-8 production seen when comparing the 24-h hyperoxia control group to either the anoxia/room air group or the

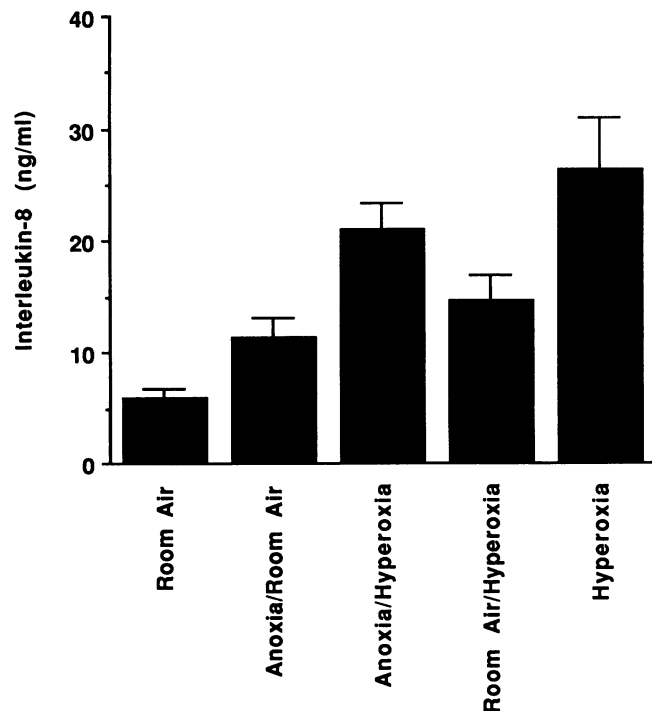


Figure 2. Monocyte exposure to oxygen stress induces the augmented production of interleukin-8.

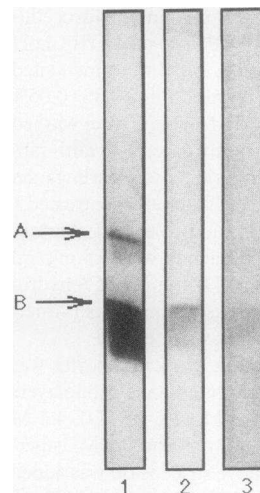
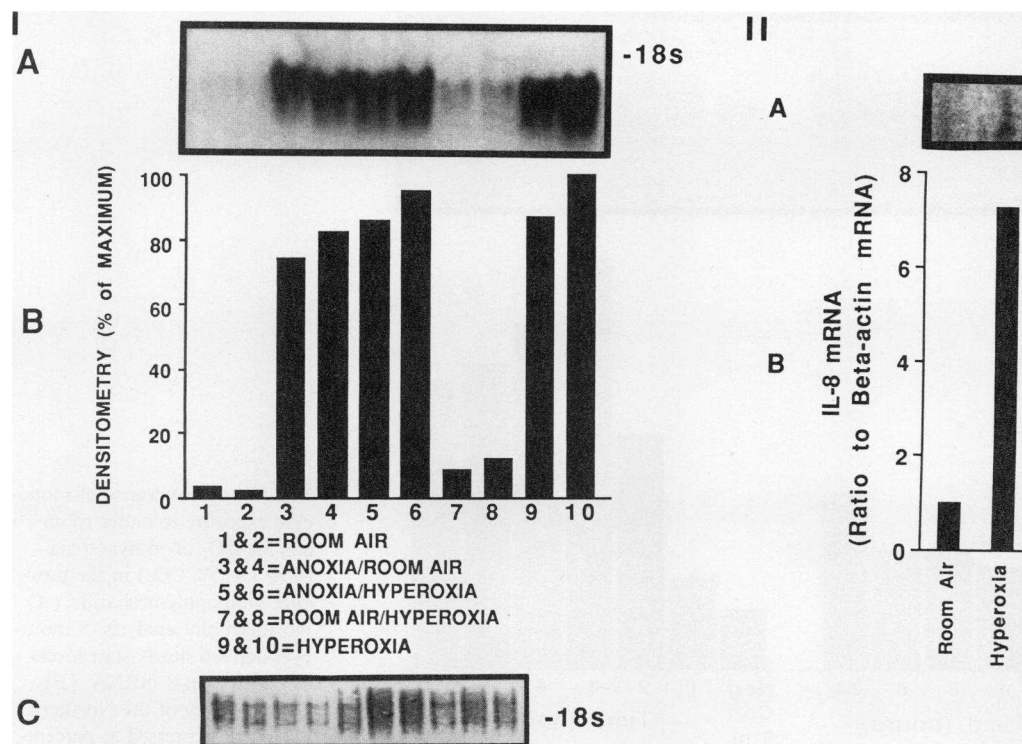


Figure 3. Western blot analysis of IL-8 antigen. Lane 1, recombinant IL-8 (72-amino acid form). Lane 2, monocyte supernatant antigenic IL-8 after hyperoxia exposure. Lane 3, monocyte supernatant antigenic IL-8 after room air exposure. A is pointing toward the dimeric form of recombinant IL-8 ( $\sim 16$  kD). B is pointing toward the monomeric form of IL-8 ( $\sim 8$  kD).

room air/hyperoxia group. Subsequent Western blot analysis of these monocyte supernatants demonstrated that the IL-8 generated under our experimental conditions occurred predominantly as a single, 72-amino acid, species (Fig. 3).

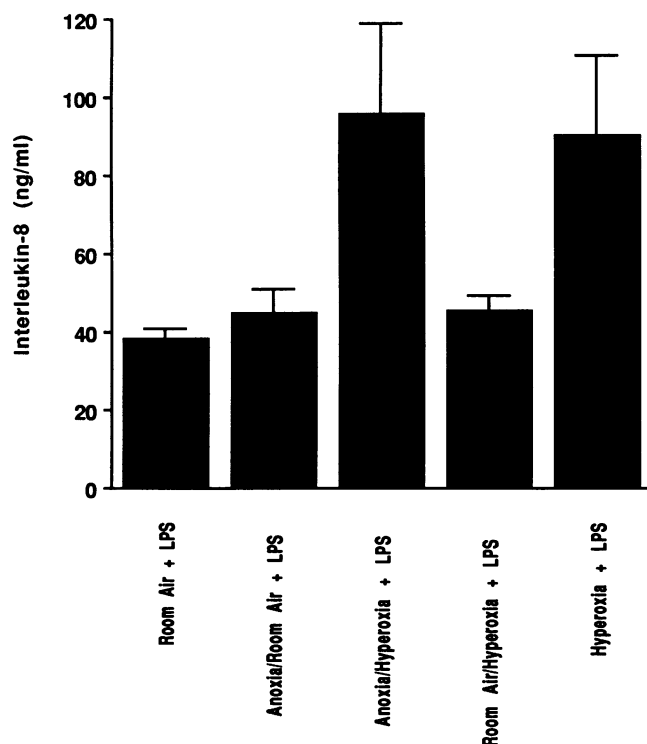
To determine whether the above results were due to elevated mRNA transcripts, we next examined the expression of steady-state IL-8 mRNA by monocyte cultures under each set of conditions. The steady-state mRNA present in cell cultures from sequential experiments was assessed by Northern blot analysis, shown in Fig. 4 (panel I A). In a parallel fashion with our preceding antigenic data, steady-state IL-8 mRNA was significantly elevated in the anoxia/hyperoxia and hyperoxia groups, as compared to the room air control group. Significant elevation of steady-state IL-8 mRNA is also seen in the anoxia/room air group. Since actinomycin D stabilization analysis of IL-8 mRNA in previous experiments suggested that these elevations of mRNA were secondary to differing rates of transcription, we then performed a nuclear transcriptional ("run-off") analysis to assess this question directly. Nuclei were isolated from cell cultures of the room air and hyperoxia control groups, and radiolabeled transcripts of IL-8 mRNA were generated, with results depicted in Fig. 4 (panel II). As shown, when standardized to the "housekeeping" gene  $\beta$ -actin, there is a nearly sevenfold difference in IL-8 mRNA between the room air and hyperoxia groups, which correlated with the above antigenic IL-8 data. Exposure to oxidant stress therefore appears to augment transcription of monocyte-derived IL-8 mRNA.

*LPS is synergistic for anoxia/hyperoxia-induced IL-8 production.* The intestine remains one of the most vulnerable sites of occult ischemic injury, with any mucosal ischemic damage potentially introducing large boluses of either gut-colonizing bacteria or their products (endotoxin) into the gut wall (23). Because various types of either focal intestinal, or diffuse mesenteric, ischemia have been well documented histologically to manifest reperfusion injury associated with a vigorous PMN infiltration (6), we next studied the effects of concomitant treatment with LPS under one of the five sets of conditions described previously. Monocyte cultures were stimulated with 100 ng/ml of LPS at time 0, and exposed to either conditions a, b, c, d, or e as described above. The dose of LPS used under these conditions represented the half-maximal concentration for the induction of monocyte-derived IL-8 (19). These condi-



**Figure 4.** Northern blot and nuclear transcriptional analysis of IL-8 mRNA. Panel *I A* is the Northern blot of steady-state levels of IL-8 mRNA. Panel *I B* is the corresponding densitometry of the Northern blot in panel *I A*, expressed as the percentage of maximum (24 h of under hyperoxic conditions). Panel *I C* is the corresponding 28s and 18s rRNA of the Northern blot in Panel *I A*. The IL-8 mRNA corresponded with a size of 1.8 kB. Panel *II A* is the autoradiograph of the nuclear transcriptional analysis (run-off) of monocyte-derived IL-8 mRNA. Panel *II B* is the densitometry of the nuclear transcriptional analysis in panel *II A*, standardized to the housekeeping gene beta-actin.

tions resulted in antigenic IL-8 production of  $38.4 \pm 2.5$  ng/ml,  $44.8 \pm 5.9$  ng/ml,  $95.7 \pm 22.9$  ng/ml,  $45.0 \pm 4.2$  ng/ml, and  $90.9 \pm 20.5$  ng/ml, for conditions *a*, *b*, *c*, *d*, and *e*, respectively

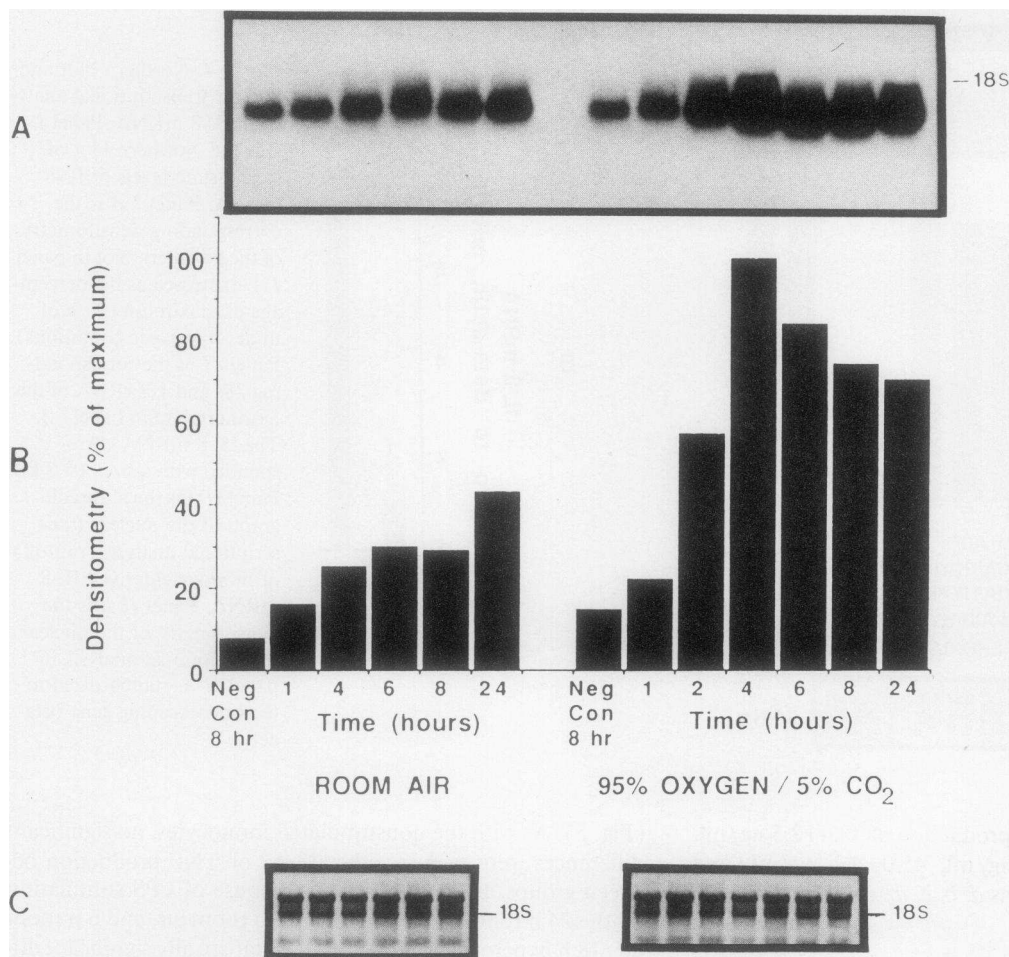


**Figure 5.** Monocyte exposure to oxygen stress in the presence of lipopolysaccharide is synergistic for the production of interleukin-8.

(Fig. 5). As with the nonstimulated monocytes, no significant differences were seen in either IL-1 $\beta$  or TNF production between groups. In examining the response of LPS stimulation on the 24 h room air, 6 h anoxia/18 h room air, and 6 h room air/18 h hyperoxia groups, previous statistically significant differences between these groups were ablated, with an apparent plateau in antigenic IL-8 generation near 40 ng/ml. Given that the latter two groups of conditions above responded similarly to the room air control, this seemed to initially imply that LPS exposure resulted in IL-8 induction of a uniform degree, irrespective of the oxidant stress concurrently invoked. However, under conditions of either 24 h hyperoxia, or 6 h anoxia/18 h hyperoxia, LPS stimulation resulted in a synergistic increase in IL-8 production (Fig. 5).

To determine whether these results were also due to elevated mRNA transcripts, we again examined whether the expression of steady-state IL-8 mRNA paralleled levels of antigenic IL-8. The steady-state mRNA present in the cells exposed to either hyperoxia or room air was assessed by Northern blot analysis. A representative Northern blot analysis of the time-course for the generation of steady-state levels of monocyte-derived IL-8 mRNA in response to LPS stimulation, at either room air or hyperoxic conditions, is shown in Fig. 6. In a parallel fashion with our preceding antigenic IL-8 data, steady-state IL-8 mRNA was significantly elevated in the LPS-stimulated hyperoxic group as compared with the LPS-stimulated room air group. Stabilization analysis with actinomycin D of IL-8 mRNA under each of the above conditions failed to demonstrate a difference in the half-life of decay of IL-8 mRNA (data not shown). Thus, exposure to oxidant stress, concomitant with an agonist (LPS) for IL-8 expression, appears to synergistically augment transcription of monocyte-derived IL-8 mRNA.





**Figure 6.** Time course of monocyte exposure to either room air/5% CO<sub>2</sub> or to hyperoxia (95% O<sub>2</sub>/5% CO<sub>2</sub>) in the presence of lipopolysaccharide. (A) Northern blot analysis of monocyte-derived steady-state levels of interleukin-8 mRNA. (B) Densitometry of the Northern blot in A, expressed as percentage of maximum (the 4-h time-point of monocyte exposure to hyperoxia in the presence of lipopolysaccharide). (C) The 28S and 18S rRNA of the Northern blot in A. The IL-8 mRNA corresponded with a size of 1.8 kB.

## Discussion

Cellular injury after reversible periods of ischemia, or hyperoxia-induced lung injury, has long been hypothesized to result from the local generation of oxygen radicals, including superoxide anion, hydroxyl radical, singlet oxygen, and hydrogen peroxide, which occurs in these settings (24). These reactive oxygen species, with their affinity for membrane phospholipids, thus promote cell damage and may explain some degree of local reperfusion injury. Similarly, under conditions of hyperoxia, the mitochondrial contributions to free radical production of whole lung can increase 15- to 20-fold (25). Large numbers of PMN have also been found to migrate into the lung during oxidant stress; this influx coincides with the beginning of cellular damage, suggesting that these cells may play an important role in potentiating lung injury (25). Since the stereotypical histologic response seen in reperfusion and hyperoxia-induced injury is leukocyte recruitment and abundant inflammation, elucidating the mechanism that leads to the cellular response seen in these disease states might be accomplished by identifying common aspects between reperfusion-induced cellular stress and the conventional inflammatory response.

We investigated whether monocytes subjected to an oxidant stress simulating ischemia-reperfusion would actively generate the neutrophil chemoattractant IL-8. Our first series of experiments involved exposure of monocytes to varying dura-

tions of anoxia before exposure to a uniform period of hyperoxia. These data showed a definite time-dependent relationship between duration of anoxic preconditioning, and subsequent antigenic IL-8 expression. 6 h represented the duration of anoxic preexposure necessary to maximally augment subsequent hyperoxia-induced changes in IL-8 expression without alterations in cellular viability.

We next examined the effects of abrupt increases in intracellular oxygen tension on antigenic IL-8 production. Of five possible sets of conditions, three (anoxia/room air, anoxia/hyperoxia, room air/hyperoxia) involved sequential increase in oxygen exposure to simulate ischemia-reperfusion events, while the other two served as positive (hyperoxia) and negative (room air) controls. These results demonstrated no difference between the anoxia/hyperoxia group and the positive control (hyperoxia) group, in the generation of antigenic IL-8. The quantity of IL-8 generated by the anoxia/hyperoxia group could not be explained on the basis of hyperoxic induction alone, since it differed significantly from the room air/hyperoxia group, despite identical durations of hyperoxic exposure. This difference, coupled with the Northern blot analysis demonstrating increased steady-state levels of IL-8 mRNA under conditions involving anoxic exposure, underscores the importance of anoxic preconditioning. Secondly, increased antigenic IL-8 production was demonstrated by all noncontrol groups; however, this increase was significantly greater in the anoxia/

hyperoxia group. This response could not be attributed to the effects of TNF or IL-1 $\beta$ , as no differences in the levels of these cytokines were seen between conditions. That the variations seen in IL-8 production represented alterations at the transcriptional level was suggested by Northern blot analysis, and confirmed by nuclear transcriptional assay. Thus, within our system, the greater the oxidant stress, the more profound the response in IL-8 production.

Another interesting finding of our study is the synergy between anoxia-hyperoxia exposure, and concomitant LPS stimulation. Earlier investigators, using animal models, have reported an amplified neutrophil response to LPS stimulation in the setting of prior hyperoxic exposure (26). Moreover, the interaction of LPS and oxidant stress is relevant in such clinical scenarios as gut ischemia-reperfusion injury, and sepsis-induced adult respiratory distress syndrome. We therefore examined the combined effects of LPS stimulation and our previous test conditions. In these experiments, previously significant differences in IL-8 generation among the room air control, anoxia/room air, and room air/hyperoxia groups are lost, with an identical response seen in the presence of LPS. In contrast, in the hyperoxia control and anoxia/hyperoxia groups, LPS stimulation results in a synergistic increase in antigenic IL-8 production. The synergistic response by the anoxia/hyperoxia group cannot be fully accounted for by the effects of hyperoxic exposure alone, since the room air/hyperoxia group failed to respond differently from the room air control. Moreover, this response could not be attributed to the influence of either TNF or IL-1 $\beta$ , as these cytokines were elevated uniformly in all LPS-stimulated groups. Further studies suggest that the increase in antigenic IL-8 levels resulted from an increase in steady-state IL-8 mRNA expression. The expression of steady-state IL-8 mRNA was seen to parallel levels of antigenic IL-8, with a threefold increase in steady-state IL-8 mRNA as compared to room air conditions. In addition, IL-8 mRNA stabilization analysis suggested that hyperoxia-induced expression of monocyte-derived IL-8 occurred at the transcriptional level.

The role of neutrophil chemotaxis in the genesis of oxygen toxicity and hyperoxia-induced tissue injury has been previously appreciated. The enhanced chemotactic activity present in bronchoalveolar lavage specimens from rats exposed to hyperoxia was first identified by Fox (27). Christman and colleagues later reported hyperoxia-induced augmentation of endotoxemia-mediated neutrophil alveolitis in a rat model, and demonstrated increased chemotactic activity of the bronchoalveolar lavage specimens from these animals (26, 28). Furthermore, the presence of neutrophil influx has been associated with ischemia-reperfusion injury. Neutrophil depletion in a canine model of myocardial ischemia attenuates the extent of subsequent reperfusion injury (4), as does interference with leukocyte-endothelial cell adhesiveness and hence PMN trans-endothelial migration (29).

In our studies, anoxic and hyperoxic conditions were imposed sequentially on an in vitro monocyte-based system, representing a simulation of the degree of oxidant stress imposed by ischemia-reperfusion conditions in vivo. Using this model, we were able to first demonstrate that anoxic preconditioning definitely augmented hyperoxia-induced IL-8 production; second, that sequential anoxic/hyperoxic exposure definitely augmented IL-8 production relative to conditions of less pronounced oxidant stress; and third, that LPS stimulation acted

synergistically with sequential anoxic-hyperoxic stress, potentiating IL-8 production. Finally, these effects resulted from increased steady-state levels of IL-8 mRNA. Given the apparent role of neutrophils in mediating reperfusion injury, the evidence for hyperoxic induction of neutrophil chemotactic factors, and the demonstration of anoxia/hyperoxia-induced IL-8, we postulate that cells exposed to an oxidant stress simulating conditions of ischemia-reperfusion can be induced to generate increased levels of IL-8.

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