Vol. 188, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1992 Pages 453-462

Regulation of Manganese Superoxide Dismutase:
IL-1 and TNF Induction in Pulmonary Artery and Microvascular Endothelial Cells

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Received September 4, 1992

Summary IL-1 and TNF are important mediators in the inflammatory response, and have been associated with endothelial cell damage in the lung. TNF and IL-1 cell-mediated injury has been proposed to occur through an increase in intracellular oxygen free radical production. However, these cytokines have also been shown to protect the lung from hyperoxia-mediated oxidant injury. In this paper we evaluated the response of the antioxidant enzymes, MnSOD and Cu/ZnSOD to IL-1, TNF, and LPS in both rat pulmonary artery and microvascular endothelial cells. These mediators produced an increase in MnSOD but not Cu/ZnSOD expression in both rat pulmonary endothelial cells. An additive effect was observed with co-treatment by the cytokines with LPS. The MnSOD mRNA induction is dependent upon a transcriptional event, but did not require de novo protein synthesis.

IL-1 and TNF have been associated with damage to the pulmonary vascular endothelium, resulting in capillary leak with an increase in interstitial cellularity, pulmonary edema, alveolar exudates, and diffuse hemorrhage (1,2). This lung response is similar to that documented following exposure to bacterial endotoxin/lipopolysaccharide (LPS) (1). In fact, many investigators have attributed TNF and in some circumstances IL-1, as mediators of LPS induced injury (1-5). Furthermore, LPS, IL-1, and TNF have been shown to induce oxygen radical production in cell culture systems (6-8), and several studies have implicated oxygen free radicals in the mechanism of LPS, IL-1 and TNF induced cell injury (6,8-10). Normal cellular defense mechanisms which protect against free radical injury begin with the antioxidant enzyme cascade. The superoxide dismutases occupy the crucial first step of this protective function.

Abbreviations: MnSOD, Manganese Superoxide Dismutase; Cu/ZnSOD, Copper Zinc Superoxide Dismutase; LPS, Bacterial Endotoxin/ Lipopolysaccharide; IL-1, Interleukin-1; TNF, Tumor Necrosis Factor; IL-6, Interleukin-6; PAEC, Pulmonary Artery Endothelial Cells; PMVEC, Pulmonary Microvascular Endothelial Cells.

0006-291X/92 $4.00

We have previously demonstrated that LPS produces a selective induction of the mitochondrial-localized manganese superoxide dismutase (MnSOD) but not the cytosolic, copper zinc (Cu/Zn) SOD in rat pulmonary epithelial cells (11) and porcine pulmonary artery endothelial cells (12). Pulmonary epithelial cells demonstrate a time-dependent induction of MnSOD mRNA in response to LPS which requires de novo transcription but not protein synthesis. In fact, blocking protein synthesis with exposure to LPS produces a superinduction of the MnSOD mRNA. In contrast, the induction of MnSOD by LPS in porcine endothelial cells requires both de novo transcription and protein synthesis. We and others have also shown that MnSOD mRNA levels are induced by the cytokines, IL-1 and TNF. To evaluate the mechanisms employed to regulate the MnSOD gene by these inflammatory mediators, we previously isolated and characterized the rat MnSOD genomic locus. With our knowledge of the rat locus, we undertook a detailed examination of MnSOD expression in response to IL-1, and TNF in both rat pulmonary artery endothelial cells (PAEC) and pulmonary microvascular endothelial cells (PMVEC).

METHODS

Tissue culture and cell preparation

Rat pulmonary artery endothelial cells (PAEC) were isolated from segments of pulmonary artery and isolating cells by mechanical methods as described (13). Isolation of rat pulmonary microvascular endothelial cells (PMVEC) was by retrograde perfusion of the lungs with microcarrier beads (14). Cells were grown in M199 with 10 mM L-glutamine, 10% fetal bovine serum (Flow Laboratories, McLean, VA.) and antibiotic solution (ABAM, Sigma Corp., St Louis, MO.) at 37°C.

Stimuli and Inhibitors

E. coli serotype 055:B5 LPS (Sigma L2637), 0.5 μg/ml, 2.0 ng/ml IL-1 (gift from the National Cancer Institute), and 10.0 ng/ml TNF (gift from Genentech, San Francisco, CA) were utilized as stimuli for the induction of MnSOD mRNA levels in rat PMVEC and PAEC. Some cells were also treated with 4 μM actinomycin D (Sigma Corp.), and 25 μM cycloheximide (Sigma Corp.).

RNA Isolation and Northern Analysis

Total RNA was isolated by the acid guandinium thiocyanate-phenol-chloroform extraction described by Chomczynski and Sacchi (15) with modifications (11). Twenty μg of total RNA was fractionated on a formaldehyde/agarose gel, electrotransferred to a noncharged nylon membrane (Genescreen, Du Pont-New England Nuclear) and hybridized for 12-18 h with a radiolabeled rat MnSOD, rat Cu/ZnSOD, or mouse B-actin cDNA (11). The radiolabeled cDNA were products of random primer extension (BRL, Gaithersburg, MD). The membranes were washed under high stringency conditions and subjected to autoradiography at -85°C. The mRNA levels were quantitated by using the Bio Image Visage 60 video densitometry system (Millipore/Bio Image, Ann Arbor, MI), and evaluated in the linear range of the camera and x-ray film.

Protein Isolation and Western Analysis

The cells were washed 3 times with 3 ml of phosphate-buffered saline (pH 7.4), and extracted in 0.5 ml of a buffer containing 20 mM Tris (pH 7.4), 0.25 M sucrose and 5 μg of aprotinin (Sigma Corp.)/ml. The cells were homogenized and the cell lysate was centrifuged at 212,000 x g for 70 min. Ten μg of total protein (as determined by a modified Lowry) was

applied to a SDS/15%-acrylamide gel for separation under reducing conditions (16). The gel was electrotransferred to nitrocellulose, stained with Amido Black, blocked in buffer containing 20 mM Tris base (pH 7.4),0.5 M NaCl, 0.05% Tween (Sigma Corp.) and 3% bovine serum albumin for 3 h, and then transferred to a suspension buffer containing 20 mM Tris base (pH 7.4), 0.5 M NaCl, and 3% bovine serum albumin. Antibody to rat MnSOD (kindly provided by Dr. Ian Burr, Vanderbilt University) was added at 1:1000 dilution and evaluated using the ECL Western Blotting Detection System (Amersham) and autoradiography. Rat MnSOD protein levels were quantified for comparative purposes by video densitometry using the Bio Image Visage system.

RESULTS

Figures 1a and b show Northern analysis of MnSOD mRNA levels in control, LPS, IL-1, and TNF treated PMVEC and PAEC following 8 h of exposure. The 5 transcripts all represent MnSOD mRNA originating by alternative polyadenylation (17). Cu/ZnSOD mRNA levels are also shown with no apparent change under these conditions.

The dose response of IL-1 and TNF in these two cell lines are shown in figures 2a-d. Figures 2a and b illustrate the effects of increasing concentrations of IL-1 at 8 h in both endothelial cell types. Figures 2c and d graphically summarize data from the two cell lines exposed to both IL-1 or TNF, respectively, where values were calculated relative to the B-

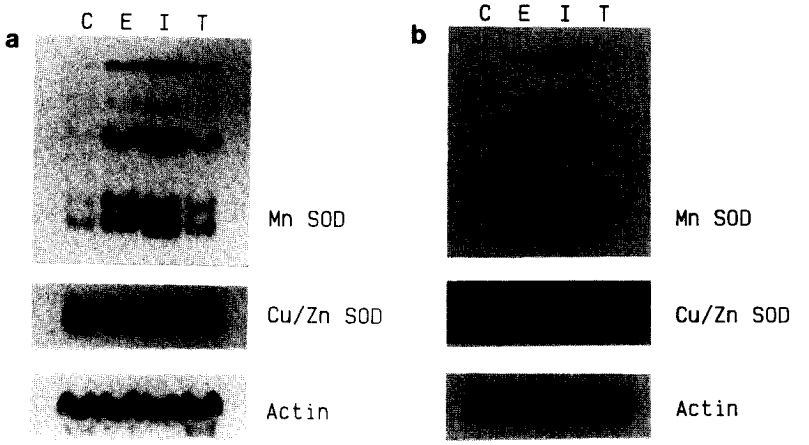

Figure 1 Northern analysis of RNA from PMVEC and PAEC exposed to inflammatory mediators.

a. PMVEC grown to confluency in M199 media with 10% fetal bovine serum at 37°C in room air/5% CO_2 were exposed to 0.5 μg/ml E. coli LPS (E), 2 ng/ml IL-1 (I), and 10 ng/ml TNF (T) for 8 h. C represents control cells which were incubated over the same time period with no exposure to a stimulus. Twenty μg of total RNA was isolated and fractionated by size and transferred to a nylon membrane. The membrane was hybridized with radiolabeled rat MnSOD, rat Cu/Zn SOD, and human ß-actin cDNAs.

b. PAEC grown to confluency in M199 media with 10% fetal bovine serum at 37°C room air/5% CO_2 were exposed to the stimuli and evaluated as described in a.

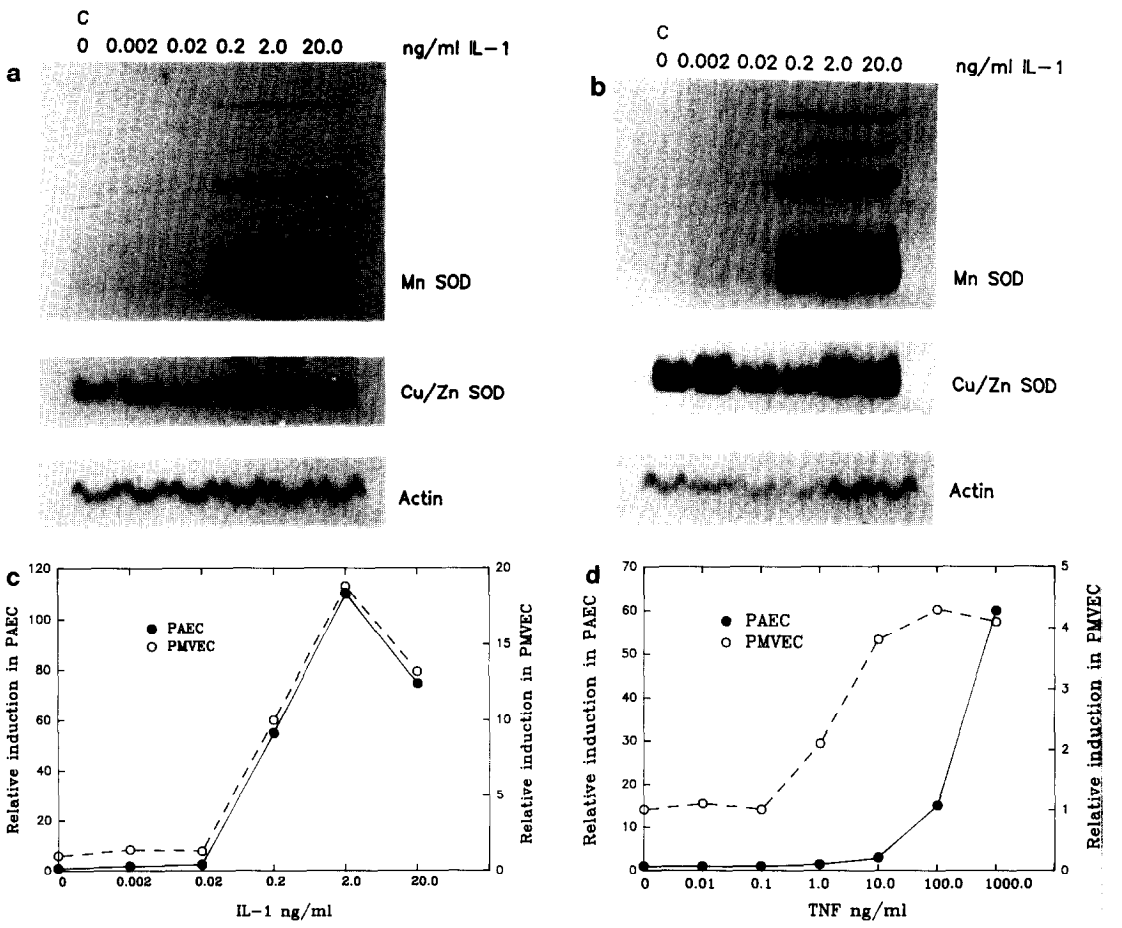

Figure 2 IL-1 and TNF dose response curve.

a. PMVEC were exposed to increasing concentrations of IL-1 for 8 h and evaluated by Northern analysis utilizing radiolabeled rat MnSOD and human ß-actin cDNAs. Lane C illustrates the basal level of expression in PMVEC whereas the remaining lanes correspond to treatment with 0.002, 0.02, 0.2, 2.0, and 20.0 ng/ml IL-1 respectively.

b. PAEC were exposed to increasing concentrations of IL-1 and evaluated as described in a.

c. This graph illustrates the relative induction of MnSOD mRNA levels for the IL-1 concentrations described in a and b based on densitometric analysis of two experiments for each cell type. The values for PAEC are illustrated on the left and PMVEC on the right.

d. This graph illustrates the relative induction of MnSOD mRNA levels for increasing TNF concentrations (0.01 to 1000.0 ng/ml) for both PAEC and PMVEC. The values are based on densitometric analysis of two experiments for each cell type and the PAEC values are illustrated on the left and PMVEC on the right.

actin signal. The maximal induction of MnSOD by IL-1 in both cell lines was first achieved at a concentration of 2.0 ng/ml (figure 2c), and this concentration was then utilized in all subsequent experiments. For PMVEC, TNF produced a saturable induction at 100 ng/ml. In contrast, the level of induction in PAEC was not saturated at 1000 ng/ml, demonstrating a

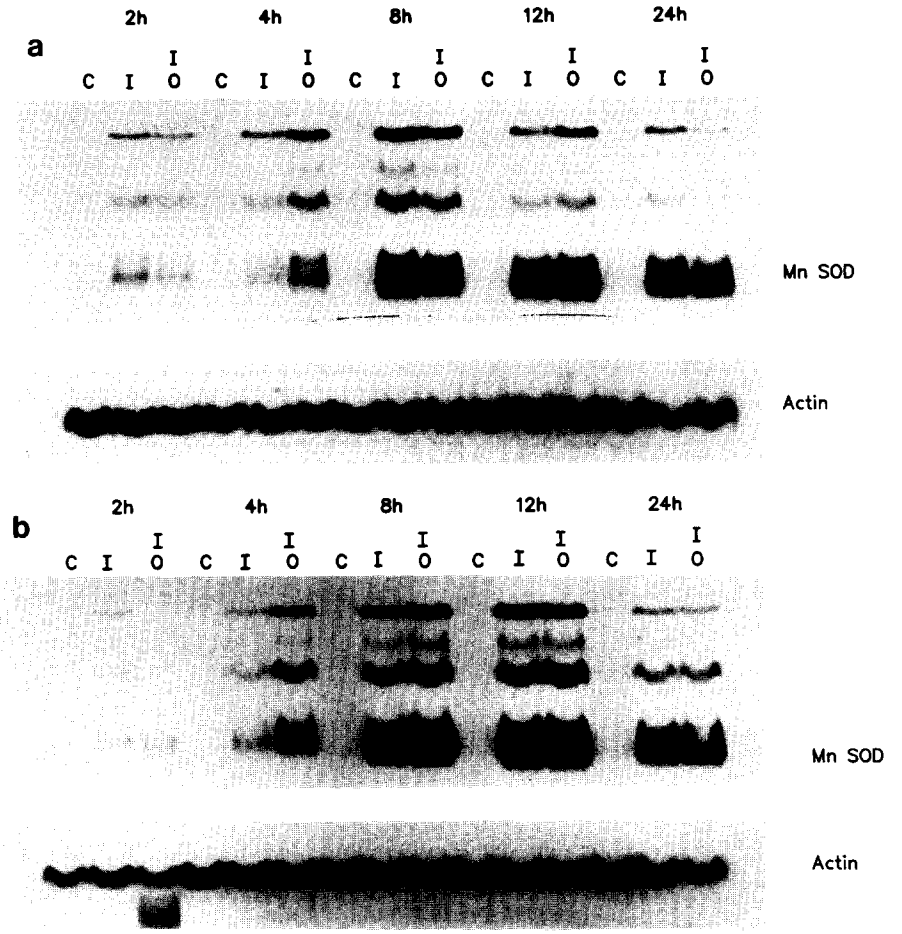

Figure 3 Northern analysis of PMVEC and PAEC in response to IL-1 and 95% oxygen.
a. Total RNA was isolated from PMVEC exposed to room air/5% CO_2 (C), 2 ng/ml IL-1 (I), or IL-1 plus 95% oxygen/5% CO_2 (IO) for 2 to 24 h. MnSOD and ß-actin mRNA levels were evaluated by Northern hybridization to radiolabeled cDNAs.
b. PAEC were treated and evaluated as described in a.

significantly different dose-dependent response to TNF. Due to the lack of physiological relevance of such high concentrations of TNF necessary in the PAEC, we limited our studies on the time dependency of this induction to IL-1.

The Northern analysis in figures 3a and b illustrate the time-dependent exposure of PMVEC (figure 3a) and PAEC (figure 3b) to IL-1 and IL-1 plus 95% oxygen. Figures 4a and b graphically represent the time course of MnSOD mRNA induction by IL-1 in these cells. For both cell types the peak induction occurs at 12 h of cytokine exposure. For comparison, figures 4a and b also summarize data from PMVEC and PAEC following exposure to only 95% oxygen, at the same time points. As these graphs illustrate, MnSOD mRNA levels are unchanged by this hyperoxic challenge, even following a 24 h exposure. A similar time-

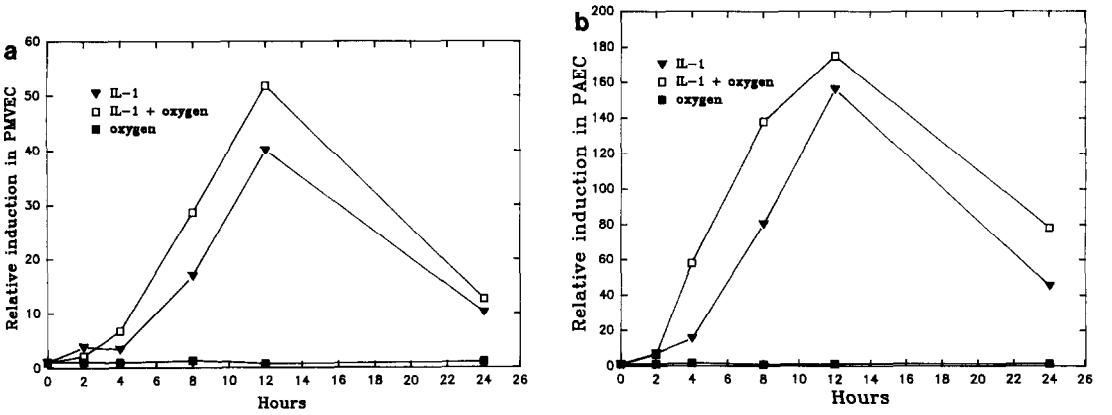

Figure 4 Graphic representation summarizing the densitometric data of two experiments as illustrated in figures 3a and b.

a. This graph summarizes the densitometric data for the relative induction of MnSOD mRNA levels in PMVEC for two experiments as described in 3a and for PMVEC exposed to 95% oxygen/5% CO_2 alone for 2 to 24 h.

b. This graph summarizes the densitometric data for two independent experiments as illustrated in 3b and PAEC exposed to 95% oxygen.

dependent response is obtained when both cell types are exposed to LPS or TNF (data not shown).

To determine if LPS-dependent changes in MnSOD mRNA levels were potentially mediated through an autocrine response due to IL-1 or TNF secretion, we co-treated PMVEC with various stimuli (LPS, IL-1, TNF) alone or in combination (LPS plus IL-1 and LPS plus TNF). The left side of figure 5 illustrates these results. When PMVEC are co-treated with LPS plus IL-1 or LPS plus TNF there is an increase in MnSOD mRNA levels greater than when the stimuli are used alone. This increase is approximately the sum of the individual treatments.

To determine if the induction of MnSOD mRNA by LPS, IL-1, and TNF in rat pulmonary endothelial cells is dependent upon a transcriptional event and/or de novo protein synthesis, cytokine stimulated cells were exposed to either actinomycin D (an RNA synthesis inhibitor) or cycloheximide (a protein synthesis inhibitor) for 8 h. The right hand portion of figure 5 illustrates the results from a Northern analysis of PMVEC.

To further address the physiological significance of the cytokine-dependent induction of MnSOD in pulmonary endothelial cells, we evaluated the relative increase in MnSOD protein levels by Western analysis. As illustrated in figure 6, MnSOD protein levels increased in response to IL-1 beginning at 48 h with a maximal induction of 9 fold after 4 days of exposure. MnSOD mRNA levels were also evaluated in identically treated PMVEC. The mRNA induction was maintained through the next four days.

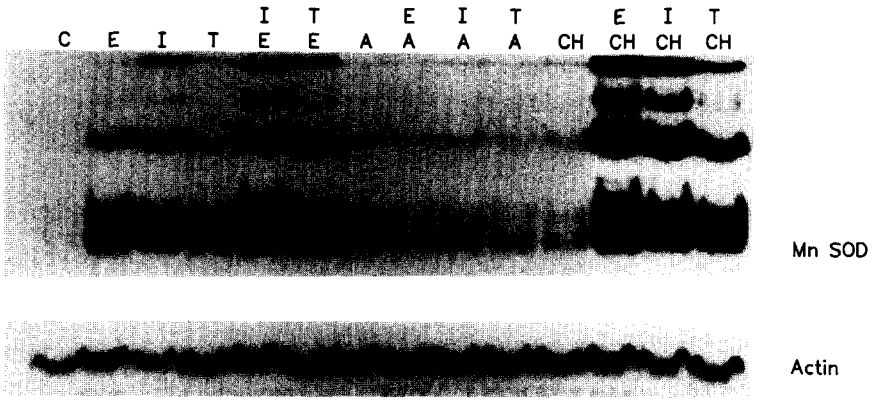

Figure 5 Effect of the mediators in combination, actinomycin D, and cycloheximide on MnSOD mRNA.

Total RNA was isolated from PMVEC under control conditions (C) or exposed to 0.5 μg/ml LPS (E), 2 ng/ml IL-1 (I), 10 ng/ml TNF (T), or in combination of IL-1 plus LPS (IE) or TNF plus LPS (TE). The cells were also treated with 20 μM actinomycin D (A) alone or co-treated with actinomycin D plus each of the stimuli: actinomycin D plus LPS (EA), actinomycin D plus IL-1 (IA), or actinomycin D plus TNF (TA). PMVEC were also exposed to 25 μM cycloheximide (CH) alone or in combination with each of the stimuli: E CH, I CH, and T CH. The exposure time for each treatment was 8 h, and the PMVEC were evaluated by Northern analysis utilizing radiolabeled MnSOD and ß-actin cDNAs.

DISCUSSION

In the lung, LPS, IL-1, and TNF have been shown to cause a significant pulmonary vascular endothelial injury (1,2,18). TNF and IL-1 have been shown to increase neutrophil adherence to endothelial cells and enhance the susceptibility of vascular endothelial cells to neutrophil-mediated killing (19). Increases in oxygen free radicals have been observed in response to IL-1 and TNF, and several studies have implicated these free radicals as

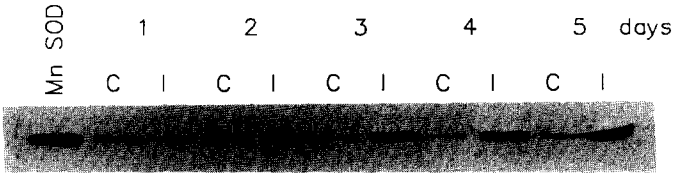

Figure 6 Western Analysis of PMVEC exposed to IL-1

PMVEC were exposed to 2 ng/ml IL-1 for 1 to 5 days and compared to control cells maintained under usual conditions for the same time periods. Ten μg of total protein was separated by size on a 15% PAGE/SDS gel and evaluated by using a rabbit derived rat MnSOD antibody. The first lane illustrates rat MnSOD protein followed by proteins isolated from control cells (C) and cells exposed to IL-1 (I) for 1 to 5 days.

mediators of TNF, IL-1, and LPS induced cell injury (6-10). Although these inflammatory mediators produce significant pulmonary toxicity, when given in sublethal doses they have been shown to be protective against oxygen toxicity. Rats pretreated with LPS, TNF or IL-1 had a higher survival rate when exposed to a hyperoxic insult (20-22).

A proposed mechanism for this protection is the induction of the antioxidant enzymes as a cellular defense mechanism against the increase in oxygen free radicals generated by high oxygen tensions. We and others have demonstrated that LPS, IL-1, and TNF produce a selective induction of MnSOD but not Cu/ZnSOD mRNA and protein levels. Furthermore, we have also demonstrated that the induction of MnSOD by these inflammatory mediators is tissue and cell specific, in that MnSOD mRNA levels in rat hepatocytes are regulated primarily by the acute phase cytokine, IL-6 (23). Conversely, IL-6 does not cause a similar induction in other cells.

To further define the cell-specific role of these inflammatory mediators on Mn SOD expression we present studies which evaluate the effects of LPS, IL-1, and TNF in pulmonary endothelial cells derived from large and small pulmonary vessels. It has been shown that endothelial cells derived from various organs or even different regions from the same organ are often structurally and functionally different (24). These studies in endothelial cells derived from the rat were also necessary because they provide vital information to properly design experiments on the molecular mechanisms governing cytokine-dependent MnSOD gene expression.

An initial observation was the higher basal MnSOD expression in PMVEC versus PAEC. This difference may be a consequence of the higher incidence of exposure in vivo of the PMVEC to oxidant and inflammatory stress. In addition, we have observed large differences in the level of cytokine-dependent induction of MnSOD expression between PMVEC and PAEC, with the largest relative induction in PAEC. Another difference was apparent when examining the dose response of each endothelial cell to TNF. PMVEC show a maximal induction of approximately 4 fold at a TNF concentration of 100 ng/ml, whereas the TNF induction in PAEC rises monotonically with a 15 fold increase at 100 ng/ml and over 60 fold at 1000 ng/ml. These quantitative differences in the dose response to TNF and IL-1 are most likely a function of receptor population differences in the two cells. This may be a consequence of either inherent sensitivity to the cytokines or possibly a function of the cell culture system.

Since LPS, IL-1 and TNF each induce MnSOD mRNA levels in pulmonary endothelial cells, we wanted to address the interrelationship between these three inflammatory mediators. The overlapping biological activities of IL-1 and TNF are often attributed to the production of one of the mediators by the other (1,25,26). For example, in endothelial cells TNF has been

shown to induce IL-1 production (18). Furthermore, LPS is also known to induce both TNF and IL-1 (17,27,28). In regard to this potential interrelatedness, we observe an increase which is approximately the sum of the individual stimuli when the stimuli are used in combination, indicating the possibility of an additive effect. This suggests that LPS and the cytokines are acting independently of each other in producing an induction of MnSOD mRNA. In addition, we show that cycloheximide does not inhibit the LPS, IL-1, or TNF dependent induction but may actually potentiate the effects of each inflammatory mediator. Together our cycloheximide and actinomycin D data illustrate that de novo protein synthesis is not required, whereas the induction of MnSOD mRNA expression by LPS, IL-1, or TNF requires a transcriptional event.

Our current hypothesis argues that LPS, IL-1, and TNF may be functioning through independent intracellular pathways. Our results also strongly support the argument that MnSOD is an unique intracellular acute phase reactant which can be activated by the full repertoire of acute phase stimuli, including LPS, IL-1, and TNF. An alternative explanation for the similarity of MnSOD responsiveness to LPS, IL-1, and TNF may stem from the activation of a common intracellular signal. For example NF-κB, a transacting DNA binding protein, is thought to be activated by a variety of stimuli including LPS, IL-1, and TNF (29,30). Recently, Schreck et. al. have indicated that reactive oxygen intermediates, specifically hydrogen peroxide, may be messengers in the activation of NF-κB (31). With the knowledge derived from these studies on rat pulmonary endothelial cells and the availability of a rat genomic clone, we are presently testing these hypotheses using promoter deletion analysis and in vivo footprinting.

ACKNOWLEDGMENTS

We would like to thank Pat Austin for assistance in preparing the manuscript. This work was aided by National Institutes of Health Grants HL39593 to HSN and HL21568, HL44204 to USR. Dr. Gary Visner was supported by a National Research Service Award (HL07820) and by a grant from the American Lung Association of Florida.

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