Extracellular superoxide dismutase is upregulated with inducible nitric oxide synthase after NF-κB activation

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Brady, Todd C., Ling-Yi Chang, Brian J. Day, and James D. Crapo. Extracellular superoxide dismutase is upregulated with inducible nitric oxide synthase after NF-κB activation. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1002-L1006, 1997.—Inflammatory cytokines have been shown to upregulate secretion of the antioxidant enzyme extracellular superoxide dismutase (EC-SOD) in dermal fibroblasts and, in other cells, to stimulate production of nitric oxide (·NO). Because superoxide rapidly scavenges ·NO, forming the injurious peroxynitrite anion (OONO−), we hypothesize that stimulated cells upregulate EC-SOD expression concurrently with ·NO release. To test for coregulation of EC-SOD and ·NO within the same cell, the timing of inducible nitric oxide synthase (iNOS) and EC-SOD transcription was measured after exposure of a rat type II pneumocyte analog, the L2 cell line, to a combination of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Upregulation of iNOS and EC-SOD transcription occurred after 6 h of exposure, and transcription of both genes was linked by activation of the transcription factor nuclear factor-κB. Both EC-SOD and iNOS were elevated in rat lung homogenates 24 h after intratracheal instillation with IFN-γ and TNF-α. The observation that EC-SOD and iNOS are temporally coregulated after cytokine exposure suggests the possibility of a critical mechanism by which cells might protect ·NO and avoid the formation of OONO− during inflammation.

interferon-γ; tumor necrosis factor-α; alveolus; lung; nuclear factor-κB

EXTRACELLULAR SUPEROXIDE DISMUTASE (EC-SOD) is a secreted antioxidant enzyme especially prevalent in lungs (12) and functions to protect cells and connective tissue from extracellular superoxide (O2·−). The dismutation of O2·− is important not only because O2·− itself is a damaging radical but also because O2·− can rapidly react with nitric oxide (·NO) to form the peroxynitrite anion (OONO−; see Refs. 19 and 20)

\[
\cdot NO + O_2 \cdot \rightarrow OONO^- 
\]

At physiological pH, OONO− decomposes into intermediates with reactivities similar to the hydroxyl radical (HO·) and NO2 (2)

\[
OONO^- + H^+ \rightarrow \cdot NO_2 + HO\cdot 
\]

Thus, by scavenging O2·−, EC-SOD may protect ·NO outside the cell. Because EC-SOD binds to the cell membrane and proteoglycans in the extracellular matrix (8), the enzyme may reduce the likelihood of extracellular OONO−, HO·, and ·NO2 formation near plasma membranes or connective tissue during ·NO release.

In environments in which increased amounts of O2·− and ·NO are produced during inflammation, such as in the alveolus, the role of EC-SOD in protecting ·NO bioavailability and preventing OONO− formation may be critical. Under basal conditions, in situ hybridization studies have demonstrated the presence of EC-SOD mRNA in a secretory alveolar epithelial cell known as the type II cell (23). Although transcription of EC-SOD after cytokine treatment has not been characterized in any cell, exposure to combinations of the inflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) has been shown to increase EC-SOD secretion in dermal fibroblasts (13). After treatment with other combinations of cytokines in different scenarios, type II pneumocytes in culture express inducible nitric oxide synthase (iNOS; see Ref. 18), an ·NO-producing enzyme synthesized in response to environmental stimuli. Because O2·− rapidly scavenges ·NO, forming OONO− and other reactive species, we hypothesize that ·NO-secreting cells such as type II pneumocytes upregulate EC-SOD expression before or concurrently with ·NO release. To test for coregulation of EC-SOD and ·NO within the same cell, the timing of EC-SOD transcription was compared with that of iNOS in a rat type II pneumocyte analog, the L2 cell line, after treatment with IFN-γ and TNF-α. Because no functional transcription factors for EC-SOD have been demonstrated, we examined the relationship between EC-SOD transcription and the activation of nuclear factor-κB (NF-κB), a transcription factor known to upregulate iNOS transcription (17, 26), to link the initiation of EC-SOD and iNOS transcription. Temporal changes in media EC-SOD and nitrite levels were also compared after cytokine treatment.

Other studies have demonstrated the presence of EC-SOD in lungs under basal conditions (12) and iNOS in stimulated lungs (7, 10). To test for concurrent upregulation of EC-SOD and iNOS expression in lung, IFN-γ and TNF-α were intratracheally instilled in rats, and protein levels were measured in lung homogenates. We present evidence characterizing the timing and NF-κB-mediated control of stimulated EC-SOD transcription and thereby establish the temporal relationship between EC-SOD and ·NO production within the same cell and animal models of inflammation.

EXPERIMENTAL PROCEDURES

Cell and animal exposure L2 cells were purchased from the American Type Culture Collection (CCL-149; Rockville, MD) and were grown in Ham’s F-12-K (Kaighn’s modification) media (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GIBCO), penicillin (10 U/ml), and...
streptomycin (10 mg/ml; Gibco). Cells were grown in 5% CO2 at 37°C and were used between the 40th and 45th passages. For transcription analysis, ~10^6 cells were plated in 2 ml of media and were grown to confluence in individual 35-mm dishes (Falcon, Lincoln Park, NJ). At confluence, media was replaced with 1 ml of F-12-K containing 1% FBS. Twenty-four hours after confluence was attained, cytokine exposures were initiated. Murine IFN-γ (2,000 U/ml; Genzyme, Cambridge, MA) and TNF-α (500 U/ml; a gift from Dr. Grace Huang, Gentech), both of which have been shown to stimulate rat cells (4, 14, 24), were added to 1 ml of 1% FBS media either with or without 100 µM pyrrolidine dithiocarbamate (PDT; Sigma, St. Louis, MO; see Refs. 1 and 9). Various times (3, 6, 12, and 18 h) after cytokine exposure, medium was removed and was stored at ~70°C until needed for nitrite determinations or EC-SOD Western blots. Cells were then lysed for RNA extraction. Eight male Sprague-Dawley rats (350 g; Charles River Laboratories, Wilmington, MA) were intratracheally instilled with 500 µl of 0.9% NaCl, either with or without 100,000 U/ml IFN-γ and 25,000 U/ml TNF-α. Twenty-four hours after instillation, rats were killed with 100 mg/kg phenobarbital sodium, a thoracotomy was performed, and the middle lobe of the right lung was excised.

RNA isolation and reverse transcriptase-polymerase chain reaction. Pure RNA was isolated by phenol extraction from a guanidium thiocyanate lysis solution (Bioline, Houston, TX). Immediately after cell lysis, the guanidium solution was frozen and was stored at ~70°C. After RNA isolation, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using recombinant T7 polymerase with magnesium and manganese acetate buffers (Perkin-Elmer, Foster City, CA). Novel primers were created for rat EC-SOD (5’-TAG CCT AGC TGC TGC GCG CAT AT; 3’-GGG CGC ACA GAG GCG ATT GA) and rat iNOS (5’-AGC ACA TGC AGA AGT AGT ACC; 3’-TGA TGC TCC CGG ACC AGG GA), RT-PCR for both EC-SOD and iNOS tubes were run simultaneously under the same thermocycling protocol: 30 min at 60°C × 1 cycle; 3 min at 94°C × 1 cycle; 20 s at 94°C followed by 30 s at 70°C × 40 cycles; and 7 min at 70°C × 1 cycle. RT-PCR samples were run in 3% agarose and were stained with ethidium bromide. Both iNOS and EC-SOD primers produced unique bands of 64 and 70 base pairs, respectively. Control RT-PCR reactions for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were run separately using novel primers (5’; GGT GTC AAC GGA TTT GGC CGT ATT; 3’; CAT GCC AGT GAG CTT CCC GTT CA), yielding a unique band of 674 base pairs.

Western blotting. Thawed media were spun at 5,000 g for 30 min at 4°C in Centricon-30 concentrators (Amicon, Beverly, MA) to yield a protein concentration of ~125 mg/ml. Lung tissue was homogenized in a mechanical homogenizer in ice-cold lysis solution [50 mM tris(hydroxymethyl)amino methane-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotonin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM NaVO3, and 1 mM NaF]. Lung homogenate was centrifuged at 14,000 revolutions/min for 10 min at 4°C, and the supernatant was stored at ~70°C. Approximately 2,500 mg of cell media or 50 µg of lung homogenate supernatant protein were loaded on a 12% polyacrylamide gel in 2-amino-2-methyl-1,3-propanediol buffers and electrophoresed on a Mini Gel apparatus (Hoefer, San Francisco, CA). Western blotting for EC-SOD was performed using a polyclonal rabbit antibody against a murine EC-SOD peptide previously sequenced (3). Blotting for iNOS was performed using a polyclonal rabbit antibody against murine iNOS (Transduction Laboratories, Lexington, KY). Chemiluminescent detection was performed using a horseradish peroxidase-conjugated secondary antibody (ECL System, Amersham, Buckinghamshire, UK).

Nitrite determinations. For nitrite determinations, growth conditions were the same as those described above except that 35-mm six-well plates (Falcon) were used instead of individual dishes. At different times after exposure, nitrite in the media of L2 cells stimulated with IFN-γ and TNF-α was detected using the Griess reagent and sodium nitrite as the standard, which has been described elsewhere (6). Nitrite determinations for each condition were performed in triplicate. Cytokine-treated wells were compared with control (nonexposed) wells at various times (12, 18, 24, and 30 h) after cytokine exposure.

Statistics. Multiple-way analyses of variance (ANOVAs) followed by one-degree-of-freedom contrasts of means (α = 0.05) were used to compare nitrite concentrations of cytokine-treated wells and control wells at all times after exposure. The means of nitrite concentrations in control wells were contrasted across time via ANOVA followed by Duncan’s multiple range test.

RESULTS

Nitrite and EC-SOD in L2 cell media are increased after cytokine exposure. L2 cells were exposed to a combination of IFN-γ and TNF-α in an attempt to compare the timing of EC-SOD and NO production within the same cell. Nitrite, which is a stable product of NO and generally correlates with the release of NO, was detected via the Griess reagent method (6) in the media of control cells and cytokine-exposed cells. At all times after exposure, the media of wells exposed to cytokines had greater amounts of nitrite than control wells (Fig. 1). These differences were statistically significant by 24 and 30 h after exposure. Nitrite did not increase significantly in control wells over time. West-
ern blot analysis of the media from exposed cells demonstrated EC-SOD in the media at 24 and 36 h after the addition of IFN-γ and TNF-α. Greater EC-SOD labeling was evident at 36 h. Media from cells not exposed to cytokines did not label for EC-SOD at incubation times up to 24 h (data not shown). Although rat and mouse EC-SOD protein are ~80% homologous (3, 25), lack of basal EC-SOD labeling in the media of control cells and at times before 24 h postexposure may be related to the use of an antibody against murine EC-SOD to detect rat protein.

iNOS and EC-SOD transcription are upregulated in L2 cells after cytokine exposure. After exposure to IFN-γ and TNF-α, L2 cell transcription of iNOS and EC-SOD mRNA was studied by RT-PCR. No iNOS mRNA was detected before cytokine exposure, but iNOS signal was evident by 6 h after cytokines were added (Fig. 2). Basal levels of EC-SOD mRNA in L2 cells were present before cytokine treatment, consistent with previous in situ studies with type II pneumocytes (23). After exposure to IFN-γ and TNF-α, EC-SOD signal increased, indicating transcriptional upregulation from basal levels. Upregulation of EC-SOD signal continued through 18 h after exposure to cytokines. No differences were observed between GAPDH mRNA amplified from control and exposed L2 cells at all times after exposure (data not shown). These studies showed that IFN-γ and TNF-α initiated iNOS transcription and upregulated EC-SOD transcription in L2 cells by 6 h after exposure, confirming the increases in media levels of nitrite and EC-SOD observed after cytokine treatment. The data suggest that upregulation of EC-SOD transcription may occur slightly before that of iNOS.

iNOS and EC-SOD transcription are regulated by NF-κB in cytokine-exposed L2 cells. iNOS transcription is initiated by activation of the transcription factor NF-κB (17, 26). The metal chelator and antioxidant PDTC has been used to inhibit NF-κB activation but does not inhibit activity of other transcription factors, such as adenosine 3',5'-cyclic monophosphate response element binding protein, activator protein-1, octamer-1, and Sp1 (21). PDTC treatment prevents cytokine-mediated induction of iNOS in a variety of cells (1, 9). As expected, addition of PDTC with cytokines to L2 cells blocked IFN-γ- and TNF-α-induced iNOS transcription (data not shown). PDTC and cytokine treatment resulted in EC-SOD transcription only at basal levels (Fig. 3). Because PDTC is an inhibitor of NF-κB activation, these data suggest that the upregulation of EC-SOD transcription and the initiation of iNOS transcription in response to IFN-γ and TNF-α are both controlled by NF-κB in L2 cells. Such regulation further supports the coexpression of EC-SOD and iNOS in this model.

iNOS and EC-SOD levels are increased in rat lung after cytokine treatment. Expression of iNOS and EC-SOD protein was studied in rat lung 24 h after intratracheal instillation of IFN-γ and TNF-α and was compared with saline-instilled rats (n = 4/condition). Western blotting indicated the presence of iNOS only in cytokine-instilled lungs (Fig. 4). EC-SOD expression was higher in the exposed lungs compared with saline-instilled lungs. These data are consistent with the increase in EC-SOD and iNOS expression observed in cytokine-treated L2 cells.

**DISCUSSION**

This report presents data demonstrating the control of EC-SOD transcription by cytokines and the temporal
correlation of iNOS and EC-SOD expression after cytokine exposure within the same cell and animal models. RT-PCR, Western blotting, and nitrite analysis of IFN-γ- and TNF-α-stimulated L2 cells suggest that, during inflammation, when IFN-γ and TNF-α are likely to be present, EC-SOD transcription and secretion are increased above basal levels concurrently with, or perhaps slightly before, upregulation of iNOS transcription and ·NO release. Induction of EC-SOD secretion and ·NO release is linked by activation of NF-κB, which simultaneously upregulates transcription of EC-SOD and iNOS. The whole lung data indicate that IFN-γ and TNF-α instillation increases both iNOS and EC-SOD expression in rat lung and are consistent with the L2 cell results. We suggest that upregulation of iNOS and EC-SOD expression occurs in the same vicinities within the lung and at approximately the same time. Further characterization of the relationship between EC-SOD and iNOS expression in response to either IFN-γ or TNF-α alone or to other cytokines present in different biological scenarios, both in cell culture and in situ, merits study.

Numerous transcription factors regulating iNOS have been characterized and include NF-κB, TNF responsive element, and IFN responsive element (17). NF-κB has been shown to be necessary for iNOS transcription in various cells (1, 9). Our results with L2 cells are consistent with these studies, since PDTC, an inhibitor of NF-κB activation, prevented transcription of iNOS. No study has previously demonstrated a functional transcription factor regulating expression of EC-SOD. This report demonstrates that PDTC also prevents upregulation of EC-SOD transcription but does not inhibit basal EC-SOD transcription, implicating activation of NF-κB in the induction of EC-SOD expression. The result indicates a potential mechanism for the observed cytokine-mediated coregulation of EC-SOD and iNOS expression, suggesting that IFN-γ and TNF-α increase EC-SOD and iNOS transcription in L2 cells via activation of NF-κB. Activated NF-κB may act directly by stimulating EC-SOD transcription or indirectly by influencing different factors that, in turn, lead to upregulation of EC-SOD. Although other transcription factors may influence EC-SOD and iNOS in L2 cells, activation of NF-κB appears to be necessary for transcription of iNOS and for increased EC-SOD transcription after exposure to IFN-γ and TNF-α. Because normal levels of EC-SOD transcription were evident during PDTC treatment, it is unlikely that PDTC prevented iNOS transcription or EC-SOD upregulation by a nonspecific response to generalized cell injury. PDTC inhibits NF-κB activation but does not affect the function of other transcription factors (21). Nonetheless, the possibility exists that the compound may diminish upregulation of EC-SOD transcription by means not associated with NF-κB activation, including acting as an antioxidant, chelating metals, or affecting other transcription factors yet to be characterized.

In the stimulated L2 cell and whole lung models, the finding that EC-SOD and iNOS upregulation are temporally linked after activation of NF-κB suggests that major amounts of ·NO do not cross the plasma membrane before EC-SOD secretion. If EC-SOD upregulation occurred late in the inflammatory response after significant ·NO release in the presence of extracellular O2–, then the cell might suffer damage from O2•− as well as from radicals derived from OONO−. Because EC-SOD binds to cell membranes and the extracellular matrix (8), released ·NO may be protected from O2– only within these areas. Further experiments will elucidate the effects of ·NO release in inflammatory environments and verify the necessity of protecting ·NO and preventing OONO− formation near cells and associated connective tissue during inflammation. ·NO may act on targets within the zone protected by EC-SOD to exert muscle relaxation (16), act as a signaling molecule (5), or modulate immune responses of nearby inflammatory cells to prevent host injury (15, 22). Alternatively, ·NO may diffuse from the cell and associated extracellular matrix (e.g., into the alveolar space) to react with O2–, forming OONO− and other injurious compounds, perhaps targeting invading organisms (11). Thus correlation of EC-SOD and iNOS may be a critical cellular response during inflammation. By synchronizing the expression of these enzymes, the cell may protect itself and the surrounding matrix from extracellular O2–, OONO−, HO•, and -NO2 while increasing the efficacy of secreted ·NO as a functional effector outside the plasma membrane.

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