Tumor Necrosis Factor-α Selectively Induces MnSOD Expression via Mitochondria-to-Nucleus Signaling, whereas Interleukin-1β Utilizes an Alternative Pathway*

Richard J. Rogers‡§, Joan M. Monnier¶, and Harry S. Nick¶¶

From the Departments of ‡Anesthesiology, §Biochemistry and Molecular Biology, and ¶Neuroscience, University of Florida College of Medicine, Gainesville, Florida 32610

Mitochondrial levels of the anti-oxidant enzyme, manganese superoxide dismutase (MnSOD), are dramatically elevated in response to stimulation with tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and lipopolysaccharide (LPS). However, the precise intracellular signaling pathways responsible for this inducible expression are poorly understood. MnSOD expression in pulmonary epithelial and endothelial cells, treated with inflammatory mediators and various inhibitors, was studied by Northern analysis. The mitochondrial electron transport chain inhibitors, antimycin A and myxothiazol, selectively blocked TNF-α-inducible expression of MnSOD but not that of IL-1β or LPS, indicating different signaling pathways. N-Acetylcysteine could reliably reduce inducible MnSOD expression by TNF-α, but not IL-1β, linking reactive oxygen species (ROS) to the TNF-α signaling pathway. Elevated levels of arachidonic acid have been demonstrated previously to generate mitochondrial ROS. A specific cytoplasmic phospholipase A2 inhibitor reduced stimulated MnSOD expression by TNF-α, but not by IL-1β, further supporting the role of ROS. Other investigators have shown that MnSOD expression may be regulated by NF-κB. Our results with a specific inhibitory kinase inhibitor indicate that NF-κB modulates IL-1β signaling but not the TNF-α pathway. Thus, we have demonstrated that although inducible MnSOD transcription may appear similar at the messenger RNA level, the intracellular signaling pathways are differentially regulated.

Manganese superoxide dismutase (MnSOD), a vital antioxidant enzyme localized to the mitochondrial matrix, catalyzes the dismutation of superoxide anions (O₂⁻) to hydrogen peroxide (H₂O₂). In aerobic cells, the mitochondrial electron transport chain is probably the most abundant source of O₂⁻ at atmospheric oxygen concentrations, it is estimated that between 1 and 3% of the O₂ reduced in the mitochondrial electron transport chain during ATP production may form O₂⁻ (1–3). Although O₂⁻ and other ROS are by-products of normal respiratory, imbalance or loss of cellular homeostasis results in oxidative stress, causing damage to cellular components (lipid membranes, proteins, and nucleic acids) (4, 5). MnSOD acts as the first line of cellular defense to detoxify these O₂⁻ (6). Various inflammatory mediators (TNF-α, IL-1 β, IL-6, and LPS) in multiple tissues have been demonstrated to elicit dramatic elevations of both the messenger RNA and protein levels of MnSOD (7–12). The increased levels of MnSOD have been shown to be cytoprotective (13–16). However, the signaling pathways responsible for MnSOD expression are numerous and are still far from being fully elucidated.

Elaborate intercommunications take place between the nucleus and mitochondria coordinating not only mitochondrial gene expression and genome maintenance but also nuclear gene expression (17). The classic view has been that mitochondria simply function as organelles responding to changes in energy demand. However, recent data would suggest a more complex picture where mitochondria also function as active signaling organelles in a number of important intracellular pathways (18–21).

TNF-α binding to membrane receptors triggers complex signal transduction cascades (22–24), some of which result in excess ROS production in the mitochondria (25, 26). The cytotoxic effect of these ROS is either direct or necessary for downstream signaling events leading to cell death. The crucial toxic role of ROS was demonstrated by the inhibition of mitochondrial electron transport at specific sites, which differentially interferes with TNF-α-mediated cytotoxicity (25) and by the correlation between sensitivity to TNF-α cytotoxicity and mitochondrial activity in the cell (26). Pharmacological experiments revealed that the mitochondrial respiratory chain is the major source of TNF-α-induced ROS (25–27). Antioxidants inhibit various actions of TNF-α (transcription factor activation, gene expression, and cytotoxicity), and exogenously added ROS mimic its biological action (28–30). Our data would agree with the literature regarding ROS and TNF-α.

In this paper, we show that inhibition of mitochondrial electron transport results in the loss of TNF-α-stimulated MnSOD expression, most likely due to the loss of mitochondria-to-nucleus signaling with ROS acting as the second messenger in the signal transduction pathway. In addition, we demonstrate that although inflammatory mediators (TNF-α, IL-1β, and LPS) may elicit similar inducible mRNA levels of MnSOD, the signaling pathways leading to this expression are very different.

EXPERIMENTAL PROCEDURES

MATERIALS—Recombinant human TNF-α (a gift from Genentech), IL-1β (a gift from NCI, National Institutes of Health), antimycin A, N-acetylcysteine, AACOCF₃, SB 203580, PD 98059 (Calbiochem), IκBα-mediated cytotoxicity (25) and by the correlation between sensitivity to TNF-α cytotoxicity and mitochondrial activity in the cell (26). Pharmacological experiments revealed that the mitochondrial respiratory chain is the major source of TNF-α-induced ROS (25–27). Antioxidants inhibit various actions of TNF-α (transcription factor activation, gene expression, and cytotoxicity), and exogenously added ROS mimic its biological action (28–30). Our data would agree with the literature regarding ROS and TNF-α.

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kinase inhibitor, Bay 11-7082 (BioMol, Plymouth Meeting, PA), bacterial lipopolysaccharide (LPS), amobarbital (Amytal), 2-heptyl-4-hydroxyquinolone $N$-oxide, oligomycin, and myxothiazol (Sigma) were used.

Cell Culture—L2 cells, a rat pulmonary epithelial-like line (ATCC CCL 149), were grown in Ham’s F12K media (Life Technologies, Inc.) with 10% fetal bovine serum (Flow Laboratories, McLean, VA), 10 mM L-glutamine, and antibiotic/antimycotic solution (ABAM, Sigma) at 37°C in air, 5% $CO_2$. VA cells, a rat pulmonary artery endothelial cell line, isolated from segments of pulmonary artery by mechanical methods (11), were grown in Medium 199 with Earle’s salts (Sigma) with sodium bicarbonate to pH 7.4, 10% fetal bovine serum, 10 mM L-glutamine, and antibiotic/antimycotic solution at 37°C in air, 5% $CO_2$. L9 cells, a mouse fibroblast antimycin-resistant mutant cell line (generously provided by Dr. Neil Howell, see Ref. 31), were grown in Ham’s F12K media (Life Technologies, Inc.) with 10% fetal bovine serum (Flow Laboratories, McLean, VA), 10 mM L-glutamine, and antibiotic/antimycotic solution (ABAM, Sigma) at 37°C in air, 5% $CO_2$. When the cells were 70–90% confluent they were exposed to inflammatory mediators (10 ng/ml TNF-$a$, 2 ng/ml IL-1, or 0.5 μg/ml LPS) and/or inhibitors. At 8 h after exposure, total RNA was isolated and evaluated by Northern analysis as described below.

RNA Isolation and Northern Analysis—Total RNA was isolated by the acid guanidium thiocyanate/phenol/chloroform extraction method described by Chomczynski and Sacchi (32) with modifications (7). Twenty micrograms of total RNA was size-fractionated on a 1% agarose gel (33) and electrotransferred to a charged nylon membrane (Zetabind, Cuno Laboratory Products, Cuno Inc., Meriden, CT) and UV covalently cross-linked. The membrane was hybridized with $^{32}$P-labeled rat manganese superoxide dismutase or rat cytochrome b (as an RNA loading control) cDNAs and subjected to autoradiography. All autoradiographs depicted in the figures throughout this paper are representative of at least three independent experiments.

Measurement of Intracellular Generation of ROS—Flow cytometric analysis of intracellular generation of ROS was performed using dihydrorhodamine 123 as a probe (34, 35). Cells were cultured in 6-well plates, and at confluence ($1 \times 10^5$ cells/well) they were treated with TNF-$a$ (10 ng/ml), antymycin A (4 μM), or a combination of TNF-$a$/antimycin A. After 8 h of incubation, dihydrorhodamine 123 (5 mM) was added, and the incubation was prolonged for an additional 30 min. The cells were harvested, washed, centrifuged for 5 min at 800 rpm, resuspended in phenol red-free M199 medium, and analyzed by flow cytometry (excitation, 488 nm; emission 530 nm).

Statistical Analysis—All results are expressed as means ± S.D. unless stated otherwise. The unpaired Student’s t test was used to evaluate the significance of differences between groups, accepting $p < 0.05$ as the level of significance.

RESULTS

Mitochondrial Electron Transport Inhibitors Modulate TNF-$a$-induced Expression of MnSOD in Pulmonary Epithelial Cells—Previous analysis of the effects of mitochondrial electron transport chain inhibitors showed that, depending on the site of action of the inhibitor, the cytotoxicity of TNF-$a$ was either increased or decreased (25). A schematic of the mitochondrial respiratory chain is shown in Fig. 1A. Based on earlier experiments demonstrating stimulated expression of MnSOD mRNA following treatment of a rat pulmonary epithelial-like cell line (L2 cells) with inflammatory mediators (7, 9), we initiated studies to evaluate the effect of TNF-$a$ on MnSOD expression in cells also treated with mitochondrial respiratory chain inhibitors. Fig. 1, B—D, illustrates the effects of treatment of L2 cells with antimycin A or amobarbital and/or TNF-$a$. The control samples exhibit a low constitutive level of expression of MnSOD mRNA. Addition of antimycin A or amobarbital at increasing concentrations (25) did not affect this constitutive MnSOD mRNA expression. Maximal induction of MnSOD mRNA levels with TNF-$a$ occurs after 8 h, and thus this time point was selected for isolation of RNA for Northern analysis. Cotreatment with TNF-$a$ and antimycin A simultaneously caused a marked decrease in MnSOD mRNA expression at both concentrations of antimycin A (Fig. 1, B and C) (25). Amobarbital, which blocks electron transfer through complex I (Fig. 1A), had minimal effect on TNF-$a$-induced expres-

Fig. 1. A, simplified scheme of the respiratory chain showing sites of substrate entry, inhibitor action, and potential sites of superoxide anion formation. Cyt, cytochrome; UQ, ubiquinone; Fe$–$S, iron-sulfur center. B, Northern analysis of RNA from pulmonary epithelial cells exposed to TNF-$a$ and/or mitochondrial inhibitors for 8 h. Inhibition of TNF-$a$-stimulated induction of MnSOD by amobarbital (50 and 400 μM) and antimycin A (12.5 and 50 μM) in rat pulmonary epithelial cells (L2 cells) either untreated or treated with mitochondrial inhibitors alone or in combination with TNF-$a$ (10 ng/ml) or with TNF-$a$ alone is shown. Control lanes 2 and 3 contain 0.1 and 0.5% ethanol, which were used as the solvent for all inhibitors not soluble in water. All figures are scanned images of autoradiographs and not from a PhosphorImager. C, densitometric analysis of autoradiographs ($n = 3$) demonstrating fold changes of TNF-inducible MnSOD levels in L2 cells relative to control and in response to inhibitors, antimycin A or amobarbital. All quantitation of Northern analysis (relative to cathepsin B as a loading control) within this paper was performed using ScionImage software and a Microtek ScanMaker 9600XL scanner with transparency adapter.
sion of MnSOD mRNA compared with antimycin A (Fig. 1, B and D). Microscopic examination at 8 h showed no apparent difference in the cellular viability between control and treated cells.

Antimycin A Strongly Decreases TNF-α-inducible MnSOD Expression in Pulmonary Endothelial Cells—With the results shown in Fig. 1B, we proceeded to evaluate the cellular specificity of the mitochondrial inhibitor, antimycin A, studying its effects on another cell type, the rat pulmonary artery endothelial cell line (VA cells, see Ref. 11). By observing that the two concentrations of antimycin A used in the pulmonary epithelial cell experiments (Fig. 1B) gave similar results in the inhibition of TNF-α-stimulated MnSOD mRNA levels and based on that fact that much lower concentrations of antimycin A are effective at inhibiting mitochondrial electron transport, we tested concentrations from 0.5 to 20 µm to find the optimum inhibitory concentration in the VA cells. Fig. 2, A and C, illustrates the effects of increasing concentrations of antimycin A on MnSOD mRNA levels. The maximal inhibition was achieved at a relatively low concentration of 4 µm. By using this concentration of antimycin A, we tested the effect on TNF-α-stimulated expression over 24 h. The maximal inhibition appears to occur between 8 and 12 h when both TNF-α and antimycin A were added simultaneously (data not shown). Furthermore, the sequence of addition of TNF-α or antimycin A to the cells was important for the TNF-α-stimulated expression of MnSOD mRNA. If TNF-α was added as little as 15 min prior to antimycin A, the diminution of the inducible expression was dramatically reduced (data not shown). Other investigators (34) have found that pretreatment of L929 cells with mitochondrial inhibitors resulted in a significant decrease in binding of TNF to cell surface receptors. Thus, all the cell treatments in our experiments were done simultaneously.

Effects of Other Mitochondrial Respiratory Chain Inhibitors on TNF-α-stimulated Expression of MnSOD—Treatment of both pulmonary epithelial and endothelial cells with the complex III inhibitor, antimycin A, showed dramatic inhibition of TNF-α induction of MnSOD mRNA. With this in mind, we decided to test whether another complex III inhibitor would give similar results. The inhibitors, antimycin A and myxothiazol, both block electron transport at the cytochrome b-c₁ segment of the mitochondrial respiratory chain but at different binding sites (37, 38). Fig. 2, B and D, shows the effects of increasing concentrations of myxothiazol on TNF-α-stimulated expression of MnSOD mRNA. The extent of inhibition by myxothiazol is very similar to the pattern observed for antimycin A, which might be expected considering the proximity of the binding of antimycin and myxothiazol in the cytochrome b-c₁ crystal structure (38). Interestingly, the inhibition of gene expression by these electron transport inhibitors is exquisitely specific in that 2-heptyl-4-hydroxyquinoline N-oxide, which also inhibits complex III but at a different site, does not alter TNF induction of MnSOD (data not shown). These results would suggest that inhibition of mitochondrial electron transport at complex III alters production of ROS that can act in retrograde communication with the nucleus.

The Signaling Pathway for TNF-α Is Different from the Pathways for LPS- or IL-1-stimulated Expression of MnSOD—We have shown previously that both lipopolysaccharide (LPS) and interleukin-1 (IL-1) also induce expression of MnSOD in both pulmonary epithelial and endothelial cells (7, 9). Maximal induction occurs at 8–12 h similar to TNF-α. To evaluate whether signaling pathways for all three inflammatory mediators were similar when mitochondrial respiration is inhibited with antimycin A, we examined the effect that increasing concentrations of antimycin A had on the LPS- and IL-1-stimulated expression of MnSOD. Inhibition of TNF-α-stimulated induction of MnSOD by antimycin A in rat pulmonary endothelial cells (VA cells) either untreated or treated with increasing concentrations of antimycin A alone or in combination with TNF-α (10 ng/ml) or with TNF-α alone for 8 h is shown. B, Northern analysis of RNA from pulmonary artery endothelial cells exposed to TNF-α and myxothiazol. Inhibition of TNF-α-stimulated induction of MnSOD in rat pulmonary endothelial cells (VA cells) either untreated or treated with increasing concentrations of myxothiazol alone or in combination with TNF-α (10 ng/ml) or TNF-α alone is shown. C and D, densitometric analysis of autoradiographs (n = 3) demonstrating fold changes of TNF-inducible MnSOD levels in VA cells relative to control and in response to inhibitors, antimycin A (C) or myxothiazol (D).
TNF-α-stimulated Expression of MnSOD Is Unaffected by Antimycin A in a Resistant Fibroblast Cell Line—To demonstrate that the effects of antimycin A are directly associated with mitochondrial inhibition and not due to other possible side effects, we obtained an antimycin-resistant mouse fibroblast mutant cell line, LA9 (31). In this mutant, the rate of respiration is normal, but electron transport through the succinate-cytochrome c oxidoreductase segment of the mitochondrial respiratory chain, which includes cytochrome b, shows resistance to inhibition by antimycin A. Fig. 3, B and E, illustrates the effect of TNF-α on the expression of MnSOD in the mutant LA9 cells. We should point out that we previously demonstrated that the five separate MnSOD mRNA transcripts in the rat result from alternative polyadenylation (39); however, there are only two murine MnSOD transcripts at 1 and 4 kilobase pairs. At concentrations of antimycin A varying from 0.5 to 20 μM, TNF-α continued to induce MnSOD mRNA levels demonstrating the specificity of the antimycin action in VA cells.

Inhibition of Mitochondrial ATPase with Oligomycin Also Represses TNF-α-stimulated Expression of MnSOD—Oligomycin, which inhibits F$_{1}$F$_{0}$-ATPase, causes uncoupling of mitochondrial respiratory electron transport and ATPase activity. Previous work by other investigators (40) has demonstrated that cells treated with TNF-α results in an increase in oligomycin-sensitive mitochondrial respiration, with the resultant increase in ROS. However, cells treated with both TNF-α and oligomycin resulted in decreased levels of cellular ATP as well as blockade of the increase in ROS generation (40). To evaluate whether oligomycin treatment of VA cells would inhibit TNF-α induction of MnSOD, we treated VA cells with increasing concentrations of oligomycin in the presence and absence of TNF-α. The results shown in Fig. 4, A and B, would indicate that oligomycin inhibits TNF-α-inducible MnSOD expression and that this inhibition may be due to the decreased mitochondrial ATP and/or ROS levels.

Reactive Oxygen Species Are Important for TNF-α-stimulated Expression of MnSOD—Since levels of ROS have been shown previously to be increased in cells undergoing oxidative stress
We made use of the antioxidant, N-acetylcysteine (NAC), to evaluate whether ROS scavenging can modulate TNF-α-inducible expression of MnSOD. NAC caused a dose-dependent decrease in the TNF-α-stimulated expression of MnSOD to baseline levels with no detectable effect on cell viability (Fig. 5, A and C). However, NAC did not cause any change in the IL-1β-inducible expression of MnSOD (Fig. 5, B and D), further evidence that the signaling pathways are different for TNF and IL-1. Of note, the concentrations of NAC capable of decreasing MnSOD expression are far below the millimolar levels used in much of the literature, demonstrating the potential importance of ROS in TNF-α-mediated signal transduction and the sensitivity of ROS in retrograde mitochondria-to-nucleus communication.

TNF-α-stimulated Expression of MnSOD in Endothelial Cells Is Dependent on Cytoplasmic Phospholipase A2 (cPLA2)—Several studies (43–46) have demonstrated the connection between arachidonic acid and mitochondrial ROS production. To explore whether inhibition of cPLA2 and thus mitochondrial ROS affect MnSOD expression, we utilized the potent and selective cPLA2 inhibitor, arachidonitrifluoromethyl ketone (AACOCF3) (44). This inhibitor caused a dose-dependent repression of MnSOD expression in TNF-α-stimulated endothelial cells (Fig. 6, A and C), whereas AACOCF3 had no effect on IL-1β-stimulated cells (Fig. 6, B and D).

TNF-α-inducible Expression of MnSOD Is Not Dependent on NF-κB—Other investigators (45) have shown that ROS activation by diverse conditions is important for gene activation by NF-κB. To investigate whether NF-κB is important in TNF-α signaling of MnSOD, we utilized the IκB inhibitor, BAY 11-7082 (47). At increasing concentrations of the IκB inhibitor, MnSOD expression in IL-1β-stimulated endothelial cells could be reduced to baseline (Fig. 7, B and E), with no effect on TNF-α-stimulated cells (Fig. 7, A and D). These data suggest that NF-κB is activated in the IL-1β signaling pathway but not in the TNF-α signaling pathway of MnSOD. Other kinase signaling pathways were also investigated by using specific...
inhibitors. The mitogen-activated protein (MAP) kinases are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus. Two of the MAP kinase pathways that have been implicated in TNF-α and IL-1β signal transduction are JNK/SAPK and p38. The c-Jun kinase/stress-activated protein kinase cascade is activated following exposure to UV radiation, heat shock, or inflammatory cytokines. The p38 kinase (reactivating kinase) is the newest member of the MAP kinase family. It is activated in response to inflammatory cytokines, endotoxins, and osmotic stress. Selective inhibitors of the MAPKs (MKK1/2 (PD 98059) (48) and p38 (SB 203580) (49) were utilized at increasing concentrations to treat endothelial cells with or without TNF-α. No differences were seen between inducible expression of MnSOD with either of the inhibitors (representative example, Fig. 7, C and F) suggesting that TNF-α signal transduction for MnSOD likely does not occur through these MAP kinase pathways.

ROS Production from Mitochondria in Response to TNF and/or Antimycin A—Intracellular generation of ROS by cultured VA cells in response to TNF-α treatment was measured by flow cytometry using the fluorescent probe, dihydrorhodamine 123. As shown in Fig. 8, treatment of cells with 10 ng/ml TNF-α for 8 h resulted in significantly higher rhodamine 123 fluorescence, indicating increased ROS generation. Of note, cells treated with 4 μM antimycin A did demonstrate increased fluorescence compared with control cells. However, cells treated simultaneously with TNF and antimycin A showed no higher fluorescence than cells treated with antimycin A alone. Cells treated with IL-1β did not have any more fluorescence than control cells.

DISCUSSION

Manganese superoxide dismutase plays an important role in the cellular defense against superoxide produced by the mitochondrial electron transport chain during normal cellular metabolism (13–16). Reduction or deficiency of MnSOD has been shown to promote cytotoxicity under conditions of oxidant stress (50, 51). A number of laboratories, including our own (51–53), have begun to understand the workings of the promoter and the intronic enhancer in causing the dramatic inducible expression of MnSOD. However, the molecular intracellular signaling pathways and the nature of the induction of MnSOD by various inflammatory mediators are still being unraveled.

Retrograde communication from the mitochondria to the nucleus likely consists of metabolic signals and transduction pathways that function across the inner mitochondrial membrane. Since ROS are very short lived molecules closely regulated by a coordinated enzyme system, they could be potential signal transducers of putative mitochondria-to-nucleus signaling pathways. Production of ROS in the mitochondria is related to changes in electron flux through the respiratory chain, brought about by various physiological conditions such as heat shock (55), variations in oxygen tension (56), and exposure to nitric oxide (57). ROS have been found to act as second messengers in cellular functions such as cell growth and differentiation (58, 59). Mitochondrial respiration has been linked to the expression of the mammalian gene, GLUT1 (60). Expression of the GLUT1 gene, one of the isoforms of the glucose transporter, is enhanced by hypoxia and by exposing cells to inhibitors of mitochondrial respiration (60). Therefore, we postulated that inhibition of mitochondrial respiration might also regulate MnSOD expression possibly through a mechanism involving intracellular levels of ROS.

Our data show that mitochondrial respiratory chain inhibitors, antimycin A (Fig. 1, B and C, and Fig. 2, A and C) and
myxothiazol (Fig. 2, B and D), as well as the F$_{1}$F$_{0}$-ATPase inhibitor, oligomycin (Fig. 4, A and B), can repress TNF-α-inducible expression of nuclear-encoded MnSOD. We have also addressed the specificity of the antimycin effects with studies in antimycin A-resistant LA9 mutant cells stimulated with TNF-α (Fig. 3, B and D). Northern analysis utilizing the antioxidant, NAC, would suggest that TNF-α-mediated pathways required intracellular ROS to function (Fig. 5, A and C), whereas the IL-1β pathway did not (Fig. 5, B and D). In addition, the rhodamine 123 fluorescence data (Fig. 8) further implicate ROS, ROS by-products, and/or lipid peroxides as likely candidates for signal transduction from the mitochondria to the nucleus in the TNF-α signal pathway. In addition, other investigators (45, 46) have shown that submicromolar concentrations of arachidonic acid cause a substantial increase in ROS production in mitochondria. The data in Fig. 6, A and C, would suggest that decreased levels of arachidonic acid, occurring as a result of selective blockade of cPLA$_{2}$ enzyme, are sufficient to inhibit the TNF-α-inducible expression of MnSOD. Thus oxidative events generated in the mitochondrion, not simply inhibition of energy-coupled processes, are crucial in TNF-α-induced MnSOD gene expression.

ROS or other mitochondrial intermediates may control both the cytotoxic and gene-regulatory effects of TNF-α, thus pro-

FIG. 7. A, Northern analysis of RNA from pulmonary artery endothelial cells exposed to TNF-α and/or IκK inhibitor (Bay 11-7082) for 8 h. Inhibition of TNF-α-stimulated induction of MnSOD in pulmonary artery endothelial cells (VA cells) either untreated or treated with increasing concentrations of IκK inhibitor alone or in combination with TNF-α (10 ng/ml) or TNF-α alone is shown. B, Northern analysis of RNA from pulmonary artery endothelial cells exposed to IL-1β and/or IκK inhibitor (Bay 11-7082). Inhibition of IL-1β-stimulated induction of MnSOD in pulmonary artery endothelial cells (VA cells) either untreated or treated with increasing concentrations of IκK inhibitor alone or in combination with IL-1β (2 ng/ml) or IL-1β alone is shown. C, Northern analysis of RNA from pulmonary artery endothelial cells exposed to TNF-α and/or MAP kinase inhibitor (PD 98059). Levels of TNF-α-stimulated induction of MnSOD in pulmonary artery endothelial cells (VA cells) either untreated or treated with increasing concentrations of the MAP kinase inhibitor (PD 98059) alone or in combination with TNF-α or TNF-α alone are shown. Also, when the MAP kinase inhibitor, SB 203580, was used in similar experiments, no alteration in TNF-α-inducible pattern of MnSOD was observed, almost identical to the above experiment with PD 98059. D–F, densitometric analysis of autoradiographs (n = 3) demonstrating fold changes of TNF-α-inducible (D and E) or IL-1-inducible (E) MnSOD levels in VA cells relative to control and in response to IκK inhibitor (D and E) or PD 98059 (F).
FIG. 9. Effect of TNF-α on intracellular generation of ROS. Cellular generation of ROS was determined by flow cytometry using rhodamine 123 fluorescence. Cells were incubated in the absence (control) or presence of 4 µM antimony A or 10 ng/ml TNF-α or 10 ng/ml TNF-α plus 4 µM antimony A or 2 ng/ml IL-1β. Results are means ± S.D. of four independent experiments. *, p < 0.01 and; **, p < 0.001 between control and experimental treatments.

FIG. 8. Model of TNF-α and IL-1β signal transduction pathways involved in inducible MnSOD expression. Mitochondrial inhibitors (antimony A and myxothiazol) and F$_{1}$F$_{0}$-ATPase inhibitor (oligomycin) are shown to negatively affect mitochondrial production of ROS, which are involved in the TNF-α signaling pathway but not the IL-1β pathway. The cPLA$_{2}$ inhibitor (AAOCPC) diminishes production of arachidonic acid (AA), which increases mitochondrial production of ROS to produce potential lipid peroxide species. IkB kinase inhibitor (Bay 11-7082) inhibits phosphorylation of the IkB subunit thus preventing separation of the IkB subunit from nuclear factor kB (NF-kB) and blocking it from translocating into the nucleus.

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Thus, our data would suggest that multiple signaling pathways result in stimulated expression of MnSOD. Clearly, inhibition of mitochondrial electron transport alters inducible expression of MnSOD by TNF-α but not IL-1β or LPS. Thus, it would appear that the TNF-α signaling pathway requires retrograde communication from the mitochondria to the nucleus, probably involving intracellular ROS. However, due to the high reactivity of ROS and their production within the mitochondrial membrane, it is much more likely that the cytoplasmic signaling molecule of the TNF-α signaling pathway may be a protein acted upon by a lipid peroxide or other ROS or even the lipid peroxide itself. Our data with the cPLA$_{2}$ inhibitor would seem to bolster this argument in the TNF-α pathway. Other investigators (46) have shown that TNF-α-induced ROS production requires cPLA$_{2}$ and 5-lipoxygenase activity but not cyclooxygenase activity in the Rac signal transduction cascade. This would suggest that ROS generation is dependent on synthesis of arachidonic acid and its subsequent metabolism to leukotrienes. In fact, exogenously applied leukotriene B$_{4}$ could increase mitochondrial ROS (46). A model of our proposed MnSOD signaling pathways is shown in Fig. 9, detailing the differences between the TNF-α and IL-1β pathways. It may be that a yet unidentified signal pathway is activated by intracel-
