0026-895X/96/020277-08$3.00/0

MOLECULAR PHARMACOLOGY, 50:277-284 (1996).

Characterization of Hepatic Nitric Oxide Synthase: Identification as the Cytokine-Inducible Form Primarily Regulated by Oxidants

DAWN L. DUVAL, DANETTE R. MILLER, JOHN COLLIER, and RUTH E. BILLINGS

Department of Environmental Health, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado 80523

Received September 29, 1995; Accepted April 15, 1996

SUMMARY

Induction of hepatic nitric oxide synthase (NOS) by tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), interferon-γ (IFNγ), interleukin-6 (IL-6), and lipopolysaccharide was assessed as activity and immunoreactive protein. Hepatic NOS activity was cytosolic and had cofactor requirements consistent with inducible nitric oxide synthase (NOS2). NOS induction by TNFα was dose dependent from concentrations of 0.06 to 60 nM and was increased 2–3-fold by IFNγ. NOS induction was reflective of total TNFα binding to hepatocyte receptors. Hepatocyte TNFα binding fit a biphasic curve with high affinity (K_d = 1.4 nM, B_{max} = 3157 sites) and low affinity (K_d = 157 nM, B_{max} = 204,948 sites) elements. NOS2 activity was induced by lipopolysaccharide, IL-1β, TNFα, and IFNγ but not by IL-6. All cytokine stimuli were inhibited by antioxidants. Oxygen radical generation was directly measured as dichlorofluoroscein fluorescence in isolated mitochondria. Mitochondria from TNFα-treated hepatocytes generated more oxygen radicals than did controls. Antioxidants reduced mitochondrial generation of oxygen radicals. Activation of the transcription factor nuclear factor-κB by TNFα, IFNγ, and IL-1β was assessed by gel shift analysis. Cytokine treatment increased nuclear factor-κB binding, and the addition of antioxidants or rotenone inhibited cytokine activation. Taken together, these data suggest that oxygen radicals, possibly generated by mitochondria, play a major role in NOS2 induction by cytokines.

NO is a highly reactive nitrogen radical with a plethora of actions, including vasodilation, neurotransmission, and cytotoxicity. NO is synthesized by three NOS isoforms. These isoforms, NOS1, NOS2, and NOS3, are the products of three different genes described originally as neuronal, inducible, and endothelial NOS based on their initial detection and cloning (1). NOS1 and NOS3 are constitutively expressed. NOS2 is induced in response to cytokines, LPS, and other stimuli (2–4) in a variety of cells, including keratinocytes, hepatocytes, endothelial cells, myocardial cells, and leukocytes (5). Although these enzymes are primarily cytosolic, the NOS3 isoform can be myristylated and localized in the membranes of endothelial cells (6). All NOS isoforms catalyze the conversion of L-arginine to citrulline and NO in a reaction requiring NADPH and O_2 in stoichiometric quantities. NOS activity is also dependent on a number of cofactors, including FAD, FMN, H_4B, and glutathione (7). In addition, calmodulin regulates NOS activity by mediating electron transfer to the heme group. Although NOS1 and NOS3 are tightly regulated by calcium-dependent calmodulin binding, NOS2 activity is not regulated in this manner because of a highly hydrophobic region that binds calmodulin even in the presence of the calcium-chelating agent EGTA (8). Alterations in the availability of these substrates and cofactors may contribute to NOS regulation. For example, in endothelial cells and macrophages, the levels of H_4B are regulated by stimulatory and inhibitory cytokines (9, 10). Although the liver has high endogenous levels of H_4B, which make it an unlikely regulator of hepatic NOS activity (11), efficient measurements of liver NOS activity required the addition of urea cycle inhibitors and substrates (12), suggesting that substrate level changes attributed to the urea cycle may affect NOS activity. Alternatively, glutathione is also necessary for maximum NOS activity, and its effect has been attributed to stabilization of the NOS enzyme (13). Because recent studies have shown

This work was supported by National Institutes of Health Grant DK44755. D.L.D. was supported by a predoctoral fellowship provided by the Pharmaceutical Manufacturers Association.

ABBREVIATIONS: NO, nitric oxide; NOS, nitric oxide synthase; NOS2, inducible nitric oxide synthase; NOS3, endothelial nitric oxide synthase; NOS1, neuronal nitric oxide synthase; IL-1β, interleukin-1β; TNFα, tumor necrosis factor-α; IFN, interferon; IL-6, interleukin-6; LPS, lipopolysaccharide; H_4B, tetrahydrobiopterin; DCF, 5- (and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; GDH, glutamate dehydrogenase; PBS, phosphate-buffered saline; NF-κB, nuclear factor-κB; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DTT, dithiothreitol; ADU, arbitrary densitometry units.

that NO causes feedback inhibition of NOS by reacting with the heme group (14), glutathione may help prevent this reaction by reacting with free NO to form nitrosothiols.

Mechanisms that control gene transcription and translation may also be critical determinants in the induction of NOS. Previous studies in our laboratory have shown that oxygen radicals and glutathione regulate the gene expression of NOS2 in cultured rat hepatocytes stimulated with TNFα (15). Because TNFα also acts on murine hepatocyte cultures to simulate the production of reactive oxygen intermediates (16), oxygen radicals may serve as a common mechanism by which this cytokine stimulates the induction of NOS. This seems especially likely because the promoter region of inducible murine macrophage NOS contains elements that bind activator protein-1 and NF-κB (17, 18). These transcription factors are activated in response to oxidative stress (17, 18). Furthermore, NOS2 induction has been linked to activation of NF-κB in macrophages, both directly and by the ability of dithiocarbamates to inhibit NOS induction (19, 20). Nevertheless, the regulatory region of the *NOS2* gene also contains consensus elements for activation by interferons and LPS (17, 18), so oxidants might play a lesser role when these agents are involved in NOS induction.

In the current experiments, rat hepatocyte NOS activity in homogenates has been characterized with respect to cofactor dependence and subcellular localization because in previous studies, these aspects of hepatic NOS activity have not been examined. Most importantly, regulation of hepatocyte NOS activity by multiple cytokines and LPS has been studied to extend the previous observation that TNFα induction of NOS activity in hepatocytes is regulated by oxidants (15). This is significant because in the intact animal, multiple cytokines are always present when immune stimulation occurs. The cytokines used in the current study (TNFα, IL-1, IL-6, and IFNγ) are the major inflammatory cytokines to which the liver is exposed and are associated with NOS2 induction (2, 21). To explore this, we used a mixture of antioxidants because these chemicals have been previously described to diminish the oxidative effects of TNFα in cultured mouse and rat hepatocytes (15, 16).

Materials and Methods

Reagents. Recombinant human TNFα (specific activity, 6.27×10^4 units/μg) was supplied by Genentech (South San Francisco, CA). ^{125}I-labeled recombinant human TNFα (40 μCi/μg) was purchased from Dupont-New England Nuclear (Boston, MA). Recombinant rat IFN-γ was purchased from GIBCO BRL (Gaithersburg, MD). Recombinant murine IL-1β was purchased from R&D Systems (Minneapolis, MN). DCF was purchased from Molecular Probes (Eugene, OR). H_4B was purchased from Schircks Laboratories (Jona, Switzerland). [^{3}H]Arginine (40–70 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). The monoclonal antibody to amino acids 961-1144 of NOS2 and the antibodies to NOS1 and NOS3 isoforms were purchased from Transduction Laboratories (Lexington, KY). Oligonucleotides used in the EMSAs were purchased from Promega (Madison, WI). All other chemicals and reagents, unless otherwise specified, were purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Animal treatment. Adult male Sprague-Dawley rats (250–400 g) were injected intraperitoneally with 10 mg/kg LPS (*Escherichia coli*, serotype 055:B5, prepared by phenol extraction; Sigma) dissolved in PBS. After 8 hr, hepatocytes were isolated by a standard collagenase perfusion method (22) and purified by differential centrifugation to give cultures of ≥90% viability and ≥95% purity. Subcellular fractions were prepared by differential centrifugation for assessment of the cellular localization of hepatic NOS.

Cell culture. Isolated hepatocytes were plated on rat tail collagen-coated culture plates (Corning, Palo Alto, CA) in Waymouth's media supplemented with serine, alanine, asparagine, penicillin, and streptomycin at a density of 7×10^5 cells/cm^2. Cells were incubated in a humidified atmosphere of 95% air/5% CO_2 at 37° for 2–3 hr to allow adherence. Nonadherent cells were removed by washing the plates twice with PBS, and cultures were treated in fresh media. Cell viability was assessed by lactate dehydrogenase leakage in cell culture. None of the treatments significantly increased lactate dehydrogenase leakage compared with control values, which were typically <20% after 24 hr.

NOS activity. Nitrite levels in media were determined colorimetrically on a Molecular Devices Thermomax plate reader (Menlo Park, CA). Total nitrite and nitrate were measured by reducing the nitrate to nitrite with NADPH:nitrate reductase, followed by reaction with Griess reagent according to a modification of previously described techniques (23). NOS activity was measured in homogenized hepatocytes using two methods: conversion of [^{3}H]arginine to [^{3}H]citrulline and production of nitrite/nitrate. Cell samples were scraped into 50 mM Tris·HCl buffer, pH 7.4, with 1 mM DTT and protease inhibitors. The cells were homogenized by forcing cells through a 21-gauge needle several times and were frozen at −80° until assay. In both assays, incubation components were 50 mM Tris·HCl, pH 7.4, 2 mM $CaCl_2$, 200 μM L-arginine, 200 μM NADPH, 50 mM valine, 1 mM citrulline, 5 mM GSH, and 10 μM H_4B. After incubation at 37°, the reactions were stopped by the addition of buffer (citrulline assay) or by heating at 100° for 2 min (nitrite/nitrate analysis). [^{3}H]Citrulline was separated from arginine by ion exchange chromatography and quantified by liquid scintillation counting (24).

Binding of TNFα to rat hepatocytes. The binding kinetics of human recombinant TNFα to isolated rat hepatocytes was measured by determining the specific binding of ^{125}I-labeled TNFα to isolated rat hepatocytes. Hepatocytes were isolated and cultured on collagen-coated plates as described in Cell culture. After adherence, hepatocyte cultures were placed in a humidified atmosphere of 95% air/5% CO_2 at 4° and treated with 0.17 pmol/ml ^{125}I-TNFα and varying concentrations of unlabeled TNFα. Nonspecific binding was defined as the amount of radiolabeled TNFα bound in the presence of 1 μM unlabeled TNFα. Hepatocytes were incubated for 4 hr at 4° to allow binding to reach equilibrium, washed four times with ice-cold PBS, and solubilized in 1 N sodium hydroxide. The data were analyzed using Radioligand (Biosoft, Cambridge, UK).

Western blot analysis of inducible NOS. Treated hepatocytes were washed and pelleted in PBS. Pellets were resuspended in 10 mM Tris·HCl, pH 7.4, and denatured by boiling for 5 min. Protein was measured using a micro-bicinchoninic acid kit (Pierce, Rockford, IL), and equal amounts of total protein were electrophoresed under reducing conditions (5% 2-mercaptoethanol) on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose paper. Blots were blocked in 1% bovine serum albumin dissolved in 10 mM Tris·HCl, pH 7.5, 100 mM sodium chloride, and 0.1% Tween-20 (wash buffer) at 4° overnight and then incubated at room temperature with 0.5 μg/ml anti-NOS2 in blocking buffer for 2 hr. The blots were washed and incubated with the F(ab')$_2$ fragment of sheep anti-mouse IgG conjugated to peroxidase in 5% nonfat milk in wash buffer for 2 hr. After washing, the blots were developed with enhanced chemiluminescence reagent and exposed to Xomat-AR film. Bands were quantified using an Imaging Densitometer (BioRad, Hercules, CA). The specificity of this antibody to the 130-kDa NOS2 was verified by running macrophage cell lysate as a standard for NOS2. Total protein from control hepatocyte cultures was also assessed for NOS1 and NOS3 by Western blot analysis. Blots were prepared as previously described but were blocked in 2.5% nonfat milk overnight at 4° before incubation for 2 hr with 1 μg/ml concentration of either anti-NOS1 or NOS3

antibody in 2.5% nonfat milk. The blots were washed and incubated with the F(ab')$_2$ fragment of sheep anti-mouse IgG conjugated to peroxidase in 5% nonfat milk in wash buffer for 2 hr before washing and development as previously described. These monoclonal antibodies are certified by Transduction Laboratories for cross-reactivity with NOS1 and NOS3 isoforms in the rat. In addition, a purchased standard of endothelial cell extract or total protein prepared from rat cerebellum was used as standards for NOS3 (140 kDa) and NOS1 (155 kDa), respectively.

Mitochondrial oxygen radical generation. Hepatocytes were harvested after 3 hr of treatment with TNFα (1 μg/ml) by trypsin digestion. Cells were pelleted and treated with 0.3 mg/ml digitonin for 2 min. Permeabilized hepatocyte suspensions were centrifuged at 13,000 × *g* for 5 min through a layer of *N*-dibutylphthalate to separate mitochondria from other cellular components. Mitochondrial pellets were resuspended and centrifuged for 2 min at 50 × *g* to remove contaminating plasma membrane. Mitochondria were incubated for 10 min at room temperature with 10 μM DCF and then pelleted and resuspended in 250 mM mannitol in PBS. Fluorescence was measured over time using a plate-reading fluorometer (Millipore, Bedford, MA) with an excitation filter of 485 nm and an emission filter of 530 nm. Antioxidants (2.5 mM Trolox, an α-tocopherol analog; 250 μM ascorbic acid; and 40 mM benzoic acid) were added to the resuspended mitochondria before fluorescence reading. Fluorescence was normalized by the specific activity of GDH, a mitochondrial marker, in each sample.

EMSAs. Nuclear extracts from hepatocyte cultures were prepared by hypotonic lysis in a buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 500 μM phenylmethylsulfonyl fluoride, 50 μM sodium vanadate, and 2 μg/ml concentrations of leupeptin, aprotonin, and pepstatin. On lysis, 0.6% Nonidet-40 was added, and samples were centrifuged for 30 min at 13,000 × *g* at 4° to remove RNA and cytosol. The pellets were resuspended in a buffer containing 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, and 1 mM EGTA and centrifuged for 4 min at 13,000 × *g* at 4° to remove cellular debris. Supernatants were collected and dialyzed before use in EMSAs. EMSAs were performed according to the method of Schreck et al. (25). Hepatocyte nuclear extracts (5 μg) were combined with a reaction buffer containing 0.4 μg/μl poly(dI/dC), 10 mM Tris·HCl, pH 7.5, 1 μg/μl bovine serum albumin, 20 mM NaCl, 1 mM EDTA, 5% glycerol, 0.2 mM DTT, and a ^{32}P-labeled oligonucleotide of the consensus sequence for the NF-κB binding element (0.333 pmol). Binding reactions were incubated at 25° for 20 min, at which point the reactions were terminated by the addition of loading dye. Samples were loaded on a 4% polyacrylamide gel that had been prerun at 150 V for 30 min and electrophoresed for 2 hr at 200 V at 2° in 1× buffer (6.8 mM Tris, 3.4 mM sodium acetate, and 1 mM EDTA, pH 7.5). The bandshift was visualized by autoradiography and quantified by densitometry. Specific NF-κB binding was determined by competition assays using unlabeled homologous and mutant oligonucleotides.

Results

Characterization of hepatocyte NOS. As reported previously (15), TNFα induced NOS activity in hepatocyte cultures as measured by quantifying nitrite and nitrate accumulation in the media. Fig. 1 shows that NOS induction is dose dependent from TNFα concentrations of 0.001 to 1 μg/ml (62.7–62,700 units/ml, 0.06–60 nM). IFNγ increased the effect of TNFα by ~2–3-fold without changing the shape of the dose-response curve. A low level of NOS activity was also observed in untreated hepatocytes.

To more accurately measure NOS enzymatic activity and study its regulation, cultured hepatocytes treated with TNFα/IFNγ were homogenized. The standard assay of NOS activity in which the conversion of [^{3}H]arginine to [^{3}H]citrulline is measured required modification. The basic components of the assay include calmodulin, NADPH, H_4B, and [^{3}H]arginine. Modulation of the urea cycle by the addition of valine to inhibit arginase and unlabeled citrulline (12) to the assay mixture increased the apparent NOS activity from 73 ± 1 to 420 ± 22 pmol of [^{3}H]citrulline/min/mg protein. Measurement of NOS activity by this optimized assay was directly comparable to measurements of the NO oxidation products nitrite and nitrate under identical assay conditions. In these experiments, citrulline formation was 193 ± 22 pmol/min/10^6 cells and nitrite and nitrate formation was 203 ± 32 pmol/min/10^6 cells, indicating that the assays measuring citrulline formation were equivalent to nitrite and nitrate formation.

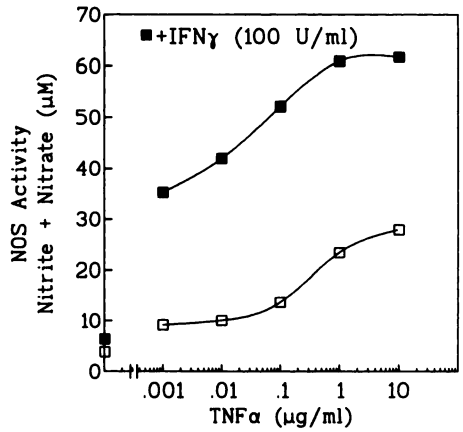

Fig. 1. Dose-response curve of NOS induction by TNFα and IFNγ. Hepatocytes were treated with varying concentrations of TNFα alone (□) or with addition of IFNγ (■, 100 units/ml). Media samples were collected at 18 hr after cytokine treatment, and NOS activity was measured by nitrite and nitrate accumulation in the media. Values represent the mean of duplicate samples in a study representative of four separate experiments.

NOS activity was found primarily in the cytosol fraction, as expected. In hepatocytes from LPS-treated rats, [^{3}H]citrulline formation (pmol/min/mg protein) was 328.5 ± 5.6 in the cytosol, 39.1 ± 5.2 in the nuclear fraction, and 13.5 ± 1.5 in the mitochondrial fraction. NOS activity was not discernible in the microsomal fraction.

The cofactor requirements for hepatocyte NOS were assessed, and the results are shown in Fig. 2. The calcium dependence was measured by omitting calcium from the incubations and adding 2 mM EGTA. This resulted in a surprisingly large decrease in activity, which may be attributed to a decreased ability of calmodulin to transfer electrons in the absence of bound calcium (26). Both trifluoperazine (400 μM), a calmodulin inhibitor, and the omission of GSH decreased NOS activity by ~40%. The omission of H_4B decreased activity by >50%. NOS inhibitors *N*-monomethyl-L-arginine and *N*-nitro-L-arginine methyl ester both decreased activity, although *N*-monomethyl-L-arginine was more effective (Fig. 2).

Receptor binding of TNFα to cultured rat hepatocytes. To determine whether NOS induction could be correlated with TNFα receptor binding, various concentrations of ^{125}I-TNFα were incubated with hepatocytes at 4°. As previously found with cultured mouse hepatocytes (27), Scatchard analysis of these data indicated that the curvilinear profile of binding was characteristic of a two-site model (Fig. 3). By

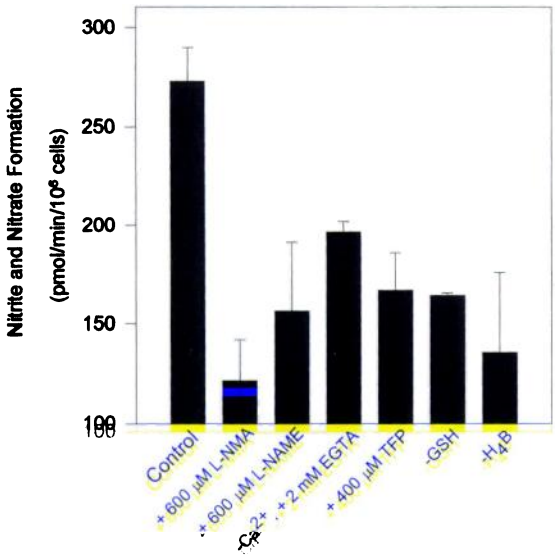

Fig. 2. Cofactor dependence and inhibition of TNFα and IFNγ induced NOS. NOS activity was measured as the formation of nitrite and nitrate in hepatocyte homogenates of cells treated with 10 μg/ml TNFα and 100 units/ml IFNγ for 21 hr. Basic assay components included 50 mM Tris·HCl, pH 7.4, 2 mM $CaCl_2$, 200 μM L-arginine, 200 μM NADPH, 50 mM valine, 1 mM citrulline, 5 mM GSH, and 10 μM H_4B. Additions (+) or omissions (−) from this standard assay are indicated. Values represent mean ± standard error of duplicate samples in a study representative of two to four separate experiments.

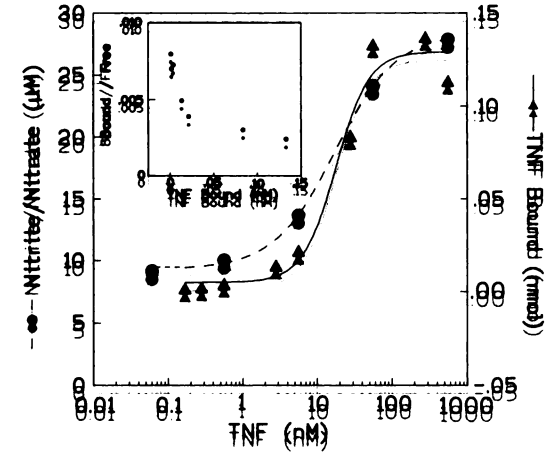

Fig. 3. NOS induction by TNFα corresponds with receptor binding. Hepatocytes were treated with varying concentrations of TNFα. Correlation of TNFα receptor binding (▲) with NOS activity (●). The amount of TNFα bound was calculated from the total amount of [^{125}I]TNF specific binding after the 4-hr incubation (i.e., at equilibrium), and the specific radioactivity of the [^{125}I]TNF was added to the incubation. Media samples were collected at 18 hr, and NOS activity was measured by nitrite and nitrate accumulation in the media. Values represent the mean of duplicate samples in a study representative of four separate experiments. Incubations for the receptor binding studies included 100,000 cpm of ^{125}I-TNFα with increasing concentrations of cold TNFα and were conducted with hepatocyte cultures at 4° in a humidified atmosphere of 95% air/5% carbon dioxide for 4 hr. Samples were collected, and radioactivity was measured as described in Materials and Methods. *Points*, mean of triplicate samples. *Inset*, Scatchard analysis of TNFα binding. Receptor incubations were conducted as described above.

LIGAND analysis, this model was assessed as two binding sites with varying affinities for TNFα. They are represented by a high affinity element (K_d = 1.4 nM) with ~3157 binding sites/cell for the TNFα trimer and a low affinity element (K_d = 157 nM) with ~204,948 binding sites/cell for the TNFα trimer. Fig. 3 shows that there is a high correlation between NOS induction by TNFα and total receptor binding. This correlation suggests that NOS induction by TNFα is limited by its receptor binding rather than subsequent signal transduction/transcription activation effects.

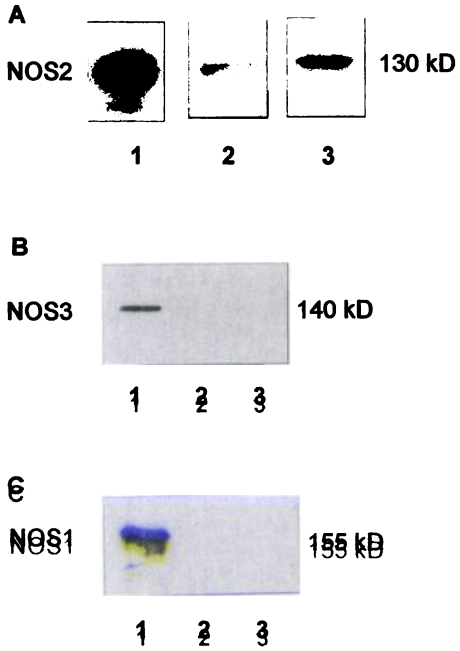

Fig. 4. Identification of immunoreactive NOS in untreated hepatocytes. Total protein was collected from untreated rat hepatocytes and assayed for NOS2, NOS3, and NOS3 protein by Western blot analysis as described in Materials and Methods. A, Analysis of NOS2. *Lane 1*, 2 μg of purchased macrophage lysate. *Lane 2*, 15 μg of hepatocyte lysate. *Lane 3*, 15 μg of hepatocyte lysate obtained from a different cell preparation. B, Analysis of NOS3. *Lane 1*, 15 μg of human endothelial cell lysate. *Lane 2*, 20 μg of hepatocyte lysate. *Lane 3*, 10 μg of hepatocyte lysate. C, Analysis of NOS1. *Lane 1*, 15 μg of rat cerebellum total protein. *Lane 2*, 10 μg of hepatocyte lysate. *Lane 3*, 5 μg of hepatocyte lysate.

Identification of NOS immunoreactive protein in untreated hepatocytes. In the course of these experiments, hepatocytes not treated *in vitro* with cytokines exhibited various levels of NOS activity. This observation led us to question whether this activity was due to expression of a constitutive form of NOS (i.e., NOS1 or NOS2). Fig. 4 shows that variable levels of NOS2 were observed in untreated hepatocytes, whereas neither NOS1 nor NOS3 was detected. Interestingly, the antibody to the NOS1 isoform recognized a hepatocyte protein of ~73 kDa (data not shown). It is likely that this protein is cytochrome P450 reductase, which is structurally similar to this NOS isoform.

Multiple cytokine induction of NOS. The liver *in vivo* is exposed to multiple cytokines, and it has been shown that several cytokines induce NOS in isolated hepatocytes (2). Therefore, experiments were conducted to determine which cytokines alone and in combination are most effective at inducing NOS and whether the induction of NOS is dependent on generation of oxidants, as previously reported to occur with TNFα alone (15). Activity was measured by total nitrite and nitrate accumulation in the culture media (Fig. 5). The cytokines IL-1β, TNFα, and IFNγ, as well as LPS, each induced NOS activity. IL-6 had no inductive effect on NOS.

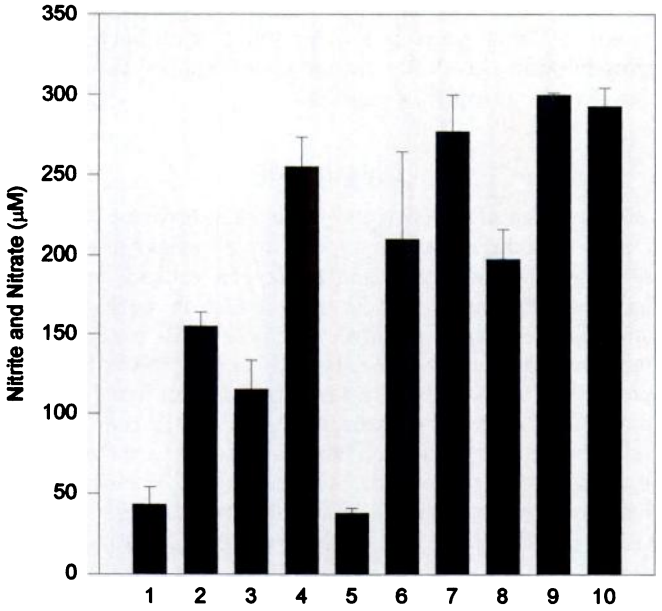

Fig. 5. Induction of NOS by cytokines and endotoxin. Hepatocytes were treated with 1 μg/ml TNFα, 100 units/ml IFNγ, 50 units/ml IL-1β, 1 nM IL-6, or 10 μg/ml endotoxin alone and in combination. Media samples were collected and assayed for total nitrite and nitrate accumulation. Values are mean ± standard error of duplicate samples in two to four experiments. *1*, Control; *2*, TNFα; *3*, IFNγ; *4*, IL-1β; *5*, IL-6; *6*, LPS; *7*, IFNγ and LPS; *8*, TNFα and IFNγ; *9*, TNFα and IL-1β; and *10*, TNFα, IL-1β, IFNγ, IL-6, and LPS.

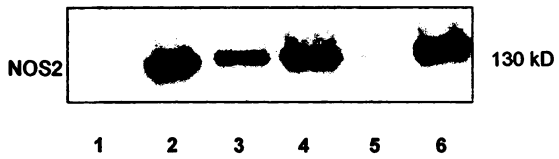

Fig. 6. Induction of NOS2 immunoreactive protein by cytokines. Hepatocytes were treated with 1 μg/ml TNFα, 100 units/ml IFNγ, 50 units/ml IL-1β, 1 nM IL-6, or 10 μg/ml LPS. Total protein was collected after 24 hr and assayed for NOS protein by Western blot analysis as described in Materials and Methods. Each lane was loaded with 15 μg of total protein. *Lane 1*, control. *Lane 2*, TNFα. *Lane 3*, IFNγ. *Lane 4*, IL-1β. *Lane 5*, IL-6. *Lane 6*, LPS.

TABLE 1
Cytokine induction of immunoreactive protein

Treatment[a]	NOS protein[b]
	Densitometry unit
Control	1
TNFα (1 μg/ml)	33
IFNγ (100 units/ml)	16
IL-1β (50 units/ml)	34
IL-6 (1 nM)	1
LPS (10 μg/ml)	36

[a] Hepatocytes were treated, and after 24 hr, total protein was collected.
[b] 15 μg of total protein was loaded. Values are densitometry units of Western blot shown in Fig. 6.

Maximal response was produced by the combination of TNFα and LPS with IL-1β or IFNγ as well as by all of the cytokines in combination.

The cytokine induction of NOS activity was verified by measurement of immunoreactive protein (Fig. 6 and Table 1). Inductions of immunoreactive protein by TNFα, IFNγ, IL-1β, IL-6, and LPS were compared. The immunoreactive protein induced by IL-1β, TNFα, and LPS was approximately equal, although the NOS protein induced by IFNγ was much lower. IL-6 did not induce immunoreactive NOS.

An antioxidant mixture consisting of Trolox (an α-tocopherol analog), ascorbic acid, and sodium benzoate was added to the incubations with the various cytokines and combinations. Individual components of this mixture have been shown to inhibit the oxidative effects of TNFα in cultured rat and mouse hepatocytes (15, 16). NOS induction was reduced by the antioxidants in response to each of the cytokines and LPS, although the degree of the inhibition varied (Fig. 7, A

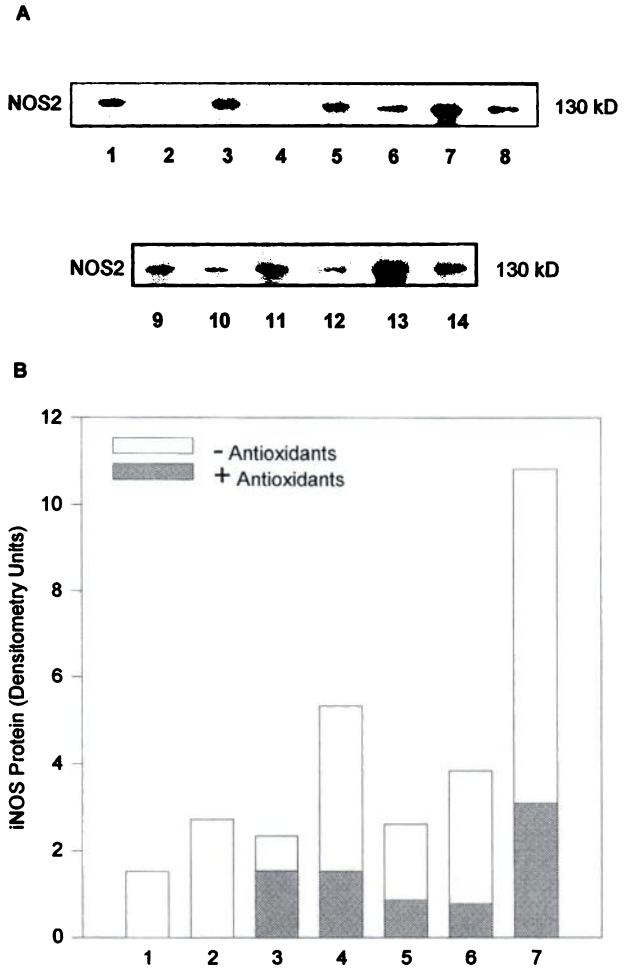

Fig. 7. Induction of NOS2 immunoreactive protein is inhibited by antioxidants. Hepatocytes were treated with 1 μg/ml TNFα, 100 units/ml IFNγ, 50 units/ml IL-1β, 1 nM IL-6, or 10 μg/ml LPS in the presence and absence of an antioxidant mixture (2.5 mM Trolox, 250 μM ascorbic acid, and 10 mM sodium benzoate). A, Total protein was collected after 24 hr and assayed for NOS protein by Western blot analysis as described in Materials and Methods. Each lane was loaded with 15 μg of total protein. *Lane 1*, control. *Lane 2*, control, antioxidants. *Lane 3*, TNFα. *Lane 4*, TNFα, antioxidants. *Lane 5*, IFN. *Lane 6*, IFN, antioxidants. *Lane 7*, IL-1. *Lane 8*, IL-1, antioxidants. *Lane 9*, LPS. *Lane 10*, LPS, antioxidants. *Lane 11*, TNFα and IFN. *Lane 12*, TNFα and IFN, antioxidants. *Lane 13*, TNFα, IFN, IL-1, IL-6, and LPS. *Lane 14*, TNFα, IFN, IL-1, IL-6, and LPS, antioxidants. B, Densitometry values for the Western blot shown in A. *Open bars*, densitometry values for immunoreactive NOS2 (*iNOS*) of samples treated with LPS or cytokines in the absence of antioxidants. *Shaded bars*, immunoreactive NOS2 stimulation in the presence of antioxidants. *1*, Control; *2*, TNFα; *3*, IFN; *4*, IL-1; *5*, LPS; *6*, TNFα and IFN; and *7*, TNFα, IFN, IL-1, IL-6, and LPS.

and B). The antioxidant mixture also effectively inhibited induction in hepatocytes treated with cytokine mixtures. These data suggested that oxygen radical generation is a common mechanism for the induction of NOS in hepatocytes.

Mitochondrial release of reactive oxygen intermediates. To verify the stimulation of mitochondrial oxygen radical generation by cytokines, we measured the fluorescence of DCF, a carboxylated analog of dichlorodihydrofluorescein, in mitochondria isolated from control cells and hepatocytes treated with TNFα (1 μg/ml) for 3 hr (Fig. 8A). TNFα was selected for these studies because it is the cytokine most commonly associated with oxygen radical generation and its inductive effects were completely blocked by antioxidants (Fig. 7). The mitochondria from TNFα-treated hepatocytes generated more oxygen radicals, as indicated by an increased rate of fluorescence development compared with control mitochondria (Fig. 8A). A mixture of antioxidants, including 2.5 mM Trolox, 250 μM ascorbic acid, and 40 mM benzoic acid, reduced fluorescence in treated mitochondria from 720 ± 111 to 186 ± 9 fluorescence units/specific activity of GDH (Fig. 8B). This result indicates that the observed increase in DCF fluorescence is attributable to TNFα stimulation of oxygen radical generation in the mitochondria. Furthermore, the antioxidant mixture that inhibits TNFα induction of NOS inhibits mitochondrial generation of oxygen radicals.

Inhibition of cytokine-stimulated NF-κB activation by antioxidants. Because NOS2 induction in macrophages has been linked to activation NF-κB (19, 20), a transcription factor that is stimulated by oxygen radicals, we examined the activation of NF-κB binding by a mixture of TNFα (1 μg/ml), IL-1β (50 units/ml), and IFNγ (100 units/ml) and explored the effect of the antioxidant mixture on this activation (Fig. 9). As expected, the cytokine mixture activated NF-κB. Most importantly, this activation was impaired by the addition of the antioxidant mixture, which decreases NOS2 induction by cytokines. Thus, there is a direct correlation between NOS induction and NF-κB activation when hepatocytes are stimulated with multiple cytokines.

Discussion

The results of the current study demonstrate that NOS activity is induced in hepatocytes by a variety of cytokines and suggest that generation of oxygen radicals may be a significant common step in the induction pathway. One source of these oxygen radicals seems to be the mitochondrial electron transport chain. Although hepatic NOS has been extensively studied, little attention has been devoted to the enzymology of the protein in terms of cofactor requirements and subcellular localization. In general, we have shown that hepatic NOS is a cytosolic enzyme with cofactor requirements consistent with the NOS2 activity identified in other cell types. All hepatic NOS activity was attributable to NOS2 because neither constitutive isoform was recognized by the antibodies used in these studies.

Dose-response curves of the induction of NOS by TNFα indicate that NOS was induced in a dose-dependent manner from 0.06 to 60 nM. Comparison of these data with TNFα binding to rat hepatocytes suggests that NOS induction by TNFα is conferred by binding to the high abundance/low affinity receptors. These data also suggest that cytokine receptor binding is the rate-limiting step in NOS induction.

Cytokines are produced in response to injury or infection in a complex pattern of mutual induction and cooperative action (21). Of these cytokines, TNFα is produced early in the response pathway, and its actions have been implicated in the cytotoxicity associated with the resulting inflammation (28). Many cellular responses to TNFα are exacerbated by expo-

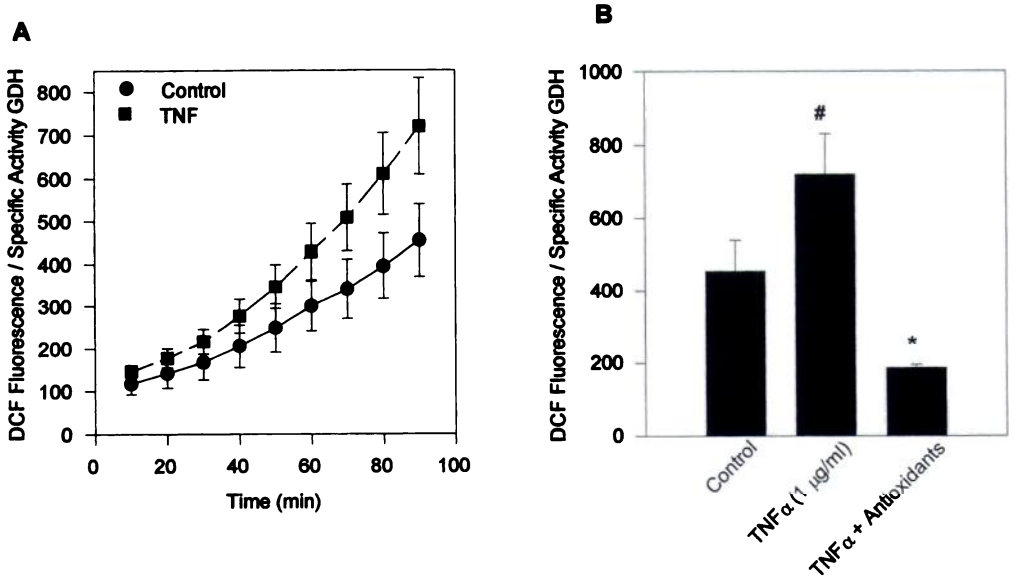

Fig. 8. Mitochondrial generation of oxygen radicals is inhibited by antioxidants. Hepatocytes were treated with 1 μg/ml TNFα for 3 hr. Hepatocytes were harvested, and mitochondria were isolated as described in Materials and Methods. Mitochondria were incubated for 10 min at room temperature with 10 μM DCF and then pelleted and resuspended in 250 mM mannitol in PBS. Antioxidants (2.5 mM Trolox, 40 mM sodium benzoate, and 250 μM ascorbate) were added where indicated, and fluorescence was measured every 10 min for 90 min. Fluorescence is expressed as fluorescence units normalized by the specific activity of GDH. A, Oxygen radical generation over time from control and TNFα-treated hepatocytes. Values represent mean ± standard deviation of triplicate samples. B, Inhibition of oxygen radical generation by antioxidants. Values represent mean ± standard deviation of triplicate samples after 90 min. *, Values significantly different from control ($p < 0.05$). #, Values significantly different from TNFα-treated hepatocytes ($p < 0.05$).

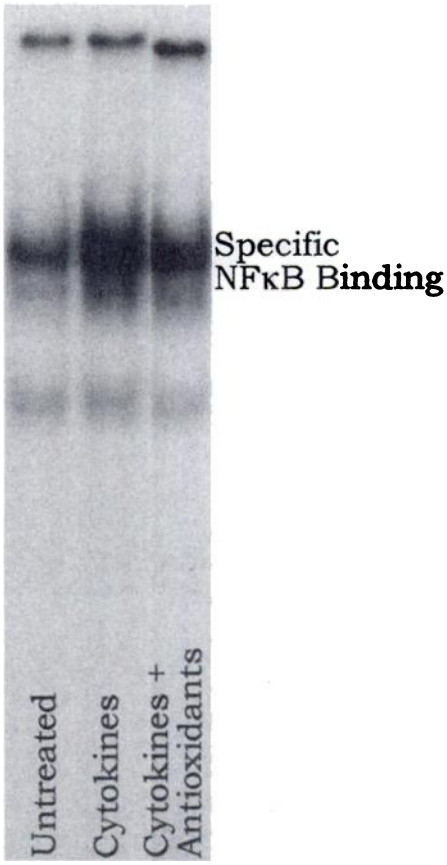

Fig. 9. Cytokine activation of NF-κB is inhibited by antioxidants. Hepatocytes were treated with 1 μg/ml TNFα, 100 units/ml IFN, and 50 units/ml IL-1 in the presence and absence of an antioxidant mixture (2.5 mM Trolox, 250 μM ascorbic acid, and 10 mM sodium benzoate). After 1 hr, hepatocytes were harvested, nuclear proteins were extracted, and NF-κB binding activity was measured as described in Materials and Methods. Specific NF-κB binding was determined as the radioactive band that was eliminated when excess unlabeled NF-κB consensus oligonucleotide was added to the incubations. This band was not affected by the addition of mutated NF-κB oligonucleotide. Densitometry values for the specific NF-κB binding were 34 ADU for control, 86 ADU for cytokines, and 58 ADU for cytokines-antioxidants.

sure to IFNγ released by T lymphocytes (29). The increase in TNFα is followed by a slower, sustained increase in IL-1β (21). TNFα and IL-1β act cooperatively to induce both each other and other cytokines, including IL-6. This immunological activation stimulates a group of proteins referred to as the acute phase proteins. IL-6 is a key mediator of the acute phase response (30). However, the failure of IL-6 to induce NOS suggests that NOS is not part of the acute phase response. Similarly, studies have shown that chemical agents that induce the acute phase response in hepatocytes do not induce NOS (31).

Previous studies in our laboratory have established the importance of reactive oxygen intermediates in TNFα induction of NOS2 in rat hepatocytes (15). In the current study, we demonstrated directly that TNFα stimulates the mitochondrial generation of oxygen radicals and that oxygen radical formation is inhibited by a specific antioxidant mixture. Components of this mixture have been shown to diminish the oxidative effects of TNFα in hepatocytes (15, 16). Additional experiments were conducted with this antioxidant mixture to explore the role of oxygen radicals in NOS2 induction by various cytokines. It was found that NOS2 induction by TNFα, IL-1β, IFNγ, and LPS was inhibited by the antioxidant mixture, although the extent of inhibition varied with the cytokine. The inhibition ranged from 100% with TNFα to <50% with IFNγ. This is consistent with reports that the regulatory region of the NOS2 gene contains consensus elements for activation by interferons and LPS (17, 18). Nevertheless, antioxidants inhibited NOS2 induction by the complete mixture of cytokines plus LPS by 71%. These results suggest that all of the cytokines tested act, at least in part, through the generation of oxidants and that antioxidants are effective inhibitors of NOS2 induction by a combination of the major inflammatory cytokines. Oxidative stress has been linked to the regulation of inducible genes by the activation of NF-κB and activator protein-1 (25, 32). Binding elements for these *trans*-acting factors are found in the promoter of murine macrophage NOS2 (17, 18), and NF-κB activation has been shown to play a role in NOS2 induction by cytokines in macrophages (33). Links between NOS2 induction in hepatocytes and NF-κB induction have not been established. In the current study, cytokines were found to activate NF-κB in cultured rat hepatocytes. Experiments to explore the mechanism of antioxidant action showed that NF-κB activation, as well as NOS2 induction, was inhibited by the antioxidant mixture that was used. One source of the oxygen radicals is the mitochondrial electron transport chain, as indirectly suggested by earlier studies (15, 34) and demonstrated in the current study. Interestingly, it has recently been shown that oxidant stress in mitochondria can promote extramitochondrial activation of NF-κB (35). How this occurs and the molecular mechanism by which cytokines enhance mitochondrial generation of oxygen radicals remain to be elucidated.

References

1. Moncada, S., and A. Higgs. The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329**:2002–2012 (1993).
2. Geller, D. A., A. K. Nussler, M. DiSilvio, C. J. Lowenstein, R. A. Shapiro, S. C. Wang, and R. L. Simmons. Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc. Natl. Acad. Sci. USA* **90**:522–526 (1994).
3. Cunha, F. Q., W. Y. Weiser, J. R. David, D. W. Moss, S. Moncada, and F. Y. Liew. Recombinant migration inhibitory factor induces nitric oxide synthase in murine macrophages. *J. Immunol.* **150**:1908–1912 (1993).
4. Radomski, M. W., R. M. J. Palmer, and S. Moncada, Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. USA* **87**:10043–10047 (1990).
5. Kroncke, K. D., K. Fehsel, and V. Kolb-Bachofen. Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. *Biol. Chem. Hoppe-Seyler* **376**:327–343 (1995).
6. Forstermann, U., J. S. Pollock, H. H. H. W. Schmidt, M. Heller, and F. Murad. Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA* **88**:1788–1792 (1991).
7. Moncada, S., R. M. J. Palmer, and E. A. Higgs. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**:109–142 (1991).
8. Abu-Soud, I. M., and D. J. Stuehr. Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc. Natl. Acad. Sci. USA* **90**:10769–10772 (1993).
9. Schoedon, G., M. Schneemann, N. Blau, C.-J. S. Edgell, and A. Schaffner. Modulation of human endothelial cell tetrahydrobiopterin synthesis by activating and deactivating cytokines: new perspectives on endothelium-derived relaxing factor. *Biochem. Biophys. Res. Commun.* **196**:1343–1348 (1993).
10. Werner, E. R., G. Werner-Felmayer, D. Fuchs, A. Hausen, G. Reibnegger, J. J. Yim, and H. Wachter. Impact of tumour necrosis factor-α and interferon-γ on tetrahydrobiopterin synthesis in murine fibroblasts and macrophages. *Biochem. J.* **280**:709–714 (1991).
11. Fukushima, T., and J. C. Nixon. Analysis of reduced forms of biopterin in biological tissues and fluids. *Anal. Biochem.* **102**:176–188 (1980).
12. Knowles, R. G., M. Merrett, M. Salter, and S. Moncada. Differential

induction of brain, lung, and liver nitric oxide synthase by endotoxin in the rat. *Biochem. J.* **270**:833–836 (1990).
13. Stuehr, D. J., N. S. Kwon, and C. F. Nathan. FAD and GSH participate in macrophage synthesis of nitric oxide. *Biochem. Biophys. Res. Commun.* **168**:558–565 (1990).
14. Griscavage, J. M., N. E. Rogers, M. P. Sherman, and L. J. Ignarro. Inducible nitric oxide synthase from a rat alveolar macrophage cell line is inhibited by nitric oxide. *J. Immunol.* **151**:6329–6337 (1993).
15. Duval, D. L., D. J. Sieg, and R. E. Billings. Regulation of hepatic nitric oxide synthase by reactive oxygen intermediates and glutathione. *Arch. Biochem. Biophys.* **316**:699–706 (1995).
16. Adamson, G. M., and R. E. Billings. Tumor necrosis factor induced oxidative stress in isolated mouse hepatocytes. *Arch. Biochem. Biophys.* **294**: 223–229 (1992).
17. Xie, Q., R. Whisnant, and C. F. Nathan. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon and bacterial lipopolysaccharide. *J. Exp. Med.* **177**:1779–1784 (1993).
18. Lowenstein, C. J., E. W. Alley, P. Raval, A. M. Snowman, S. H. Snyder, S. W. Russell, and W. J. Murphy. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon γ and lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* **90**:9730–9734 (1993).
19. Adcock, I. M., C. R. Brown, O. Kwon, and P. J. Barnes. Oxidative stress induces NFκB DNA binding and inducible NOS mRNA in human epithelial cells. *Biochem. Biophys. Res. Commun.* **199**:1518–1524 (1994).
20. Mulsch, A., B. Schray-Utz, P. I. Mordvintcev, S. Hauschildt, and R. Busse. Diethyldithiocarbamate inhibits induction of macrophage NO synthase. *FEBS Lett.* **321**:215–218 (1993).
21. Ulich, T. R., K. Guo, B. Irwin, D. G. Remick, and G. N. Davatelis. Endotoxin-induced cytokine gene expression *in vivo*. II. Regulation of tumor necrosis factor and interleukin-1α/β expression and suppression. *Am. J. Pathol.* **137**:1173–1185 (1990).
22. Ku, R. H., and R. E. Billings. The role of mitochondrial glutathione and protein sulfhydryls in formaldehyde toxicity in glutathione-depleted rat hepatocytes. *Arch. Biochem. Biophys.* **247**:183–189 (1986).
23. Ding, A. H., C. F. Nathan, and D. J. Stuehr. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J. Immunol.* **141**:2407–2412 (1988).
24. Bush, P. A., N. E. Gonzalez, J. M. Griscavage, and L. J. Ignarro. Nitric oxide synthase from cerebellum catalyzes the formation of equimolar quantities of nitric oxide and citrulline from L-arginine. *Biochem. Biophys. Res. Commun.* **185**:960–966 (1992).
25. Schreck, R., P. Rieber, and P. A. Baeuerle. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription and HIV-1. *EMBO J.* **10**:2247–2258 (1991).
26. Cho, H., Q. Xie, J. Calaycay, R. Mumford, K. Swiderek, T. Lee, and C. F. Nathan. Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.* **176**:599–604 (1992).
27. Adamson, G. M., and R. E. Billings. Tumor necrosis factor: receptor binding and expression of receptors in cultured mouse hepatocytes. *J. Pharmacol. Exp. Ther.* **269**:367–373 (1994).
28. Colletti, L. M., D. G. Remick, G. D. Burtch, S. L. Kunkel, R. M. Strieter, and D. A. J. Campbell. Role of tumor necrosis factor-α in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. *J. Clin. Invest.* **85**:1936–1943 (1990).
29. Sheehan, K. C. F., and R. D. Schreiber. *The Synergy and Antagonism of Interferon-gamma and TNF* (B. Beutler, ed.). Raven Press, New York, 145–178 (1992).
30. Hattori, M., A. Tugores, J. K. Westwick, L. Veloz, H. L. Leffert, M. Karin, and D. A. Brenner. Activation of activating protein 1 during hepatic acute phase response. *Am. J. Physiol.* **264**:G95–G103 (1993).
31. Geller, D. A., P. D. Freeswick, D. Nguyen, A. K. Nussler, M. DiSilvio, R. A. Shapiro, S. C. Wang, R. L. Simmons, and T. R. Billiar. Differential induction of nitric oxide synthase in hepatocytes during endotoxemia and the acute-phase response. *Arch. Surg.* **129**:165–171 (1994).
32. Matthews, J. R., N. Wakasugi, J. Virelizier, J. Yodoi, and R. T. Hay. Thioredoxin regulates the DNA binding activity of NF-KB by reduction of a disulphide bond involving cysteine 62. *Nucleic Acids Res.* **20**:3821–3830 (1992).
33. Sherman, M. P., E. E. Aeberhard, V. Z. Wong, J. M. Griscavage, and L. J. Ignarro. Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem. Biophys. Res. Commun.* **191**:1301–1308 (1993).
34. Schulze-Osthoff, K., R. Beyaert, V. Vandevoorde, G. Haegeman, and W. Fiers. Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *EMBO J.* **12**:3095–3104 (1993).
35. Garcia-Ruiz, C., A. Colell, A. Morales, N. Kaplowitz, and J. C. Fernandez-Checa. Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial function and activation of transcription factor nuclear factor-*kappa* B: studies with isolated mitochondria and rat hepatocytes. *Mol. Pharmacol.* **48**:825–834 (1995).

Send reprint requests to: Dr. Ruth E. Billings, Department of Environmental Health, College of Veterinary Medicine and Biomedical Sciences, Environmental Health Building, Colorado State University, Fort Collins, CO 80523. E-mail: rbillings@vines.colostate.edu