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Regulation of Hepatic Nitric Oxide Synthase by Reactive Oxygen Intermediates and Glutathione[1]

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Regulation of induced nitric oxide synthase in rat hepatocyte primary cultures was explored. Nitric oxide synthase (NOS) induction by tumor necrosis factor-α (TNFα) is synergized by interferon-γ, and both NOS activity and gene expression are maximal by 10 h and maintained through 24 h. Glutathione depletion by diethylmaleate, which conjugates reduced glutathione, 1,3-bis(chloroethyl)-1-nitrosourea (BCNU), a glutathione reductase inhibitor, or buthionine sulfoxamine, a glutathione synthesis inhibitor, abolishes or reduces NOS induction in TNF α-treated hepatocytes, whereas *N*-acetylcysteine has little effect. Thus, reduced glutathione is critical to NOS mRNA induction and activity in TNFα-treated hepatocytes. NOS induction in TNFα-treated cells is reduced by rotenone, a mitochondrial complex 1 inhibitor. Concurrent treatment with TNFα and the antioxidant, Trolox, or the iron-chelating agent, desferrioxamine, also reduces NOS activity. Dithiothreitol, a thiol antioxidant, reduced TNFα induction of NOS. Trolox and BCNU, combined, blocked TNFα stimulation of NOS greater than either agent alone. These results suggest that TNFα increases mitochondrial production of reactive oxygen intermediates (ROI), which contributes to NOS induction. Hepatocytes exposed to extracellular ROI generation through a xanthine/xanthine oxidase superoxide-generating system expressed increased NOS activity and mRNA levels. NOS induction by superoxide also requires reduced glutathione since diethylmaleate blocks induction by xanthine/xanthine oxidase while *N*-acetylcysteine elevates NOS expression. Thus, the generation of ROI by cytokines or other physiological processes stimulates the induction of NOS and this process is regulated by cellular levels of reduced glutathione. © 1995 Academic Press, Inc.

Key Words: **nitric oxide synthase; tumor necrosis factor-α; glutathione; superoxide; liver; gene expression.**

Injury initiated by chemicals, sepsis, or ischemia is exacerbated by the infiltration of leukocytes which release endogenous mediators such as the macrophage-derived cytokine, TNFα.[3] Many of the responses elicited by these mediators are due to the direct actions of induced proteins, including nitric oxide synthase (NOS). NOS isoforms have been grouped into two broad categories: constitutive NOS (cNOS), which includes neuronal and endothelial subtypes, and induced NOS (iNOS), which appears to be a single form expressed in a wide variety of cells (1). iNOS tightly binds calmodulin without elevation of the cellular calcium concentration (2, 3), whereas cNOS is activated by calcium-dependent binding (1, 4, 5). Both types of NOS convert arginine to citrulline and nitric oxide in a reaction dependent upon NADPH, O_2, FAD, FMN, tetrahydrobiopterin, and thiols (1, 6). Maximal induction of iNOS depends upon synergistic combinations of stimuli, and the most effective stimuli vary with the cell type. These stimulatory compounds include TNFα, interleukin-1β, interferon-γ, and lipopolysaccharide, as well as gamma irradiation and phorbol

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[3] Abbreviations used: TNFα, tumor necrosis factor-α; IFNγ, interferon-γ; NOS, nitric oxide synthase; iNOS, induced nitric oxide synthase; cNOS, constitutive nitric oxide synthase; ROI, reactive oxygen intermediates including superoxide anion, hydrogen peroxide, and hydroxyl radical; NO, nitric oxide; BCNU, 1,3-bis(chloroethyl)-1-nitrosourea; BSO, buthionine sulfoximine; DFO, desferrioxamine; RT-PCR, reverse transcriptase–polymerase chain reaction; DEM, diethylmaleate; DTT, dithiothreitol; NAC, *N*-acetylcysteine, X/XO, xanthine/xanthine oxidase; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide.

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esters (7–11). The induction of NOS can be blocked by other mediators including interleukin-10, transforming growth factor-beta, epidermal growth factor, glucocorticoids, and pyrrolidine dithiocarbamate (7, 12–15). The promoter region of murine macrophage iNOS has at least 22 identifiable regulatory elements which include AP-1, NF-κB, TNFα, IFNα, and IFNγ responsive elements (16, 17).

Nitric oxide is a crucial cellular mediator with physiological activities which include vasodilation, neurotransmission, regulation of platelet aggregation, and cytotoxicity (1). In hepatocytes, nitric oxide has been shown to inhibit enzymes of the mitochondrial electron transport chain and alter protein synthesis and may ultimately protect against the cytotoxic effects resulting from endotoxemia (18–20). However, NOS induction is not involved in the cytotoxic or oxidative effects of TNFα in murine hepatocytes (21).

The activities of TNFα and other cytokines have been intimately linked with the production of oxidative stress. In addition to leukocyte-generated oxidative bursts, intracellular ROI may be generated due to TNFα inhibition of mitochondrial electron transport or activation of arachidonic acid metabolism (22). Interleukin-6 (23), an acute-phase protein inducer, interleukin-8 (24, 25), a neutrophil chemoattractant and activator, and vascular cell adhesion molecule-1 (26) are all induced in response to oxidative stress. The role of nitric oxide in oxidative damage is equivocal since NO can scavenge superoxide anion to decrease ROI toxicity (27), but it subsequently forms peroxynitrite radical which can either degrade to the highly reactive hydroxyl radical (28) or rapidly interconvert to relatively nontoxic nitrate depending upon cellular conditions (27). NO scavenging can decrease superoxide-mediated leukocyte adhesion (29, 30) and reduce the cytotoxic effects of extracellular ROI generation (27).

Thiol levels are closely associated with NOS activity. Thiols are necessary for maximal NOS activity and enzyme stability (6) and NO reacts with thiols to form stable reactants or alter protein function (31, 32). In addition, studies of NF-κB and AP-1 regulation and transcription factor binding sites found in the promoter region of inducible murine macrophage NOS gene (16, 17) implicate both oxidative stress and reduction by thioredoxin and nonprotein sulfhydryl compounds in their activation (33–37). In studies of hepatocytes, AP-1 activation has been associated with hepatocyte mitogen treatment or cytokine stimulation of the acute phase response (38) and iNOS induction in insulin-producing cells is preceded by increases in the AP-1 subunit c-*fos* (39). Since murine macrophage iNOS is 94% homologous to the rat hepatocyte gene (40), studies of its promoter region may be applicable to studies of hepatocyte NOS regulation. Therefore, the present studies explore the induction of nitric oxide synthase in hepatocytes and examine the role of thiols as well as ROI in the regulation of iNOS activity and gene expression.

MATERIALS AND METHODS

Reagents. Recombinant human tumor necrosis factor-α (specific activity 6.27×10^4 units/μg) was supplied by Genentech (South San Francisco, CA). Recombinant rat interferon-γ was purchased from Gibco-BRL (Gathersburg, MD). 1,3-Bis(chloroethyl)-1-nitrosourea (BCNU) was purchased from Bristol Laboratories (New York, NY). Desferrioxamine mesylate (DFO) was purchased from Ciba-Geigy (Basel, Switzerland). Trolox was purchased from Aldrich (Milwaukee, WI). Tetrahydrobiopterin was purchased from Schircks Laboratories (Jona, Switzerland). [^{3}H]Arginine and ECL reagent were purchased from Amersham (Arlington Heights, IL). The monoclonal antibody to amino acids 961–1144 of the macrophage inducible NOS was purchased from Transduction Laboratories (Lexington, KY). Micro BCA protein assay reagent was purchased from Pierce (Rockford, IL). All other chemicals and reagents, unless specified, were purchased from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA).

Cell culture. Hepatocytes were isolated from male Sprague–Dawley rats (250–400 g) by collagenase perfusion (41) and purified by differential centrifugation to give cultures of at least 90% viability and 95% purity. Hepatocytes were plated on rat tail collagen-coated culture plates (Corning, Palo Alto, CA) in Waymouths medium 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°C for 2–3 h to allow adherence. Nonadherent cells were removed by washing the plates twice with phosphate-buffered saline and cultures were treated in fresh medium.

Nitric oxide synthase activity. Nitrite levels in medium were determined colorimetrically on a Molecular Devices Thermomax plate reader. Total nitrite/nitrates were measured by reducing the nitrate to nitrite with NADPH:nitrate reductase followed by reaction with Griess reagent in a modification of previously described techniques (42, 43). 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 dithiothreitol and protease inhibitors (44). The cells were homogenized and frozen at −80°C 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 tetrahydrobiopterin. After incubation at 37°C, the reactions were stopped by addition of buffer (citrulline assay) or by heating at 100°C for 2 min (nitrite/nitrate analysis). [^{3}H]Citrulline was separated from arginine by ion exchange chromatography and quantified by liquid scintillation counting (5, 9).

NOS messenger RNA regulation. NOS mRNA in hepatocyte samples was measured by semiquantitative RT-PCR. Total RNA was extracted (45) and first-strand cDNA was synthesized using random hexamer primers with recombinant Moloney murine leukemia virus reverse transcriptase (RT) (USB, Cleveland, OH). NOS cDNA was amplified in a reaction consisting of 0.25 mM each of dCTP, dATP, dGTP, and dTTP, 60 mM Tris–HCl, 15 mM ammonium sulfate, 2 mM $MgCl_2$, 10% dimethyl sulfoxide (DMSO), 0.4 μM upstream and downstream primers, and 1.25 units of Amplitaq DNA polymerase (Perkin–Elmer) at pH 9.5 in 50 μl total volume. The PCR reaction (55°C annealing, 72°C extension, 94°C melting) was conducted in a Perkin–Elmer 9600 GeneAmp system. The NOS PCR reaction was optimized at pH 9.5 and 2 mM $MgCl_2$ using a PCR optimizing kit from InVitrogen (San Diego, CA). Dimethyl sulfoxide was added at a final concentration of 10% to improve specificity of the primers. The RT-PCR was optimized at 0.2–0.4 μg total RNA by varying the total RNA added to establish a linear range of PCR product. Aliquots were collected at various cycles to set a range of increasing product. Incubations omitting mRNA or RT were used to control for genomic DNA or mRNA

contamination. iNOS primers were designed from the murine macrophage NOS sequence avoiding regions in which the hepatocyte amino acid sequence varied (40, 46, 47) and synthesized by DNA Express (Fort Collins, CO). The downstream primer was 1778–1800 bp (5'-CTTCAGAGTCTGCCCATTGC-3') and the upstream primer was 1261–1286 bp (5'-CAGAAGCAGAATGTGACCATCATGGA-3'). Reaction aliquots were run on 3% Nu-Sieve agarose gels, visualized by ethidium bromide staining, and negative images were analyzed with a Bio-Rad imagining densitometer. Products were quantified by normalizing the densitometric values of NOS PCR product against those of β-actin. β-Actin PCR products of 245 bp were synthesized from duplicate RNA samples using murine β-actin primers purchased from Stratagene. The authenticity of NOS PCR products was verified by fragment size, restriction enzyme digestion, and Southern blot analysis using an oligonucleotide probe directed toward the 1746 to 1769 bp region of the rat NOS gene (DNA Express).

Western blot analysis of inducible nitric oxide synthase. Treated hepatocytes were washed and pelleted in phosphate-buffered saline. Pellets were resuspended in 10 mM Tris–HCl, pH 7.4, and denatured by boiling for 5 min. Protein was measured using a micro BCA kit and equal amounts of total protein were electrophoresed under reducing conditions (5% 2-mercaptoethanol) on a 7.5% polyacrylamide–SDS gel and transferred to nitrocellulose blots. Blots were blocked in 1% BSA dissolved in 10 mM Tris–HCl, pH 7.5, 100 mM sodium chloride, and 0.1% Tween 20 (wash buffer) overnight and then incubated with 0.5 μg/ml of anti-NOS in blocking buffer for 2 h. 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 h. After washing, the blots were developed with ECL reagent and exposed to Xomat-AR film. Bands were quantified using an Imaging Densitometer (BioRad).

RESULTS

Cytokine induction of nitric oxide synthase. The induction of NOS activity in primary rat hepatocyte cultures by TNFα (3 μg/ml) and IFNγ (100 U/ml) is maximal by approximately 10 h of treatment and is maintained up to 24 h (Fig. 1). There is a very close correlation between iNOS activity and gene expression. Media nitrite concentrations are proportional to nitrite plus nitrate and these assays of the spontaneously formed oxidation products of NO correlated with assays which measure the conversion of [^{3}H]arginine to the stable NOS product, [^{3}H]citrulline (Fig. 1). TNFα (3 μg/ml) synergizes with IFNγ (100 U/ml) in the induction of NOS. At 24 h, cultures treated with TNFα alone had 12.7 ± 1.98 μM nitrite, IFNγ-treated cultures had 2.89 ± 1.82 μM nitrite, and cultures treated with both cytokines had 31.07 ± 1.94 μM nitrite.

Reductive/oxidative regulation of TNF-α-induced nitric oxide synthase activity. To explore the role of oxidation/reduction in TNFα induction of nitric oxide synthase activity, hepatocyte cultures were treated with mediators which alter cellular thiol stores or inhibit cellular generation of ROI and with various antioxidant compounds. Preliminary studies explored the effects of these mediators on NOS activity as measured by nitrite release (Table I). Depletion of cellular thiol levels by treatment with BCNU (an inhibitor of glutathione reductase), BSO (an inhibitor of glutathione synthesis), or

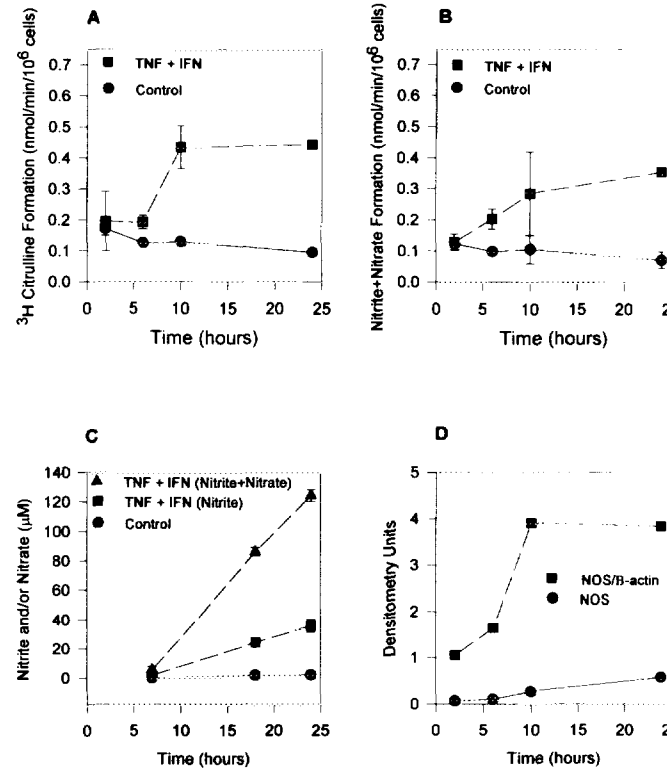

FIG. 1. Time course of induction of NOS activity and mRNA induction by TNFα (3 μg/ml) and IFNγ (100 U/ml). Hepatocyte cultures were treated and media, cell homogenates, or total RNA samples were collected and assayed for NOS activity or mRNA induction. (A) NOS activity in hepatocyte homogenates measured by conversion of [^{3}H]arginine to [^{3}H]citrulline. (B) NOS activity in hepatocyte homogenates measured by formation of nitrite/nitrate. (C) NOS activity measured by nitrite or nitrite/nitrate release into the media of treated and control hepatocyte cultures. (A, B, and C) Data points represent the mean ± standard deviation of two independent experiments. (D) NOS mRNA induction measured by semiquantitative RT-PCR. Values are arbitrary units from densitometry of ethidium bromide stained gels of PCR products representative of data from two independent experiments. NOS PCR products (●). The ratio of densitometry of NOS to β-actin products in duplicate samples (■).

DEM (which conjugates reduced glutathione) decreased TNF-α induction of activity (Table I). BCNU and BSO reduction of nitrite release was dose dependent (data not shown) and the combination completely abolished the effects of TNFα (Table I). DEM treatment completely blocked NOS activity, while supplementation of cellular glutathione with 1 mM *N*-acetylcysteine (NAC) only slightly stimulated NOS activity (Table I). None of these treatments caused significant cytotoxicity during the 24-h incubation period.

Since coenzyme Q of the mitochondrial electron transport chain is a potential source of oxygen radical generation, inhibitors of complex 1 were tested for their ability to inhibit NOS induction. Both rotenone and amobarbital diminished nitric oxide production in response to TNFα (Table I). Hepatocytes were treated

TABLE I

Nitric Oxide Synthase Activity in Treated Rat Hepatocytes

Treatment	NOS activity nitrite (μM)[a]
Control	5.56 ± 3.23
TNFα (1 μg/ml)	16.12 ± 4.97[b]
+NAC (1 mM)	16.53 ± 2.10
+DEM (1 mM)	−0.10 ± 0.49[c]
+BCNU (100 μM)	9.09 ± 5.58[d]
+BSO (200 μM)	8.82 ± 4.44[c]
+BCNU (100 μM) + BSO (200 μM)	6.76 ± 3.30[c]
+Rotenone (10 μM)[e]	1.39 ± 0.98[c]
+Amobarbital (400 μM)	6.51 ± 2.92[c]
+Trolox (500 μM)	6.38 ± 0.52[c]
+Trolox (1 mM)	4.82 ± 0.52[c]
+DFO (2.5 mM)	10.4 ± 3.46[d]

[a] Nitrate values are mean ± standard deviation of media samples taken at 18 h of 2–12 different experiments.

[b] Significantly different from control using a one-tailed Student *t* test, $P < 0.0001$.

[c] Significantly different from TNFα treated using a one-tailed Student *t* test, $P < 0.05$.

[d] Significantly different from TNFα treated using a one-tailed Student *t* test, $P < 0.1$.

[e] These incubations included 5 mM fructose to supply an energy alternative to mitochondrial electron transport. Fructose did not effect NOS induction.

with nonthiol antioxidants and TNFα to further elucidate the role of ROI in NOS induction. Trolox, a water-soluble α-tocopherol analog, reduced NOS activity in a dose-dependent manner (Table I). Desferrioxamine, an iron chelator which prevents iron-catalyzed Haber–Weiss production of hydroxyl radicals, also reduced nitric oxide production in TNFα-treated hepatocytes (Table I), but was less effective.

Reductive/oxidative regulation of TNFα-induced nitric oxide synthase messenger RNA. The studies demonstrating that TNFα-induced nitric oxide synthase activity is responsive to oxidative/reductive regulation were extended to examine the effects of these same modulators of cellular thiol stores and cellular generation of ROI on TNFα induction of iNOS mRNA levels. Because thiols and reactive oxygen intermediates have direct effects on nitric oxide synthase activity, it was important to determine if the observed effects were due to alterations of iNOS activity or gene expression. Depletion of cellular thiol levels by treatment with DEM completely blocked TNFα-stimulated iNOS gene induction while NAC treatment modestly elevated mRNA levels (Fig. 2, Table II). Both BSO and BCNU alone decreased gene induction and these mediators together blocked iNOS mRNA induction (Fig. 2, Table II).

Treatment of hepatocyte cultures with the mitochondrial complex 1 inhibitor, rotenone, reduced iNOS mRNA induction by TNFα (Fig. 3, Table III). These

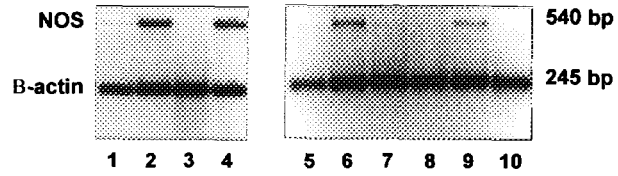

FIG. 2. The effect of glutathione levels on TNFα induction of NOS mRNA. NOS and β-actin PCR products from RT-PCR of treated hepatocyte cultures. Cells were treated with 1 mM DEM diluted in DMSO (final concentration 0.3%), 1 mM NAC, 200 μM BSO, or 100 μM BCNU with TNFα (1 μg/ml). Total RNA samples were collected after 18 h and RT-PCR was conducted as described. PCR products were visualized by ethidium bromide staining and densitometry of negative images was performed. Lane 1, control; lane 2, TNFα; lane 3, TNFα and DEM; lane 4, TNFα and NAC; lane 5, control; lane 6, TNFα; lane 7, TNFα and BSO; lane 8, TNFα and BSO plus BCNU; lane 9, TNFα and BCNU; lane 10, BSO plus BCNU.

data implicate mitochondrial generation of ROI in the induction of iNOS mRNA expression.

The antioxidant, Trolox, had minimal effects on TNFα-stimulated NOS mRNA expression (data not shown). Trolox (2.5 mM) in combination with BCNU (100 μM) reduced TNFα stimulation of NOS mRNA by 68%, a greater reduction than either BCNU (47%) or Trolox alone. The thiol antioxidant, dithiothreitol, decreased TNFα-stimulated induction in a dose-dependent manner, reducing mRNA levels by 23% at 100 μM and 86% at 1 mM.

Regulation of NOS-immunoreactive protein. Since NOS activity is dependent upon glutathione and nitrite levels can be altered by increased oxidation to nitrate, the stable oxidation product of nitric oxide, it was important to separate these effects from events which regulate induction of NOS protein. To do this, NOS-immunoreactive protein was quantified by Western blot anal-

TABLE II

Effect of Glutathione Levels on TNFα Induction of NOS

Treatment	NOS activity[a] nitrite (μM)	NOS mRNA[b] ratio (NOS/β-actin)
Control	3.23 ± 0.40	0.013
TNFα (1 μg/ml)	17.4 ± 1.39	0.410
+DEM (1 mM)	0.43 ± 0.40	0
+NAC (1 mM)	23.4 ± 4.35	0.429
Control	5.75 ± 0.79	0
TNFα (1 μg/ml)	15.3 ± 0.14	0.292
+BSO (200 μM)	13.9 ± 2.40	0.069
+BSO + BCNU	9.27 ± 0.74	0.031
+BCNU (100 μM)	13.0 ± 1.99	0.155
BSO + BCNU	2.23 ± 0.14	0

[a] Data represent means ± standard deviations of triplicate samples in an experiment representative of at least two separate experiments.

[b] Values are ratios of densitometric values of bands in Fig. 2.

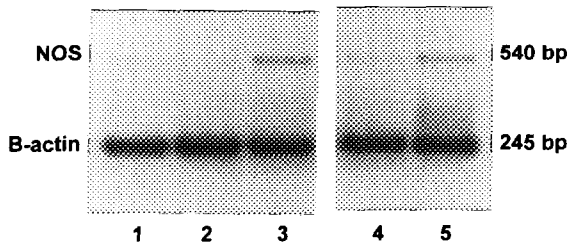

FIG. 3. The effect of rotenone on TNFα induction of NOS mRNA. NOS and β-actin PCR products from RT-PCR of treated hepatocyte cultures. Cells were treated with 10 μM rotenone dissolved in ethanol (final concentration 1%) and TNFα (1 μg/ml). Fructose (5 mM) was added to samples as an alternative energy source. Total RNA samples were collected after 18 h and RT-PCR was conducted as described. PCR products were visualized by ethidium bromide staining and densitometry of negative images was performed. Lane 1, control; lane 2, rotenone; lane 3, TNFα; lane 4, TNFα and rotenone; lane 5, TNFα and fructose.

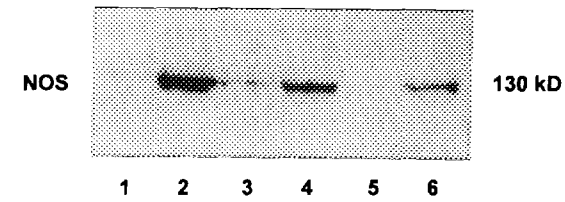

FIG. 4. Regulation of TNFα induction of iNOS-immunoreactive protein. Western blot analysis of iNOS-immunoreactive protein. Hepatocytes were treated 100 μM BCNU and 200 μM BSO, 1 mM NAC, 1 mM DEM, or 10 μM rotenone with TNFα (1 μg/ml). Total hepatocyte protein was collected at 20 h and 4 μg of each sample was used. Lane 1, control; lane 2, TNFα; lane 3, TNFα and BCNU and BSO; lane 4, TNFα and NAC; lane 5, TNFα and DEM; lane 6, TNFα and rotenone.

ysis, and the results are shown in Fig. 4 and Table IV. Control hepatocytes had only slight amounts of NOS-immunoreactive protein which was increased 25-fold by TNFα treatment. TNFα induction of immunoreactive protein was reduced or abolished by the BSO/BCNU combination, DEM, as well as rotenone. In this particular experiment, NAC caused a small decrease in both NOS immunoreactive protein and activity.

ROI induction of NOS. To explore the ability of other sources of ROI to directly stimulate iNOS gene expression, hepatocyte cultures were treated with 500 μM xanthine and varying concentrations of xanthine oxidase to generate extracellular superoxide radical anion. This resulted in a dose-dependent increase in NOS mRNA and activity (Fig. 5, Table V, Fig. 6). Altering cellular glutathione levels with DEM and NAC had effects similar to those seen with TNFα induction; DEM blocked iNOS induction while NAC enhanced superoxide stimulated induction (Fig. 5, Table V). The time dependence of the glutathione effects was examined to help determine if glutathione levels are important in the initiation of iNOS gene transcription initiation or message stability. Hepatocyte cultures were treated with DEM or NAC simultaneously with xanthine/xanthine oxidase and after a 30-minute preexposure to xanthine/xanthine oxidase. Both inhibition of iNOS induction by DEM and stimulation by NAC were most pronounced when the stimuli were added together while a 30-min preexposure to xanthine/xanthine oxidase before NAC or DEM slightly attenuated the response (Fig. 5, Table V).

DISCUSSION

The data presented in this paper support the hypothesis that hepatocyte nitric oxide synthase is regulated by glutathione and reactive oxygen intermediates. The time course of induction of iNOS mRNA by TNFα and IFNγ in hepatocytes correlates closely with the increase in activity. Therefore, in this case, translational regulation is not apparent. This is further supported by the

TABLE III

Inhibition of TNFα Induction of Nitric Oxide Synthase by Rotenone

Treatment	NOS activity[a] nitrite (μM)	NOS mRNA[b] ratio (NOS/β-actin)
Control	5.39 ± 1.27	0.045
Rotenone (10 μM)[c]	−0.48 ± 0.69	0.043
TNFα (1 μg/ml)	23.2 ± 3.49	0.182
+Rotenone	0.62 ± 0.68	0.065
+Fructose	13.0 ± 1.05	0.171

[a] Data represent means ± standard deviations of triplicate samples in an experiment representative of at least two separate experiments.

[b] Values are ratios of densitometric values of bands in Fig. 3.

[c] Hepatocytes treated with rotenone also had 5 mM fructose as an energy alternative.

TABLE IV

Regulation of TNFα Induction of iNOS-Immunoreactive Protein

Treatment	NOS activity[a] nitrite (μM)	NOS protein[b] densitometry units
Control	4.29 ± 1.46	0.04
TNFα (1 μg/ml)	36.9 ± 4.93	1
+BCNU (100 μM) and BSO (200 μM)	16.0 ± 1.96	0.12
+NAC (1 mM)	27.5 ± 2.92	0.57
+DEM (1 mM)	−2.11 ± 1.09	0
+Rotenone (10 μM)	3.67 ± 2.87	0.27

[a] Nitrite values are means ± standard deviations of triplicate samples representative of at least two experiments. Samples were collected at 20 h of treatment.

[b] Hepatocytes were harvested at 20 h. Four micrograms of total protein was loaded/well. Values represent densitometry of bands in Fig. 4.

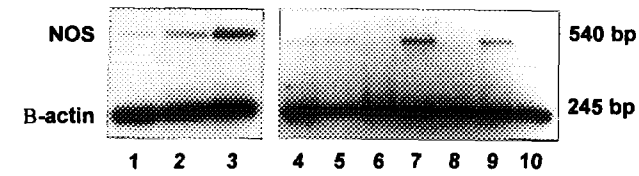

FIG. 5. Xanthine/xanthine oxidase induction of NOS mRNA. NOS and β-actin PCR products from RT-PCR of treated hepatocyte cultures. Cells were treated with xanthine oxidase and xanthine (500 μM) with 1 mM DEM or 1 mM NAC concurrently or 30 min following the treatment with xanthine/xanthine oxidase. Total RNA samples were collected after 18 h and RT-PCR was conducted as described. PCR products were visualized by ethidium bromide staining and densitometry of negative images was performed. Lane 1, control; lane 2, xanthine/xanthine oxidase (2.5 mU/ml); lane 3, xanthine/xanthine oxidase (10 mU/ml); lane 4, control; lane 5, xanthine/xanthine oxidase (10 mU/ml); lane 6, X/XO and DEM (concurrently); lane 7, X/XO and NAC (concurrently); lane 8, X/XO and DEM (30 min after X/XO); lane 9, X/XO and NAC (30 min after X/XO); lane 10, NAC.

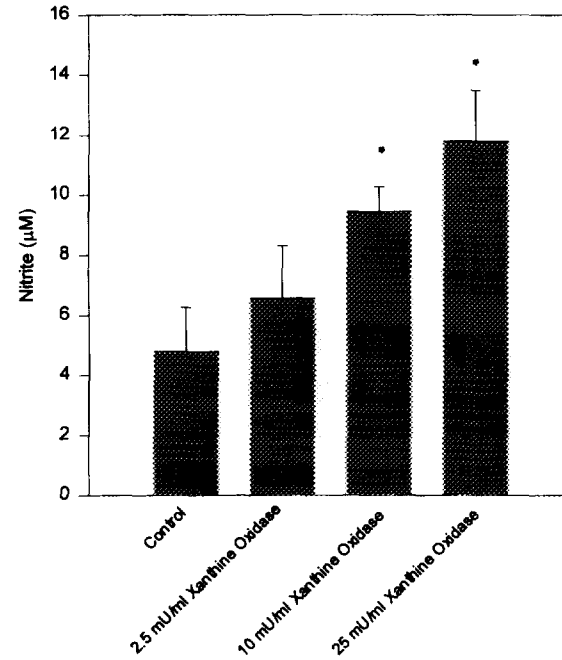

FIG. 6. Xanthine/xanthine oxidase induction of NOS activity. Hepatocytes were treated with 500 μM xanthine and increasing concentrations of xanthine oxidase. Media samples were collected at 18 h and nitrite was measured by colorimetric analysis. Values are the mean ± SEM of four experiments. *Values significantly different from control using Student's t test, $P < 0.01$.

close correlation between immunoreactive protein, mRNA, and activity levels. Because of these close correlations, NOS activity was subsequently assessed by nitrite accumulation in hepatocyte culture medium, although activity may be underestimated under oxidative conditions. While the time course studies were conducted with both TNFα and IFNγ, subsequent induction mechanism studies were conducted with TNFα alone since this cytokine is primarily associated with oxidative stress and studies with multiple cytokines may confound data interpretation. TNFα induces oxidative stress in cultured mouse hepatocytes (48) and in rat hepatocytes as measured by efflux of oxidized glutathione (data not shown). The mitochondrial electron transport chain was explored as one source of ROI frequently associated with TNFα exposure. Inhibitors of complex I, rotenone and amobarbital, reduce ROI generation by partially blocking the flow of electrons to coenzyme Q, which can subsequently donate electrons to oxygen to generate superoxide (49). Rotenone and amobarbital both reduced iNOS induction by TNFα. While the exact mechanism of TNFα's effects on mitochondria has yet to be elucidated, this is consistent with reports that ROIs generated from mitochondrial electron transport are involved in TNFα induction of interleukin-6 (23). Oxidative stress also stimulates the induction of interleukin-8 (24, 25) and vascular cell adhesion molecule (26). When hepatocyte cultures were concurrently treated with TNFα and antioxidants, iNOS induction was reduced. Trolox, the water-soluble analogue of α-tocopherol was more effective at blocking the increase in NOS activity than desferrioxamine, an iron chelator which blocks the iron catalyzed Haber–Weiss conversion of hydrogen peroxide and superoxide to hydroxyl radical. This suggests that while hydroxyl radical may be involved, it is not the primary mediator of the oxidative response in this system. Dithiothreitol, a thiol antioxidant, also effectively reduced the induction of NOS by TNFα. These studies show that iNOS gene transcription in response to TNFα is regulated by a transduction pathway that is sensitive to antioxidants.

TABLE V

Induction of Nitric Oxide Synthase by a Superoxide Generating System

Treatment[a]	NOS activity[b] nitrite (μM)	NOS mRNA[c] ratio (NOS/β-actin)
Control	9.37 ± 1.98	0.043
X/XO (2.5 mU/ml)	7.28 ± 0.72	0.148
X/XO (10 mU/ml)	11.29 ± 0.16	0.323
Control	9.99 ± 1.53	0.038
X/XO	11.90 ± 0.27	0.070
+DEM (concurrent)	0.59 ± 0.57	0.007
+NAC (concurrent)	15.56 ± 0.42	0.191
+DEM (after 30 min)	1.23 ± 0.27	0.020
+NAC (after 30 min)	16.10 ± 0.96	0.142
NAC	11.72 ± 1.41	0

[a] Hepatocytes were treated with 500 μM xanthine and 10 mU/ml xanthine oxidase. NAC and DEM were added at a final concentration of 1 mM.

[b] Data represent means ± standard deviations of triplicate samples in an experiment representative of at least two separate experiments.

[c] Values are ratios of densitometric values of bands in Fig. 5.

To determine if other sources of ROI would effectively induce iNOS, hepatocyte cultures were exposed to extracellular superoxide generated by xanthine/xanthine oxidase. iNOS mRNA and activity were induced in hepatocytes by this source of extracellular superoxide. These studies showed that ROI alone are capable of inducing iNOS. Because of NO's potential to both protect against and stimulate ROI cytotoxicity by scavenging superoxide, iNOS induction may act to regulate the effects of ROI generation. This may be especially important in the oxidative burst and leukocyte recruitment of ischemia–reperfusion events. In fact, the addition of NO-generating compounds to a myocardial ischemia–reperfusion model reduced the area of damage. This protection was attributed to decreased neutrophil infiltration (50, 51).

Reduced thiols are also required for iNOS induction since agents which deplete GSH block induction by both TNFα and superoxide generation. These data suggest a two-step mechanism of induction, one requiring ROI generation and the other reduced glutathione. Since ROI generation oxidizes cellular thiols, and thiols, including glutathione, act as antioxidants this two-step mechanism is linked by the balance of prooxidants and antioxidants. This may allow for very precise tuning of the inductive process. Our studies indicate this sensitivity by the apparent inconsistency of the effects of thiol additions. While under normal conditions, the addition of NAC had variable effects, causing slight increases or decreases in NOS activity or induction, under the highly oxidative conditions of the superoxide generating system, NAC addition stimulated NOS induction. Conversely, the addition of dithiothreitol, a more direct thiol antioxidant, consistently decreased NOS induction. Because the combination of Trolox and BCNU inhibits both steps of the induction process, the decrease in NOS induction is greater than with either agent alone.

Although additional experiments, such as nuclear run-on analysis, are necessary to establish whether steady-state mRNA increases are due to altered mRNA transcription or stability, recent studies have linked oxidative stress, thiols, and antioxidants to the regulation of inducible genes by AP-1 and NF-κB transcription factors. The Fos and Jun subunits of AP-1 are induced in response to oxidative stress in endothelial cells (33), while increased DNA binding by AP-1 occurs in response to NAC and pyrrolidine dithiocarbamate and induction of thioredoxin (35). Similarly, NF-κB is activated by oxidative stress to shed its inhibitory protein and translocate to the nucleus for DNA binding (52). Mediators which increase cellular thiol levels like *N*-acetylcysteine and α-lipoic acid block activation of NF-κB (35, 36). This is in direct contrast to studies in which reduction of NF-κB cysteines is crucial for DNA binding (34). It may be that activation of transcription by these transcription factors is a two-step process, in which the first step requires oxidative stress for activation or expression of these transcription factors and the second step requires reduced thiols for DNA binding. Consequently, if activation is blocked by the addition of a strong antioxidant it makes evaluation of subsequent DNA binding difficult. Both of these transcription factors have been linked to NOS induction. IL-1β induction of NOS in insulin-producing cells is preceded by c-*fos* expression and pyrrolidine dithiocarbamate decreases NOS activity in murine macrophages (15, 39). The increased response of hepatocytes treated with NAC together with xanthine/xanthine oxidase compared to those treated with NAC 30 min later suggests that glutathione is important in the initiation of iNOS gene transcription. Since the promoter region of macrophage iNOS contains both AP-1 and NF-κB elements, these mechanisms help to explain the duality of NOS induction in hepatocytes by ROI and thiols. However, to define the direct effects of these mediators on induction, studies of their effects on the *cis*- and *trans*-acting elements of transcription must be conducted.

The role of nitric oxide in cell injury is still unclear, but its ability to scavenge superoxide anion to eliminate the cytotoxicity of ROI (27), as well as its vasculature effects and ability to decrease leukocyte adherence (24, 25), suggests that NOS induction may protect against immunologically induced oxidative bursts which occur in response to injury. However, the reaction of nitric oxide with ROI to produce peroxynitrite (28) may contribute to cell injury—especially macrophage-mediated cell killing. It is possible that intra- and extracellularly generated ROI can react with NO to exert different effects. Additional experiments are required to adequately appreciate the significance of the present observations that ROI regulate the gene expression of iNOS.

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