Serine/threonine phosphorylation regulates HNF-4α-dependent redox-mediated iNOS expression in hepatocytes

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Guo, Hongtao, Jumping Wei, Yusuke Inoue, Frank J. Gonzalez, and Paul C. Kuo. Serine/threonine phosphorylation regulates HNF-4α-dependent redox-mediated iNOS expression in hepatocytes. Am J Physiol Cell Physiol 284: C1090–C1099, 2003. First published December 4, 2002; 10.1152/ajpcell.00394.2002.—Nitric oxide (NO), endogenously synthesized by inducible NO synthase (iNOS), serves antioxidant and antiapoptotic functions in settings characterized by oxidative stress and proinflammatory cytokines such as sepsis and shock. However, the redox-sensitive mechanisms regulating hepatocyte expression of iNOS are largely unknown. In interleukin-1β (IL-1β)-stimulated hepatocytes exposed to superoxide, we demonstrate that hepatocyte nuclear factor-4α (HNF-4α) acts as an activator of redox-associated hepatocyte iNOS expression at the level of protein, mRNA, and promoter activation. In the absence of HNF-4α, this redox-mediated enhancement is ablated. HNF-4α functional activity is associated with a unique serine/threonine kinase-mediated phosphorylation pattern. This suggests that a redox-sensitive kinase pathway targets HNF-4α to augment hepatocyte iNOS expression. Previous studies have not addressed a redox-dependent kinase signaling pathway that targets HNF-4α and enhances hepatocyte iNOS gene transcription. A unique pattern of phosphorylation determines HNF-4α activity as a trans-activator of IL-1β-mediated hepatocyte iNOS expression in the presence of oxidative stress. kinase; phosphorylation; nitric oxide; Cre-lox; transcription; inducible nitric oxide synthase; hepatocyte nuclear factor-4α.

IN THE PRESENCE OF OXIDATIVE STRESS, the hepatocellular redox state upregulates inducible nitric oxide synthase (iNOS) expression as an antioxidant function. In IL-1β-treated rat hepatocytes, we have demonstrated that iNOS gene transcription and promoter activity are increased by oxidative stress mediated by peroxide, superoxide, or acetaminophen. (10, 23, 24, 26, 31) Subsequently, in IL-1β-stimulated rat hepatocytes exposed to superoxide, we identified a redox-sensitive DR1 cis-acting activator element (nt −1,327 to nt −1,315) in the iNOS promoter: AGGTCAGGGG(T/A)CA. It also binds several different coactivators in the absence of exogenously added ligand (7, 9, 11, 16, 17, 20, 21, 37, 40). HNF-4α DNA binding activity and transactivation potential are tightly regulated by its state of phosphorylation and acetylation. Although HNF-4α activity is regulated by posttranslational modification, redox-mediated posttranslational phosphorylation of HNF-4α has not been examined in the context of hepatocyte iNOS expression. In this article, we characterize the function of HNF-4α in redox-dependent hepatocyte iNOS expression and demonstrate a crucial functional role for the HNF-4α phosphorylation state. Our results suggest that the redox-sensitive increase in hepatocyte iNOS expression is mediated through a kinase pathway that targets HNF-4α as a transcriptional activator.

MATERIALS AND METHODS

Materials. The rat hepatocyte iNOS promoter (GenBank X95629) was a gift from Prof. W. Eberhardt (University of Basel, Switzerland). The HNF-4α expression vector was a gift from Dr. Francis M. Sladek (University of California, Riverside, CA). Hepatocytes from conditional HNF-4α knockout mice (HNF-4α<sup>fl/fl AlbCre<sup>+/−</sup></sup>) were isolated in the laboratory of Drs. Yusuke Inoue and Frank J. Gonzalez (National Institutes of Health, Bethesda, MD). Dominant-negative (DN)-HNF-4α that exhibits defective DNA binding as the result of a mutation at thymine-316 was a gift from Dr. Haiyan Wang, Geneva, Switzerland.

Cell culture. Male NIH mice fed water and chow ad libitum were used for hepatocyte isolation as described by Schuetz et al. (36). Hepatocyte purity was assessed by leukocyte esterase staining and CD68 immunohistochemistry, whereas viability was assessed by trypan blue exclusion. Preparations were routinely >90% viable and >99.5% pure. ANA-1 mac-

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roporphages were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Induction of NO synthesis. IL-1β (1,000 U/ml) was used in the absence of FCS to induce NO synthesis. In selected instances, interferon-γ (IFN-γ; 100 U/ml) or tumor necrosis factor (TNF-α; 500 U/ml) was substituted for IL-1β as alternative induction agents for iNOS. 1,2,5-benzenetriol (BZT; 100 µM), an autocalytolytic source of superoxide at pH 7.4, was added to induce oxidative stress. After incubation for 6 h at 37°C in 5% CO₂, the supernatants and cells were harvested for assays.

Assay of NO production. NO released from cells in culture was quantified by measurement of the NO metabolite, nitrite. Cell culture medium (50 µl) was removed from culture dish and centrifuged; the supernatants were mixed with 50 µl of sulfanilamide (1%) in 0.5 N HCl. After a 5-min incubation at room temperature, an equal volume of 0.02% N-(1-naphthyl) ethylenediamine was added. After incubation for 10 min at room temperature, the absorbance of samples at 540 nm was compared with that of an NaNO₃ standard on a Micro-plate reader.

Immunoblot analysis. Cells or cell nuclei were lysed in buffer (0.8% NaCl, 0.02 KCl, 1% SDS, 10% Triton X-100, 0.5% sodium deoxycholic acid, 0.144% Na₂HPO₄, and 0.024% KH₂PO₄, pH 7.4) and centrifuged at 12,000 g for 10 min at 4°C. Protein concentration was determined by absorbance at 650 nm using protein assay reagent (Bio-Rad). Membranes were incubated with rabbit polyclonal antibody directed against human HNF-4α (Santa Cruz Biochemicals, Santa Cruz, CA), rabbit polyclonal antibody directed against human iNOS (Santa Cruz Biochemicals), phosphotyrosine antibodies, PY350 (Santa Cruz Biotechnology) and 4G10 (Upstate Biotechnology, Waltham, MA), or 61 antibody, PY350 (Santa Cruz Biotechnology) and 4G10 (Upstate Biotechnology, Waltham, MA), or 61 antibody, PY350 (Santa Cruz Biotechnology) and 4G10 (Upstate Biotechnology, Waltham, MA), or 61 antibody, PY350 (Santa Cruz Biotechnology) and 4G10 (Upstate Biotechnology, Waltham, MA), or 61 antibody, PY350 (Santa Cruz Biotechnology) and 4G10 (Upstate Biotechnology, Waltham, MA), or 61 antibody, PY350 (Santa Cruz Biotechnology) and 4G10 (Upstate Biotechnology, Waltham, MA). Following incubation with the primary antibody, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After an additional three washings, bound peroxidase activity was detected by the enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ).

RNA preparation and Northern blot analysis. Total RNA was isolated using TRIzol reagent (GIBCO BRL, Rockville, MD). The RNA samples (10 µg/lane) were fractionated by electrophoresis on a 1% agarose formaldehyde gel and transferred to a Hybond-C nylon membrane (Amersham Pharmacia). A 32P-dATP-labeled probe was constructed based on the rat iNOS cDNA sequence (GenBank AJ230461). Hybridization was performed at 42°C for 24 h in ULTRAhyb hybridization buffer (Ambion, Austin, TX). After hybridization, filters were washed twice and subjected to autoradiography. cDNA probes were prepared by random primer labeling, followed by purification using a Sephacry G-50 mini-column (BioMax, Odenton, MD).

Transient transfection analysis of the iNOS promoter. ANA-1 macrophages and hepatocytes were transfected using the lipofectamine technique (29). After cells were washed twice with medium, 10 µg of plasmid DNA containing the iNOS promoter construct (1,845 bp; GenBank X95629) was added per 10⁶ cells in 1 ml of medium without serum. In selected instances, an HNF-4α expression vector (10 µg) or a DN-HNF-4α expression vector was cotransfected with the iNOS promoter plasmid construct. The supernatant was assayed for CAT activity using a CAT ELISA technique (Boehringer Mannheim, Indianapolis, IN). Transfection efficiency was normalized by cotransfection of a β-galactosidase reporter gene with a constitutively active early SV40 promoter. All values are expressed as pg CAT/mg protein.

Mutagenesis of iNOS promoter. PCR-based site directed mutagenesis was performed on the two NF-κB sites (NF-κB site 1 at nt −114 and NF-κB site 2 at nt −1044) and the HNF-4α site in the context of the full-length iNOS promoter plasmid to generate mutant plasmids. The mutations were GGGGACCTC to GaaagCTC at NF-κB nt −1044, GGGGATTT to GaagTTT at NF-κB nt −114, and AGGTCAAGGGGACA to AGGTCAAGatACA at the HNF-4α site.

Chromatin immunoprecipitation assay. Chromatin from hepatocytes was fixed and immunoprecipitated using the ChIP assay kit (Upstate Biotechnology) as recommended by the manufacturer. The purified chromatin was immunoprecipitated using 10 µg of anti-HNF-4α (Santa Cruz) or 5 µl of rabbit nonimmune serum. The input fraction corresponded to 0.1 and 0.05% of the chromatin solution before immunoprecipitation. After DNA purification, the presence of the selected DNA sequence was assessed by PCR. The primers used were as follows: CCAATTCAGCTGATGGTGA and GCTCATGTGGGAGATGCT, and the PCR product was 275 bp in length. The PCR program was: 94°C × 4 min, followed by 94°C × 45 s, 55°C × 45 s, and 72°C × 45 s for a total of 28 cycles, and then 72°C × 7 min. PCR products were resolved in 10% acrylamide gels. The average size of the sonicated DNA fragments subjected to immunoprecipitation was 500 bp as determined by ethidium bromide gel electrophoresis. ChIP assays at addressing NF-κB nt −1,044 utilized PCR primers: TGTACCCTTAGACAAGGAAACA and TGAGTTCTAGAGACCAACTAGGGCT, while NF-κB nt −114 utilized AACTCGAAAATGAGAGAACAGACAG and ATGGTCATATGCATCTCGTGG.

One-dimensional phosphopeptide mapping. Cells were grown to a subconfluent state and incubated in the presence of [γ-32P]ATP (0.3 mCi/ml). HNF-4α was immunoprecipitated, as previously described, and subjected to SDS-PAGE (10, 23). The relevant band was excised, digested with S. aureus V8 protease (5 µg/slice), and separated on an 8–15% polyacrylamide gradient gel. Autoradiography was then performed.

Statistical analysis. Data are expressed as means ± SE. Analysis was performed using the Students t-test. P values <0.05 were considered significant.

RESULTS

HNF-4α and redox-enhanced iNOS expression in hepatocytes. In a model of rat hepatocytes, we have previously demonstrated that IL-1β-mediated iNOS expression is significantly increased in the presence of superoxide- or peroxide-induced oxidative stress (23–25). To determine the role of HNF-4α in this redox-enhanced iNOS expression, we utilized a mouse model to take advantage of an HNF-4α Cre-lox conditional knockout (KO) system (12). HNF-4α KO is otherwise embryonically lethal. Hepatocytes were isolated from wild-type (WT) and HNF-4α Cre-lox knockout mice by the technique of retrograde vena caval perfusion. Cells were stimulated with IL-1β (1,000 U/ml) in the presence and absence of BZT (100 µM), an autocatalytic source of superoxide at physiological pH. After a 6-h period of incubation, culture medium levels of the NO metabolite, nitrite, and cellular expression of iNOS protein and mRNA were determined (Fig. 1).
Unstimulated cells served as controls. In both WT and HNF-4 KO animals, IL-1β stimulation produced medium levels of nitrite that were eightfold higher than controls. Addition of BZT with IL-1β doubled nitrite expression in WT animals only. BZT alone did not alter nitrite levels in either WT or HNF-4 KO cells. Similarly, IL-1β induced expression of iNOS protein and mRNA in both WT and HNF-4 KO hepatocytes. However, IL-1β + BZT significantly augmented iNOS protein and mRNA levels in WT cells only. In the absence of HNF-4α, BZT did not augment IL-1β-mediated hepatocyte iNOS expression.

To examine the contribution of HNF-4α to redox-sensitive iNOS promoter activity, a CAT reporter plasmid construct containing the full-length rat hepatocyte iNOS promoter was transfected into WT and HNF-4 KO murine hepatocytes using the lipofectamine technique (Fig. 2). In WT and HNF-4 KO hepatocytes, IL-1β stimulation increased CAT expression by tenfold. In contrast, in WT cells only, IL-1β + BZT treatment increased CAT expression by fivefold over that noted with IL-1β. In the HNF-4 KO cells, IL-1β + BZT did not alter iNOS promoter activity compared with
that of IL-1β stimulation alone. BZT alone did not alter CAT expression. This indicates that HNF-4α is essential for redox-mediated enhancement of hepatocyte iNOS promoter activity. These data from WT murine hepatocytes duplicate our previous observations in primary rat hepatocytes.

To further corroborate the role of HNF-4α in this system, the DN-HNF-4α expression vector was transfected into WT murine hepatocytes. DN-HNF-4α exhibits defective DNA binding as the result of a mutation at thymine-316. NO synthesis, as normalized for transfection efficiency, was measured in cells exposed to IL-1β (1,000 U/ml) in the presence and absence of BZT (100 μM). Unstimulated cells served as controls. In the absence of DN-HNF-4α, NO production was 6.3 ± 2.1, 43.2 ± 4.7, 5.5 ± 3.1, and 93 ± 6.7 nmol/mg protein in control, IL-1β, BZT, and IL-1β + BZT cells (P < 0.01 IL-1β vs. control, BZT, and IL-1β + BZT cells; P < 0.01 IL-1β vs. IL-1β + BZT; n = 4). In the presence of DN-HNF-4α, NO production in control, IL-1β, and BZT stimulated cells was not statistically different from those noted in the absence of DN-HNF-4α. However, NO production in IL-1β + BZT cells with DN-HNF-4α was 36.2 ± 4.6, a value that is threefold less than that noted in the absence of DN-HNF-4α (P < 0.01).

DN-HNF-4α was cotransfected with the iNOS-CAT promoter construct into WT murine hepatocytes (Fig. 2). Similar to that noted in WT hepatocytes without DN-HNF-4α, CAT expression in the presence of DN-HNF-4α was not statistically different in control, IL-1β-, and BZT-treated cells. However, in IL-1β + BZT cells with DN-HNF-4α, CAT expression was increased by only 1.5-fold over IL-1β cells (P < 0.02). The BZT-associated augmentation of IL-1β-induced iNOS promoter activation was decreased by threefold in the presence of DN-HNF-4α (P < 0.02). These data suggest that HNF-4α is required for redox enhancement of iNOS promoter activity in murine hepatocytes.

HNF-4α and iNOS activity in ANA-1 macrophages. Additional studies were performed in ANA-1 murine macrophages to support the role of HNF-4α in the upregulation of iNOS promoter activity in the setting of IL-1β and BZT stimulation. ANA-1 cells do not express HNF-4α under control, IL-1β, BZT, and/or IL-1β + BZT treatment conditions. In ANA-1 macrophages, NO production was 10.2 ± 1.7, 24.3 ± 3.2, 9.1 ± 1.9, and 28.4 ± 4.3 nmol/mg protein in unstimulated controls, IL-1β (1,000 U/ml), BZT (100 μM), and IL-1β and BZT cells, respectively. In ANA-1 cells, cotransfection assays were then performed with 1) the iNOS-CAT promoter construct alone, 2) iNOS-CAT and the HNF-4α expression vector, or 3) iNOS CAT with the DN-HNF-4α expression vector (Fig. 3). In iNOS-CAT alone, iNOS + HNF-4, and iNOS + DN-HNF-4 transfection conditions, IL-1β stimulation of ANA-1 cells increased CAT expression by over eightfold compared with unstimulated controls. In the presence of IL-1β + BZT, CAT expression is increased fourfold in iNOS + HNF-4 cells only. In the presence of BZT alone, CAT expression for all three transfection conditions is not significantly different from that of control cells. These data indicate that constitutive HNF-4α expression in ANA-1 cells significantly augments iNOS promoter trans-activation in the setting of IL-1β + BZT stimulation. Interestingly, HNF-4α expression in ANA-1 cells treated with only IL-1β does not increase CAT expression compared with that noted in the absence of HNF-4α expression. This result suggests that oxidative stress is a necessary component of the signal transduction pathway by which HNF-4α augments cytokine-induced iNOS promoter trans-activation.

Mutagenesis of NF-κB and HNF-4α binding sites in the iNOS promoter. To determine whether HNF-4α acts independently of NF-κB, an essential transcription factor for iNOS transcription, the CAT reporter containing the iNOS promoter was mutated at both NF-κB binding sites (nt −1044: GGGGATTTCG to GaaagTTTTCC; nt −114: GGGGACTCTCC to GaaagTTTTCC) and transfected into hepatocytes exposed to IL-1β, BZT, and IL-1β + BZT. Under all treatment conditions, CAT activity was not different from that of unstimulated controls. These data indicate that NF-κB sites are essential for iNOS activation; HNF-4α functions as an activator that is dependent on NF-κB (data not shown).

Another potential mechanism for the action of HNF-4α may lie in augmentation of DNA binding of
NF-κB at either of its two DNA binding sites, nt −965 and nt −109. ChIP assays were performed in WT hepatocytes to examine in vitro NF-κB binding in the hepatocyte iNOS promoter (Fig. 4). NF-κB binding was exhibited at both binding sites; at each site, there was no difference noted between IL-1β and IL-1β + BZT cells. Compared with the extent of NF-κB binding noted in IL-1β cells, this indicates that HNF-4α does not augment NF-κB DNA binding in the presence of IL-1β + BZT. Also, BZT does not alter IL-1β-mediated NF-κB binding. As expected, no NF-κB binding was found in unstimulated controls and BZT cells.

The HNF-4α binding site in the iNOS CAT promoter was mutated, AGGTCA G GGGACA to AGGTCA G catACA, to ablate HNF-4α homodimer binding. Transient transfection assays were then repeated in WT hepatocytes. With the use of this mutated iNOS promoter vector, CAT expression in IL-1β + BZT was not statistically different from that of IL-1β cells (20.3 ± 3.1 vs. 23.2 ± 4.2 pg CAT/mg/β-galactosidase activity). These data indicate that mutation of the HNF-4α DNA binding element ablates the increased iNOS promoter activity seen in the presence of IL-1β + BZT.

Nuclear localization of HNF-4α. HNF-4α is primarily localized in the hepatocyte nucleus (38). To determine whether IL-1β and/or BZT stimulation alters nuclear localization of HNF-4α, immunoblots were performed with nuclear protein isolated from control-, IL-1β-, BZT-, and IL-1β + BZT-treated hepatocytes (Fig. 5). Nuclear levels of HNF-4α were not altered by IL-1β or BZT stimulation at 6 or 12 h after treatment. These data demonstrate that nuclear localization of HNF-4α is not altered by IL-1β and/or BZT stimulation. Cytoplasmic expression of HNF-4α was undetectable at all time points and treatment conditions (data not shown).

One-dimensional phosphopeptide mapping of HNF-4α. It is well known that tyrosine and serine/threonine phosphorylation (or dephosphorylation) of transcription factors alters activator subcellular localization, DNA binding properties, and transactivation potential. To determine whether distinctive phosphorylation patterns of HNF-4α are present among the various treatment conditions, one dimensional phosphopeptide mapping of HNF-4α was performed in control, IL-1β-, BZT-, and IL-1β + BZT-treated hepatocytes in the presence of [γ-32P]ATP. Immunoprecipitated HNF-4α was then partially digested with V8 protease and separated with 15% SDS-PAGE (Fig. 6). These results demonstrate that HNF-4α is constitutively phosphorylated in unstimulated cells, and the phosphorylation pattern of HNF-4α is unaltered in the presence of IL-1β or BZT stimulation. In contrast, IL-1β + BZT

Fig. 6. One-dimensional phosphopeptide map of HNF-4α V8 protease digest. Cells were grown to a subconfluent state and incubated in the presence of [γ-32P]ATP (0.3 mCi/ml). Cells were stimulated with IL-1β (1,000 U/ml) in the presence and absence of BZT (100 μM). Unstimulated cells served as controls. After 6 h, HNF-4α was immunoprecipitated and subjected to SDS-PAGE (10). The relevant band was excised, digested with S. aureus V8 protease (5 μg/slice), and separated on an 8–15% polyacrylamide gradient gel. Autoradiography was then performed. Blot is representative of 4 experiments. Arrows designate new sites of phosphorylation.
stimulation dramatically alters the pattern and extent of HNF-4α phosphorylation.

The functional consequence of this unique pattern of phosphorylation was then determined. Hepatocytes were stimulated with IL-1β + BZT in the presence and absence of the tyrosine kinase inhibitor, tyrphostin B46 (TYR; 40 μM), and the serine/threonine kinase inhibitor, staurosporine (STA; 1 μM). As generalized kinase inhibition may have untoward effects on transcriptional machinery independent of effects on HNF-4α binding to the iNOS promoter, transient transfection assays were not utilized. Instead, ChIP assays were performed to determine in vivo HNF-4α DNA binding (Fig. 7). In IL-1β + BZT-stimulated hepatocytes, significantly decreased in vivo HNF-4α binding to the iNOS promoter was noted in the presence of kinase inhibition. One-dimensional phosphopeptide mapping of HNF-4α partially digested with V8 protease was then performed to determine the effect of STA and TYR on the IL-1β + BZT pattern of phosphorylation (Fig. 8). These results indicate that the distinct pattern of phosphorylation in IL-1β + BZT cells is ablated in the presence of kinase inhibitors and is not different from that of IL-1β cells. These results indicate that in vivo HNF-4α DNA binding 1) is associated with a specific phosphorylation pattern, and 2) requires serine/threonine and/or tyrosine phosphorylation. Further one-dimensional phosphopeptide mapping of HNF-4α was performed using STA alone or TYR alone in the presence of IL-1β + BZT stimulation. These results suggest that the unique pattern of HNF-4α phosphorylation is inhibited in the presence of the STA-mediated serine/threonine kinase inhibition.

After hepatocyte treatment with IL-1β, BZT, or IL-1β + BZT, HNF-4α was immunoprecipitated and subjected to immunoblot analysis using phosphotyrosine antibodies, PY350 (Santa Cruz Biotechnology) and 4G10 (Upstate Biotechnology, Waltham, MA), or phosphoserine/threonine antibodies, 61–8,100 antiphosphoserine antibody (Zymed) and 71–8,200 antiphosphothreonine antibody (Zymed) (Fig. 9). In the presence of phosphotyrosine antibodies, no differences were noted. In contrast, a tenfold increase in labeling in IL-1β + BZT cells was noted in the presence of the antiphosphoserine and antiphosphothreonine antibodies, suggesting that the unique HNF-4α phosphorylation pattern noted in the presence of IL-1β + BZT stimulation is the result of serine/threonine phosphorylation.
To confirm that serine/threonine phosphorylation mediates HNF-4α DNA binding after cytokine and oxidant stimulation, hepatocytes were stimulated with IL-1β + BZT in the presence and absence of the serine/threonine kinase inhibitor, STA (1 μM). ChIP assays were repeated, as above. In this setting, STA treatment ablated IL-1β + BZT induced HNF-4α DNA binding to the iNOS promoter (Fig. 7).

**NO and HNF-4α-enhanced iNOS expression.** To determine whether NO plays a role in HNF-4α-enhanced iNOS promoter activity, transient transfection studies with the iNOS promoter in WT hepatocytes were repeated in the presence of a competitive substrate inhibitor of iNOS, L-N(1-iminoethyl)lysine hydrochloride (L-NIL; 100 μM) (Fig. 10). In this setting, inhibition of iNOS activity did not alter the enhanced iNOS promoter activity noted in the presence of IL-1β + BZT. Also, in IL-1β + BZT cells, ChIP assays did not demonstrate a significant difference in HNF-4α binding in the presence or absence of L-NIL (data not shown). These results suggest that NO does not play a feedback regulatory role in redox enhanced hepatocyte iNOS promoter activity or HNF-4α DNA binding.

**Oxidative stress and alternative inducers of hepatocyte iNOS.** We have previously demonstrated that alternative forms of oxidative stress, such as acetaminophen and peroxide, can augment IL-1β-mediated iNOS promoter activity in hepatocytes (25, 31). To determine the potential role of HNF-4α in TNF-α- and/or IFN-γ-induced iNOS expression, NO production was measured in WT murine hepatocytes exposed to TNF-α or IFN-γ in the presence or absence of BZT. In selected instances, the serine/threonine kinase inhibitor STA (1 μM) was added, or the DN-HNF-4α and cytokine + BZT or TNF-α + STA; ‡P < 0.01 vs. cytokine + BZT.

**Table 2. TNF and IFN-induced NO production in murine hepatocytes**

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<th>TNF-α</th>
<th>IFN-γ</th>
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<tr>
<td>Unstimulated</td>
<td>5.3 ± 2.1</td>
<td>4.7 ± 1.9</td>
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<tr>
<td>Cytokine-stimulated</td>
<td>36.1 ± 4.2*</td>
<td>24.9 ± 2.8*</td>
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<td>Cytokine + BZT</td>
<td>75.9 ± 6.3†</td>
<td>49.5 ± 5.8†</td>
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<tr>
<td>Cytokine + BZT + DN-HNF-4α</td>
<td>41.2 ± 3.8‡</td>
<td>27.9 ± 2.7‡</td>
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<tr>
<td>Cytokine + BZT + STA</td>
<td>34.9 ± 3.1§</td>
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Hepatocytes were isolated from wild-type mice by the technique of retrograde vena caval perfusion. Cells were stimulated with tumor necrosis factor-α (TNF-α) (500 U/ml) or interferon-γ (IFN-γ) (100 U/ml) in the presence and absence of BZT. In selected instances, the serine/threonine kinase inhibitor, staurosporine (STA; 1 μM) was added, or the dominant-negative HNF-4α (DN-HNF-4α) expression vector was transfected. After a 6-h period of incubation, medium levels of nitrite, the NO metabolite, were determined. Data are expressed as means ± SE of 3 experiments performed in triplicate. *P < 0.01 vs. unstimulated cells; †P < 0.01 vs. cytokine-stimulated, cytokine + BZT + DN-HNF-4α, and cytokine + BZT + STA; ‡P < 0.01 vs. cytokine + BZT.

**DISCUSSION**

In this study, we demonstrate that HNF-4α acts as an activator of redox-associated hepatocyte iNOS expression at the level of protein, mRNA, and promoter activation. In the absence of HNF-4α, this redox-mediated enhancement is ablated, as demonstrated by the HNF-4 KO murine hepatocytes and the ANA-1 macrophage HNF-4α/DN-HNF-4 cotransfection studies. In addition, in the setting of IL-1β + BZT, HNF-4α functional activity is associated with a unique serine/threonine phosphorylation pattern that is independent of NO. This would indicate that there exists a redox-sensitive serine/threonine kinase pathway that targets HNF-4α to augment hepatocyte iNOS expression.
the liver (Timothy R. Billiar, unpublished observation). At Duke University Hospital, administration of intravenous methylene blue, an inhibitor of NO function, prior to reperfusion of hepatic allografts (n = 6) is associated with a fourfold increase in peak transaminase values, suggesting increased hepatocyte injury (Jacques Somma, Duke University Medical Center, unpublished observations). However, despite its importance, little is known about redox regulation of hepatocyte iNOS expression.

In the presence of oxidative stress, the hepatocellular redox state upregulates iNOS expression as an antioxidant function. In IL-1β-treated rat hepatocytes, we showed that iNOS gene transcription and promoter activity are increased by oxidant stress mediated by peroxide, superoxide, or acetaminophen (10, 23, 24, 26, 31). Subsequently, in IL-1β-stimulated rat hepatocytes exposed to superoxide, we identified a redox-sensitive DR1 cis-acting activator element (nt −1,327 to nt −1,315) in the iNOS promoter: AGGTCA G GGGACA. The corresponding transcription factor was isolated by DNA affinity chromatography, sequenced, and identified to be HNF-4α (10, 23).

Although fatty acyl coenzyme A thioesters have been proposed as ligands for HNF-4α, they do not affect binding of coactivator or corepressor in vitro, and it remains unclear whether they are truly ligands (4, 13, 14, 37). As a result, HNF-4α remains classified as an orphan nuclear receptor. HNF-4α exhibits distinct functional domains typical of nuclear hormone receptors. In the NH2-terminal region, AF-1 functions as a constitutive activator of transcription, binds multiple protein targets, and may recruit general transcription factors and chromatin remodeling proteins. A highly conserved DNA binding domain (DBD), composed of two zinc-coordinated modules, is responsible for specific binding to cognate response elements. A flexible hinge region separates the DBD and the putative ligand-binding domain (LBD). The adjacent region also contains the dimerization interface and the transactivation function AF-2. In the COOH terminus, the F domain is a highly variable repressor region (7, 9, 11, 20, 21, 37, 40). Posttranslational modification by phosphorylation is known to alter HNF-4α DNA-binding activity, transactivation potential, nuclear translocation, and/or degradation (18, 28, 39, 41, 42).

Cellular stress such as ROS and proinflammatory cytokines regulate intracellular signal transduction cascades and modulate transcription factor activity through calcium signaling, protein kinase, and protein phosphatase pathways. ROS may directly activate kinases by altering thiol-dependent protein-protein interactions, inhibiting phosphatase activity by oxidation of an active site cysteine residue and/or stimulating proteolysis of kinase regulatory proteins. Ultimately, redox regulated tyrosine- and serine/threonine-phosphorylation of transcription factors through tyrosine kinase and stress-activated protein kinase (SAPK) activities alter transcription factor subcellular localization, DNA binding properties, and transactivation potential. In particular, the SAPKs (ERK-1/2,
BMK1, JNK, and p38 isoforms) are often the ultimate (and best-characterized) regulatory proteins in a series of sequential kinase reactions that target transcription factor modification in the setting of cellular stresses similar to those of our model (1, 2, 8, 22).

Redox-mediated posttranslational modification of HNF-4α has never been previously addressed. HNF-4α DNA binding activity and transactivation potential are tightly regulated by its state of phosphorylation and acetylation. HNF-4α potentially contains 21 serine, 6 threonine, and 7 tyrosine phosphorylation sites (3, 18). In COS 7 cells, serine/threonine phosphorylation of HNF-4α increases affinity and specificity of DNA binding by altering its tertiary structure (18). Tyrosine phosphorylation of HNF-4α is required for DNA binding, transactivation, and subnuclear localization in primary cultures of rat hepatocytes (21). In contrast, protein kinase A-dependent phosphorylation within the DBD inhibits DNA binding in HepG2 and Cos 1 cells (41). In vivo experiments support the functional importance of HNF-4α phosphorylation state. In a murine model of 15% burn injury, hepatocyte HNF-4α DNA binding is enhanced by serine phosphorylation (Ref. 5 and Peter A. Burke, M.D., personal communication). Dietary protein restriction or overnight fasting decreases hepatic HNF-4α DNA binding activity as a result of decreased serine/threonine phosphorylation (38, 41). Acetylation of HNF-4α is crucial for proper nuclear retention, DNA binding, and promoter activation in COS 1 and NIH 3T3 cells (18). Although HNF-4α activity is certainly regulated by posttranslational modification, redox-mediated posttranslational phosphorylation or acetylation of HNF-4α has not been examined.

In summary, experimental findings in models of sepsis and shock suggest that NO synthesis serves an antioxidant function that is redox modulated. However, the relationship between oxidative stress and iNOS gene transcription remains unexplored. In IL-1β-stimulated hepatocytes, we have demonstrated that 1) an HNF-4α functions as a redox-sensitive trans-activator of iNOS transcription, and 2) HNF-4α exhibits a unique redox-dependent phosphorylation pattern. Future experiments are required to characterize the molecular regulatory pathway by which HNF-4α integrates multiple extra- and intra-cellular signals mediated by kinase cascades to precisely regulate redox-dependent hepatocyte iNOS gene expression. These considerations are crucial to our understanding of HNF-4α as a novel and, as yet, poorly described redox-sensitive mechanism that regulates hepatocyte iNOS expression as an antiapoptotic and antioxidant function in the setting of sepsis and shock.

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