Hepatocyte Nuclear Factor-4α Mediates Redox Sensitivity of Inducible Nitric-oxide Synthase Gene Transcription

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The underlying redox-sensitive mechanisms that regulate hepatocyte expression of inducible nitric-oxide synthase (iNOS) and its antioxidant functions are largely unknown. We have demonstrated previously that oxidative stress induced by benzenediazonium-mediated superoxide production increases interleukin-1β-induced iNOS protein synthesis, steady state iNOS mRNA expression, NO production, and trans-activation of the iNOS promoter in primary cultures of rat hepatocytes (9, 10). Using a transient expression assay in IL-1β- and BZT-stimulated hepatocytes, we had identified a cis-regulatory element (ARE) in the rat hepatocyte iNOS promoter, in addition to an ARE-like regulatory element trans-activated by the nuclear factor-4α (HNF-4α) transcription factor. In the present study, we have shown that binding of HNF-4α to the ARE is increased by superoxide production and that HNF-4α is the transcription factor responsible for redox regulation of the iNOS promoter.

This article has been retracted by the publisher. The same data were reused to represent different experimental conditions. Specifically, in Fig. 1, lane 3 was reused in lane 11, and lanes 9 and 10 were reused in lanes 17 and 18. Additionally, lane 3 from Fig. 1 was reused in lane 2 from Fig. 3. Finally, lanes 2–4 from Fig. 1 were flipped horizontally and reused in lanes 5–7 from the left panel of Fig. 4.
induce oxidative stress. After incubation at 12 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. 50 μl of cell culture medium were removed from culture dish and centrifuged; the supernatants were mixed with 50 μl of sulfanilamide (1%) in 0.5% HCl. After a 5-min incubation at room temperature, an equal volume of 0.2% (N-1-naphthyl)ethylendiamine was added. Following incubation for 10 min at room temperature, the absorbance of samples at 540 nm was compared with that of an NaNO₂ standard on a MAXLine™ microplate reader.

**Gel Shift Assay**—Gel shift assays were performed using 12 μg of nuclear cell extract, purified chromatographic fraction, or HNF-4α peptide. In competitive binding assays, unlabeled mutant oligonucleotides were added to 200 x excess. Supershift assays were performed by the addition of 2 μg of affinity-purified goat polyclonal antibody directed against human HNF-4α (Santa Cruz Biochemicals). Probe was prepared by end-labeling the wild-type 28-bp double-stranded ARE with [γ-32P]ATP (2500 Ci/mmol) using T4 polynucleotide kinase, followed by gel purification on 15% polyacrylamide. Twenty-bp oligonucleotides used as competitors were synthesized to contain double point mutations in relation to the wild-type sequence.

**Purification of ARE Transcription Factor**—The ARE transcription factor was isolated by reacting the biotinylated DNA-protein complex with streptavidin paramagnetic particles (Dynal Biotech Inc.). Nuclei were pelleted by centrifugation (11,700 g) for 10 min at 4 °C, and eluted with one volume of 0.15 M NaCl. The resulting fraction was diluted to 0.1 M NaCl, applied to a DNA cellulose column, and excess nonbinding poly(dI-dC) competitor DNA was added. Following a 5-min incubation at room temperature, an equal volume of 2 M NaCl and excess nonbinding poly(dI-dC) competitor DNA was added. Following a 10-min incubation at 4 °C, the fraction was centrifuged at 12,000 x g for 10 min at 4 °C. Protein concentration was determined by absorbance at 650 nm using protein assay reagent (Bio-Rad). Cell lysate (50 μg/lane) were separated by 12% SDS-PAGE, and the products were electrotransferred to polyvinylidene difluoride membrane (Amersham Biosciences, Inc.). The membrane was blocked with 5% skim milk, PBS, 0.05% Tween for 1 h at room temperature. After being washed three times, blocked membranes were incubated with rabbit polyclonal antibody directed against HNF-4α (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, washed three times in PBS plus 0.05% Tween, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After an additional three washes, bound peroxidase activity were detected by the ECL detection system (Amersham Biosciences, Inc.).

**Co-immunoprecipitation**—Cell culture medium was removed and plates rinsed with PBS at room temperature. All the following steps were performed using ice-cold buffers. 0.6 ml of radiolabeled nuclear lysate (50 μg/lane) were separated by 12% SDS-PAGE, and the products were electrotransferred to polyvinylidene difluoride membrane (Amersham Biosciences, Inc.). The membrane was blocked with 5% skim milk, PBS, 0.05% Tween for 1 h at room temperature. Following washing three times, blocked membranes were incubated with rabbit polyclonal antibody directed against HNF-4α (Santa Cruz Biotechnology). Resuspended protein and the tubes incubated at 4 °C on a rocker to achieve a 1 h equilibrium. The pellet was collected by centrifugation at 60 min at 4 °C, and the supernatant discarded. The pellet rinsed with cold PBS, washed, distributed to 100-mm plates, each with about 500 μg/glane) were separated by 12% SDS-PAGE, and the products were electrotransferred to polyvinylidene difluoride membrane (Amersham Biosciences, Inc.). The membrane was blocked with 5% skim milk, PBS, 0.05% Tween for 4 h at room temperature, washed three times in PBS plus 0.05% Tween, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After an additional three washes, bound peroxidase activity were detected by the ECL detection system (Amersham Biosciences, Inc.).

**Transient Transfection Analysis of the Rat iNOS Promoter**—ANA-1 macrophages and rat hepatocytes were transfected using the DEAE-dextran technique (13). After cells were washed twice with medium, 10 μg of recombinant plasmid DNA containing the iNOS promoter (1.1 kb, GenBank® accession no. X95629) coupled to a chloramphenicol acetyltransferase (CAT) reporter gene was added per 10⁶ cells in 1 ml of medium without serum prewarmed to 37 °C and containing DEAE-dextran (250 μg/ml) and 50 mM Tris, pH 7.4. In selected instances, an HNF-4α expression vector (10 μg) or the mutant HNF-4α (mHNF-4) (S256) was co-transfected with the iNOS promoter plasmid construct. The HNF-4α expression vector was constructed by ligation of the BamHI-HindIII HNF-4α cDNA fragment from pLEN4 ligated into pcDNA3 (Invitrogen). Using the wild-type HNF-4α expression vector, the mutant HNF-4α vector in which aspartate was substituted for a Tyr critical to PC4 binding was prepared using PCR-mediated mutagenesis (14, 15). The suspension was incubated at 37 °C for 45–60 min, followed by a 1-min shock with 100 μg/melSO at room temperature. The fractions were then removed and electrophoresed on a 100–200-μg scale, each with about 5 x 10⁶ cells in 10 ml of complete medium, and incubated at 37 °C in 5% CO₂. At least 24 h later, the medium was changed, and IL-1β or IL-1β + BZT was added. Approximately 14 h later, the cells were washed with ice-cold PBS, resuspended in 0.25 mM Tris, pH 7.8, and subjected to three cycles of freezing and thawing. Lysates were centrifuged (11,700 x g for 10 min at 4 °C); the supernatant was heated at 65 °C for 10 min to inac-
tivate CAT inhibitors and then centrifuged as above. The supernatant was assayed for CAT activity using a CAT enzyme-linked immunosorbent assay technique (Roche Molecular Biochemicals). Transfection efficiency was normalized by co-transfection of a β-galactosidase reporter gene with a constitutively active early SV40 promoter. All values are expressed as picograms of CAT/mg of protein.

**Statistical Analysis**—Data are expressed as means ± S.E. Analysis was performed using Student’s t test. p values less than 0.05 were considered significant.

**RESULTS**

**Mutagenesis of the ARE Binding Site**—Utilizing nuclear protein isolated from rat hepatocytes treated with IL-1β and BZT, gel shift assays with a 32-bp double-stranded DNA probe derived from the iNOS rat hepatocyte promoter (nt −1353 to nt −1322) were performed for the identification of the ARE transcription factor. These probes contain the sequence, AGGTCAAGGGGACA, previously identified as a high affinity binding site for the ARE transcription factor. These probes contains the sequence, AGGTCAGGGGACA, previously identified as a high affinity binding site for the ARE transcription factor (9, 10).

**Isolation and Characterization of ARE Transcription Factor Protein**—Bound ARE complex previously resolved by gel shift analysis was UV cross-linked to a radiolabeled ARE DNA probe. Subtracting the molecular mass of the DNA probe indicates that the molecular mass of the ARE transcription factor protein is ~45–50 kDa. Utilizing the biotin-streptavidin DNA affinity technique with the identified ARE DNA binding sequence, ARE transcription factor was then purified and isolated from nuclear extract isolated from rat hepatocytes stimulated with IL-1β and BZT. A representative Western blot of purified extract is depicted in Fig. 2. Three major bands were identified. A Southwestern blot was performed using purified nuclear extract and radiolabeled DNA probe containing the ARE binding sequence; this demonstrated binding to band 1 alone (Fig. 2). Bands 1, 2, and 3 were excised, renatured, and analyzed by gel shift analysis (Fig. 3). Only band 1 comigrates with the native ARE complex.

**ARE Protein Sequencing and Identification**—Band 1 was excised and subjected to protein sequencing. Analysis of two separate trypsin digests of Band 1 yielded two protein sequences: QCVVDKDKRNQ and TMGNDTSPSEGAN. Both of these peptides were identical matches with HNF-4α (GenBank accession no. P22449). The molecular weight of HNF-4α corresponds to the approximate molecular weight determined from our UV cross-linking studies using the ARE transcription factor and its DNA binding sequence. Gel shift analysis utilizing radiolabeled DNA probe containing the ARE binding sequence was then performed using crude nuclear extract, purified extract (bands 1–3), purified protein (band 1), and a peptide fragment of HNF-4α in the presence and absence of HNF-4α antibody (Fig. 4). Nuclear extract, purified extract (bands 1–3), and purified protein (band 1) from IL-1β- and

**TABLE I**

| Competitor | Wild type | Mutant 1 | Mutant 2 | Mutant 3 | Mutant 4 | Mutant 5 | Mutant 6 | Mutant 7 | Mutant 8 | Mutant 9 | Mutant 10 | Mutant 11 |
|------------|-----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|-----------|-----------|
| Wild type  | TG TG GA GG GC TC AG AG GA CA AT TT | Yes | Yes | Yes | Yes | Yes | No | No | No | No | Yes | Yes |

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**FIG. 1.** Mutational analysis of ARE binding site. Gel shift competition studies were performed using nuclear extract prepared from unstimulated rat hepatocytes and those stimulated with IL-1β (1000 units/ml) and/or BZT (10 μM). Sequence of competitors are listed in Table I. Utilizing nuclear protein isolated from rat hepatocytes treated with IL-1β and BZT, gel shift assays with a 32-bp double-stranded DNA probe derived from the iNOS rat hepatocyte promoter (nt −1353 to nt −1322) were performed for the identification of the ARE transcription factor. These probes contains the sequence, AGGTCAAGGGGACA, previously identified as a high affinity binding site for the ARE transcription factor. Gel shift assays were performed using 12 μg of nuclear cell extract, purified chromatographic fraction, or HNF-4α peptide. In competitive binding assays, unlabeled mutant oligonucleotides were added at 200 μM excess. Supershift assays were performed by the addition of 2 μg of affinity-purified goat polyclonal antibody directed against human HNF-4α (Santa Cruz Biochemicals). Probe was prepared by end-labeling the wild-type 28-bp double-stranded ARE with [γ-32P]ATP (2500 Ci/mmol) using T4 polynucleotide kinase, followed by gel purification on 15% polyacrylamide. Twenty-bp oligonucleotides used as competitors were synthesized to contain double point mutations in relation to the wild-type sequence.
BZT-stimulated cells have identical DNA binding mobilities and are all supershifted in the presence of HNF-4α antibody. Antibody specificity was confirmed in supershift studies using HNF-4α peptide. No shift of HNF-4α was noted in the presence of nonspecific sera. Isolated protein band 1 and HNF-4α were both specifically recognized by HNF-4α antibody. In combination with the protein sequencing data, these data indicate that HNF-4α is the ARE transcription factor protein. Sequencing of bands 2 and 3 were also performed. Band 2 is an immunoglobulin component, whereas band 3 corresponds to the transcriptional coactivator, PC4.

Co-immunoprecipitation of HNF-4 and PC4—To examine the potential interaction between HNF-4α and PC4, co-immunoprecipitation experiments were performed using nuclear protein (Fig. 5). In control, IL-1β-, and BZT-stimulated cells, there was no detectable PC4 protein. In contrast, in the presence of both IL-1β and BZT, PC4 was readily detected. Immunoblot analysis of nuclear HNF-4α in control, IL-1β-, BZT-, and IL-1β + BZT-treated cells was also performed to normalize for HNF-4α expression. Equivalent amounts of HNF-4α were noted among the four treatment groups (data not shown). These data suggest that a nuclear HNF-4α-PC4 protein complex occurs exclusively in the presence of both IL-1β- and BZT-induced oxidative stress.

Transient Transfection Analysis of iNOS Promoter Activity—To corroborate the functional role of HNF-4α in the up-regulation of iNOS promoter activity in the setting of IL-1β and BZT stimulation, a CAT reporter plasmid construct containing full-length rat hepatocyte iNOS promoter was transfected into rat hepatocytes and ANA-1 murine macrophages. ANA-1 cells were selected because HNF-4α is not expressed in control, IL-1β-, BZT-, and/or IL-1β + BZT-treated cells, as determined by immunoblot and Northern blot analysis (data not shown). In rat hepatocytes, NO production, as determined by media levels of nitrite, was 8.8 ± 2.1, 45.3 ± 6.9, 8.7 ± 1.2, and 85.8 ± 6.1 nmol/mg of protein in unstimulated controls, IL-1β (1000 units/ml), BZT (10 μM), and IL-1β and BZT cells, respectively. 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 of protein in unstimulated controls, IL-1β (1000 units/ml), BZT (10 μM), and IL-1β and BZT cells, respectively.

Transient transfection analysis was then performed with the iNOS promoter plasmid construct alone (Fig. 6). In rat hepatocytes, IL-1β stimulation resulted in a 10-fold increase in CAT expression (p < 0.01 versus unstimulated control). The combination of IL-1β and BZT treatment increased CAT expression by 4-fold over that noted with IL-1β alone (p < 0.01 versus IL-1β). BZT alone did not alter CAT expression in comparison to that of unstimulated control cells. Similarly, ANA-1 cells also exhibit significantly increased CAT expression in the setting of IL-1β stimulation, ~8-fold greater than controls (p < 0.01 versus controls). However, in ANA-1 cells, addition of both IL-1β and BZT does not significantly alter CAT expression in comparison to IL-1β treatment alone. BZT treatment alone does not induce significant CAT expression. These data suggest that BZT-induced oxidative stress does not augment either IL-1β-induced iNOS promoter trans-activation or NO production in ANA-1 cells, which do not express HNF-4α. In contrast, oxidative stress significantly increases IL-1β-mediated iNOS promoter activation and synthesis of NO in rat hepatocytes expressing HNF-4α.
were isolated and precipitated with four volumes of cold acetone. Purified proteins were preincubated with the nuclear proteins. Supershift analysis was performed using a peptide (Santa Cruz) to demonstrate specificity of HNF-4 binding to the ARE sequence derived from the iNOS rat hepatocyte promoter. In selected instances, rabbit HNF-4 antibody, bound peroxidase activity were detected by the ECL detection system (Amersham Biosciences, Inc.). Following incubation with horseradish peroxidase-conjugated secondary antibody, bound peroxidase activity were detected by the ECL detection system (Amersham Biosciences, Inc.). Protein concentration was determined, separated by 12% SDS-PAGE, and the products were electrotransferred to polyvinylidene difluoride membrane (Amersham Biosciences, Inc.). Co-immunoprecipitation of HNF-4 and PC4. To examine the potential interaction between HNF-4α and PC4, co-immunoprecipitation experiments were performed using nuclear protein from cells treated with control, IL-1β (1000 units/ml), and/or BZT (10 μM). Whole cell lysate was preclarified and the supernatant incubated with primary antibody (polyclonal rabbit HNF-4α antibody, Santa Cruz Biotechnology). Protein concentration was determined, separated by 12% SDS-PAGE, and the products were electrotransferred to polyvinylidene difluoride membrane (Amersham Biosciences, Inc.). Blocked membranes were then incubated with goat PC4 polyclonal antibody (Santa Cruz). Following incubation with horseradish peroxidase-conjugated secondary antibody, bound peroxidase activity were detected by the ECL detection system (Amersham Biosciences, Inc.).

Co-transfection assays with the iNOS promoter construct and the HNF-4α expression vector were also performed in ANA-1 murine macrophages exposed to IL-1β and/or BZT (Fig. 6). In this setting, IL-1β stimulation of ANA-1 cells again increases CAT expression by over 8-fold (p < 0.01 versus unstimulated control). In the presence of IL-1β + BZT, CAT expression was increased over 3-fold in comparison to that noted in IL-1β-treated cells (p < 0.01 versus IL-1β). In the presence of BZT alone, CAT expression was not significantly different from that of control cells. Interestingly, HNF-4α expression in ANA-1 cells treated with only IL-1β did not increase CAT expression in comparison to 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.

In a parallel series of experiments, the mutant HNF-4 expression vector was co-transfected with the iNOS promoter-reporter construct. This mutant was selected because an amino acid (Asp for Tyr) has been substituted in the location critical for PC4 binding to iNOS promoter activity. 10 μg of plasmid DNA containing the iNOS promoter construct (1845 bp) was co-transfection of wild type HNF-4 expression vector (which asparagine for tyrosine) and the mutant HNF-4α vector with which aspartate for tyrosine was normalised by co-transfection of a β-galactosidase reporter gene with a constitutively active early SV40 promoter. All values are expressed as picograms of CAT/mg of protein.

Fig. 4. Supershift analysis of ARE protein. Bands 1, 2, and 3 were isolated and precipitated with four volumes of cold acetone. Precipitated protein was dissolved in 8 ml urea in D-100 buffer and incubated at 4 °C for 30 min. The protein was then reprecipitated by dialysis against 1 liter of 1 M urea in D-100 buffer, followed by dialysis against serial changes of D-100 buffer. Gel shift analysis was then performed using preincubated proteins from bands 1–3, crude nuclear extract, and purified nuclear extract from rat hepatocytes stimulated with IL-1β (1000 units/ml) and BZT (10 μM). The probe was a 32P labeled, single stranded DNA sequence derived from the iNOS rat hepatocyte promoter (nt –1353 to nt –1322) containing the sequence GACA, previously identified as a high affinity binding site for an HNF-4α transcription factor. In selected instances, rabbit HNF-4α antibody (polyclonal rabbit HNF-4α antibody, Santa Cruz) was preincubated with the nuclear protein from cells treated with control, IL-1β (1000 units/ml), and/or BZT (10 μM). Following incubation with horseradish peroxidase-conjugated secondary antibody, bound peroxidase activity were detected by the ECL detection system (Amersham Biosciences, Inc.).

Fig. 5. Co-immunoprecipitation of HNF-4α and PC4. To examine the potential interaction between HNF-4α and PC4, co-immunoprecipitation experiments were performed using nuclear protein from cells treated with control, IL-1β (1000 units/ml), and/or BZT (10 μM). Whole cell lysate was preclarified and the supernatant incubated with primary antibody (polyclonal rabbit HNF-4α antibody, Santa Cruz Biotechnology). Protein concentration was determined, separated by 12% SDS-PAGE, and the products were electrotransferred to polyvinylidene difluoride membrane (Amersham Biosciences, Inc.). Blocked membranes were then incubated with goat PC4 polyclonal antibody (Santa Cruz). Following incubation with horseradish peroxidase-conjugated secondary antibody, bound peroxidase activity were detected by the ECL detection system (Amersham Biosciences, Inc.).

Fig. 6. Functional role of HNF-4α expression in redox-mediated iNOS promoter activity. Transient transfection assays were performed in ANA-1 macrophages exposed to IL-1β (1000 units/ml) and/or BZT stimulation. The iNOS promoter construct (1845 bp) was coupled to a CAT reporter gene was constructed and the iNOS promoter plasmid construct. The HNF-4α expression vector was constructed by ligation of the 32P labeled, single stranded DNA fragment from pLEN4 ligated into the XbaI site of the mutant HNF-4α vector in which asparagine for tyrosine was substituted. CAT activity was assayed using a CAT enzyme-linked immunosorbent assay technique (Roche Molecular Biochemicals). Transfection efficiency was normalized by co-transfection of a β-galactosidase reporter gene with a constitutively active early SV40 promoter. All values are expressed as picograms of CAT/mg of protein.
HNF-4α, PC4, and Redox Regulation of iNOS Expression

DISCUSSION

In settings of inflammation and oxidative stress, hepatocyte iNOS expression is hepatoprotective and redox-regulated (9, 10, 16–18). The underlying redox-sensitive mechanism is not well defined. We have previously performed a functional analysis of the rat hepatocyte iNOS promoter in the setting of IL-1β and BZT stimulation, identified the ARE transcription factor, and established a functional role for ARE transcription factor in redox-mediated up-regulation of iNOS gene expression (9, 10). In this study utilizing rat hepatocytes in primary culture stimulated with IL-1β and BZT, we have established the sequence specificity and binding of nuclear protein to the previously described ARE binding site, isolated and identified the ARE transcription factor as HNF-4α, and confirmed the functional role of HNF-4α in mediating redox-sensitive iNOS promoter trans-activation. In addition, we have established the necessity of an association between HNF-4α and PC4 for increasing iNOS promoter activity in response to IL-1β and BZT. HNF-4α is a member of the steroid hormone receptor superfamily and is critical for development and liver-specific gene expression (15, 19–22, 24). In adult rodents and humans, HNF-4α mRNA is expressed in liver, small intestine, kidney, colon, pancreas, and testis. It is known to act, alone or in combinatorial association with other tissue-specific or basal transcription factors, to promote the transcription of a wide variety of target genes. These include cytochrome P450 CYP2 family members, blood coagulation factors, adipoproteins, erythropoietin, transthyretin, complement factor B, medium chain acyl-CoA dehydrogenase, HNF-1, α1-microglobulin, thione transcarbamylase, liver prolactin, and retinol-binding protein. It also activates human immunodeficiency virus type 1 long terminal repeat and phosphoenolpyruvate carboxykinase promoters. Mutations in HNF-4α are associated with juvenile-onset diabetes of the young. Peroxisome proliferator-activated receptors (PPARs) and RXR dimers bind to the DR1 motif 5′-AGGTCA-3′ in a hexameric repeat of AGGTCA separated by a nucleotide spacer. Although the identified binding sequence in our studies differs from the consensus sequence, Fraser has demonstrated previously (25) that these substitutions do not markedly alter HNF-4α binding.

The relationship between HNF-4α and HNF-1α does not appear to be regulated via an ARE. The expression of HNF-1α in cultured hepatoma cells is not affected by IL-1β and BZT stimulation (26), and HNF-1α does not bind to the DR1 motif. However, the role of HNF-1α in mediating redox-regulated iNOS promoter trans-activation is not known.

PC4 is a 15-kDa polypeptide that serves as a potential coactivator in standard reconstituted in vitro transcription systems (23, 29, 30). It mediates activator-dependent transcription by RNA polymerase II through interactions with the transcriptional activator and basal transcription machinery. PC4 binds double-stranded DNA in a sequence-independent manner. It is subjected to in vivo phosphorylation events that negatively regulate its coactivator functions. The vast majority (95%) of PC4 is phosphorylated and inactive in vivo (23, 29, 30). Interestingly, the 24 N-terminal residues of HNF-4α (AF-1) constitute a critical structural element that has been demonstrated to bind to PC4 (14, 15). Our data suggest that HNF-4α binds with PC4 under conditions of IL-1β and BZT stimulation and that this is essential for redox-mediated increase in iNOS promoter trans-activation. Co-transfection of a mutant HNF-4α in which a critical PC4 binding residue has been substituted demonstrates ablation of redox-mediated iNOS promoter activation. It is unknown whether these stimulation conditions alter HNF-4α or PC4 to facilitate this interaction. However, given the dependence of PC4 activity on its phosphorylation status and the participation of various mitogen-activated protein kinase activities in the cellular response to oxidative stress, it is tempting to speculate that PC4 may be the target. Alternatively, PC4 stimulation may enhance binding of PC4 to HNF-4α and structurally alter its DNA binding domain, thereby increasing binding. These are currently the subject of investigation in our laboratory. These conserved critical elements of the rat hepatocyte iNOS promoter, identified in this study, establish the functional role of HNF-4α as a transcription factor that mediates redox-sensitive iNOS promoter trans-activation. In addition, we demonstrated two levels of redox-sensitive regulation of iNOS in the presence of oxidative stress and IL-1β stimulation. It is essential for increased iNOS promoter activity in this setting. Our results indicate that HNF-4α is the transcriptional factor that mediates redox regulation of hepatocyte iNOS gene transcription.

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