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 benzenetriol-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 have identified a novel ARE nuclear protein, which in the setting of oxidative stress binds to the binding site, isolating the ARE promoter activity in the trans-activation of the iNOS promoter in rat hepatocytes (9, 10). Using a transient expression assay in IL-1β- and BZT-stimulated hepatocytes, we have identified a novel ARE nuclear protein, which in the setting of oxidative stress binds to the binding site, isolating the ARE promoter activity in the trans-activation of the iNOS promoter in rat hepatocytes (9, 10).

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induce oxidative stress. After incubation for 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 medium was measured by determination 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 sulphydrylamine (1% in 0.5% HCl). After a 5-min incubation at room temperature, an equal volume of 0.2% N-(1-naphthyl)ethylenediamine 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 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 [γ32P]ATP (2500 Ci/mmol) using T4 polynucleotide kinase, followed by gel purification on 15% polyacrylamide. Twenty-bp oligonucleotides were isolated from rat hepatocytes treated with IL-1β and BZT as described previously (10). Nuclei were resuspended in 10 mM Tris, pH 7.9, 100 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM NaMetaBis, and 0.2 mM PMSF, followed by Dounce homogenization on ice. Homogenized nuclei were mixed with 0.06× packed nuclear volume of 4 M ammonium sulfate, pH 7.9, and extracted with gentle mixing at 4 °C for 1 h. Extracted nuclei were precipitated by centrifugation at 25,000 × g for 20 min. The supernatant was then dialyzed for 20 min against 20 mM HEPS, pH 7.9, 20% glycerol, 100 mM KCl, 2 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM NaMetaBis, and 0.2 mM PMSF. Following dialysis, the extract was centrifuged at 25,000 × g for 4 °C for 20 min. Protein concentration of the nuclear extract was determined using the Bio-Rad protein assay system (Bio-Rad). Nuclear transcription factor activity was assayed by gel shift assay.

IL-1β- and BZT-treated nuclei (Amersham Biosciences, Inc.) and eluted with one volume of 0.55 × NaCl, washed with 0.55 × NaCl, and eluted with 0.4 × NaCl and excess nonbinding poly(dI-dC)·poly(dI-dC) DNA was added. Following a 10-min incubation at 4 °C, the solution was centrifuged at 12,000 × g for 10 min. The resulting supernatant was incubated for 5 min at 25 °C with reverse phase HPLC-purified biotinylated 40-mer oligonucleotide encompassing the ARE binding site (5’-CACATGTGGAGGTCAGGG-3‘) bound to Dynabeads M280 streptavidin in TGED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.01% Triton X-100, and 100 mM NaCl in diethyl pyrocarbonate-treated water). The magnetic beads were then washed three times with TGED buffer in 100 mM NaCl containing 0.55 × NaCl, washed with 0.55 × NaCl, and eluted with 0.4 × NaCl. The resulting fractions were individually eluted to 0.1 × NaCl, and eluted with 0.05 × NaCl and excess nonbinding poly(dI-dC)·poly(dI-dC) DNA was added. Following a 10-min incubation at 4 °C, the solution was centrifuged at 12,000 × g for 10 min. The resulting supernatant was incubated for 5 min at 25 °C with reverse phase HPLC-purified biotinylated 40-mer oligonucleotide encompassing the ARE binding site (5’-CACATGTGGAGGTCAGGG-3‘) bound to Dynabeads M280 streptavidin in TGED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.01% Triton X-100, and 100 mM NaCl in diethyl pyrocarbonate-treated water). The magnetic beads were then washed three times with TGED buffer in 100 mM NaCl containing 0.55 × NaCl, washed with 0.55 × NaCl, and eluted with 0.4 × NaCl. The resulting fractions were individually eluted to 0.1 × NaCl, and eluted with 0.05 × NaCl and excess nonbinding poly(dI-dC)·poly(dI-dC) DNA was added. Following a 10-min incubation at 4 °C, the solution was 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). Cell lysate (50 μg/lane) were separated by 12% SDS-PAGE, and the products were electrophoretically transferred 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.).

Immunoblot Analysis—ANA-1 cells were lysed in buffer (0.8% NaCl, 0.02 KCl, 1% SDS, 10% Triton X-100, 0.5% sodium deoxycholate, 0.144% Na, KHPO₄, and 0.024% KH PO₄) at pH 7.4 for 10 min at 4 °C. The protein was then renatured by dialysis against 1 liter of 1 M urea in D-100 buffer, followed by dialysis against serial changes of D-100 buffer.

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 plasmid DNA containing the iNOS promoter construct (1845 bp; GenBank® accession no. X95629) coupled to a chloramphenicol acetyltransferase (CAT) reporter gene was added per 10⁷ 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-4α) 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 Tyr⁶ 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 10% Me₂SO at room temperature. The transfection supernatants were incubated with 100–500 μg/ml of each combination of ANA-1 cells 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 8.0, and subjected to three cycles of freezing and thawing. Lysates were centrifuged (11,700 × 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 ␤-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-1␤ 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 a 32-bp DNA sequence, AGGTCAGGGGACA, previously identified as a high affinity binding site for the ARE transcription factor. Gel shift results for mutants 2–11 are shown in Fig. 1. Mutants 1–4 disrupt the ARE binding site by mutation in the wild-type DNA sequence and disrupt the ARE binding site for the ARE transcription factor. These probes contain the sequence, AGGTCAGGGGACA, previously identified as a high affinity binding site for the ARE transcription factor. Gel shift assays were performed using nuclear protein isolated from rat hepatocytes treated with IL-1␤ 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, AGGTCAGGGGACA, previously identified as a high affinity binding site for the ARE transcription factor.

**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-1␤ 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: QCVDKDKRKNQ and TMGNTSPSEGAP. 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-1␤- and
BZT-stimulated cells have identical electrophoretic mobilities and are all supershifted in the presence of an 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 the 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.
Precipitated protein was dissolved in 8 M urea in D-100 buffer and incubated at 4 °C for 30 min. The protein was then reanimated 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 reanimated 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 double-stranded DNA sequence derived from the iNOS rat hepatocyte promoter (nt –1353 to nt –1322) containing the sequence 5′-GACA, previously identified as a high affinity binding site for the ARE transcription factor. In selected instances, HNF-4α antibody (polyclonal rabbit, Santa Cruz Biotechnology, Santa Cruz, CA) was preincubated with the purified nuclear extract from rat hepatocytes stimulated with IL-1β and/or BZT (Fig. 4). For supershift analysis, an HNF-4α-PC4 protein complex in ANA-1 cells was also demonstrated by the same antibody, an HNF-4α-PC4 complex was not detected (data not shown). These data indicate that formation of an HNF-4α-PC4 complex in ANA-1 cells is required for augmentation of iNOS promoter trans-activation.

In a parallel series of experiments, the mutant HNF-4α expression vector was co-transfected with an iNOS promoter reporter construct. This mutant was selected because an amino acid (Asp for Tyr6) has been substituted in the location critical for PC4 binding to HNF-4α (14, 15). At least 24 h after medium was changed, and IL-1β or IL-1β + BZT was added. Enzyme 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.

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.
PC4 is a 15-kDa polypeptide that serves as a potent 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-4 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, HNF-4α stimulation may enhance binding of PC4 to its DNA recognition domain, or structurally alter its DNA binding domain. These are currently the subject of ongoing studies in our laboratory. These considerations support the hypothesis that HNF-4α is the transcription factor that mediates redox regulation of hepatic iNOS gene transcription.

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