Nitric Oxide-dependent Negative Feedback of PARP-1 trans-Activation of the Inducible Nitric-oxide Synthase Gene

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Nitric oxide (NO) participates in a variety of physiologic and pathophysiologic processes in diverse tissues, including the kidney. Although mechanisms for cytokine induction of inducible nitric-oxide synthase (iNOS) have been increasingly clarified, the controls for termination of NO production remain unclear. Because excessive NO production can be cytotoxic to host cells, feedback inhibition of iNOS transcription would represent a means of cytoprotection. Many of the cGMP-independent functions of NO are mediated by S-nitrosylation of cysteine thiols of target proteins. We hypothesized that NO-mediated S-nitrosylation of transcription factors might serve to feedback inhibit their trans-activation potential and deactivate iNOS gene transcription. Transient transfection of murine mesangial cells with iNOS promoter deletion-luciferase deactivates iNOS gene transcription. Transient transfection of sized that NO-mediated nitrosation of transcription factors might serve to feedback inhibit their trans-activation potential and deactivate iNOS gene transcription. Transient transfection of murine mesangial cells with iNOS promoter deletion-luciferase constructs revealed the region –915 to –849 to be NO sensitive with respect to IL-1β-induced promoter activity. In vitro DNase I footprinting identified a footprint at –865/–842 in the absence of NO, but not in the presence of endogenous or exogenously delivered NO. Southwestern blotting using this probe coupled with partial peptide sequencing of the protein bands revealed that poly(ADP-ribose) polymerase isoform 1 (PARP-1) bound the probe in a sequence-specific manner. Gel shift/supershift experiments and chromatin immunoprecipitation assay analysis confirmed this binding in vitro and in vivo. Functionally, mutation of the –859/–850 site to prevent PARP-1 binding or PARP-1 knockdown by RNA interference relieved the inhibitory effects of NO on iNOS promoter activity. Biotin-switch assays and co-immunoprecipitation with an anti-nitrotyrosine antibody indicated that PARP-1 was S-nitrosylated. We conclude that NO feedback inhibits iNOS gene transcription by S-nitrosylating the trans-activator PARP-1 and decreasing its binding and/or action at the iNOS promoter.

Inducible nitric-oxide synthase (iNOS) is expressed in numerous cell types in mammals after induction by cytokines and/or lipopolysaccharide, and once expressed, it is active at resting levels of intracellular Ca2+ (1). NO exerts its actions by chemically modifying targets, preferentially interacting with thiol groups, transition metals, and free radicals. Reaction of NO with superoxide forms peroxynitrite with resultant protein thiol nitrosylation, tyrosine nitration, DNA damage, and excessive activation of poly(ADP-ribose) polymerase (PARP) (2).

PARP-1 is the prototypical and most highly expressed member of the PARP gene family, which contains at least seven members in mammalian species (3, 4). The second most abundant non-histone nuclear protein, PARP-1 is a DNA nick sensor that uses NAD as a substrate to catalyze the addition of poly(ADP-ribose) to acceptor proteins, in particular histones, several transcription factors, and PARP itself, thereby regulating their activities and functions (4, 5). PARP-1 activated by DNA breaks facilitates transcription, replication, and DNA base excision repair. Excessive activation of PARP-1 by peroxynitrite-induced DNA damage during oxidative and nitrosative stress in ischemia-reperfusion injury, inflammation, and diabetes mellitus can cause cell death by NAD+/ATP depletion (2, 6, 7). In addition, activation of PARP-1 plays an important role in the up-regulation of inflammatory cascades via a functional association with mitogen-activated protein kinases (8) and several transcription factors (9–12), including NF-κB (13–16), or its direct binding to gene-regulating DNA sequence (17), augmenting production of pro-inflammatory mediators. PARP-1 knock-out mice, for example, are resistant to ovalbumin-induced airway inflammation, and this protection is associated with reduced iNOS gene expression (18).

NO participates in redox chemistry to provide surrogate NO-like bioactivity, which functions in the cGMP-independent control of numerous cellular functions (19). The formation of S-nitrosothiols, for example, results in allosteric receptor modification, inhibition of the activities of enzymes containing sulphydryl groups, and down-regulation of transcriptional activators (20–23). S-nitrosylation of relevant protein thiols inhibits the DNA binding activities of the transcription factors NF-κB (24), hepatocyte nuclear factor-4 (25), heterogeneous nuclear ribonucleoprotein A/B (26), p53 (27), and hypoxia-inducible factor-1 (28). S-nitrosylation appears to be a regulated process, influencing growth factors, and developmental transitions in diverse tissues (29). The range of transcription factors potentially subject to regulation by S-nitrosylation during oxidative or nitrosative stress has not been catalogued.

The excessive production of NO during activated states can injure host tissues by an “innocent bystander” effect. Therefore, NO-dependent negative feedback regulation of iNOS gene transcription would offer an efficient mechanism to terminate high output NO production. For example NF-κB, a key transactivator of the iNOS gene, is subject to NO-mediated S-nitrosylation, which inhibits its ability to bind the iNOS promoter and results in down-regulation of iNOS gene transcription (24). Similarly, NO up-regulates the iNOS inhibitor osteopontin, resulting in enhanced osteopontin-mediated inhibition of iNOS expression (26). In this study, we have demonstrated that PARP-1 is a NO-sensitive
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activate of cytokine-induced iNOS transcription in glomerular mesangial cells and that NO-mediated S-nitrosylation of PARP-1 occurs and may account for feedback inhibition of the iNOS promoter.

MATERIALS AND METHODS

Cell Culture and Reagents—Mouse mesangial cells (ATCC CRL-1927) were maintained in Ham’s F12 plus Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 5% fetal bovine serum. Vehicle, IL-1β (10 ng/ml), i-NAME (100 μg/ml), i-NIL (1 mM), i-arginine (10 μM), S-nitroso-N-acetylpenicillamine (SNAP; 100 μM), S-nitroso-glutathione (GSNO; 10 μM), or 2–4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; 10 μM) was added to the cells as indicated in the text and figure legends. Mouse recombinant IL-1β was from R & D Systems (Minneapolis, MN). Oligonucleotides were custom synthesized by Genosys (The Woodlands, TX). Lipofectamine 2000 reagent was from Invitrogen. The Dual-Luciferase™ reporter assay system and the luciferase vectors pGL3-Basic and pRL-SV40 were from Promega. The BCA protein estimation kit was from Pierce Chemical, and ECL reagents were from Amersham Biosciences. Mammalian short hairpin RNA expression plasmid pKD-PARP-v2, which contains the PARP-1 targeting sequence (sense strand) 5′-GAGGCTCAC-GAGTTGTCTTT-3′, and its negative control plasmid pKD-NegCon-v1, which contains the unrelated sequence (sense strand) 5′-AGT- CATCGACTGCTTACTT-3′, were purchased from Upstate (Lake Placid, NY). Anti-nitrotyrosine and anti-PARP-1 antibodies were from A. G. Scientific (San Diego, CA) and Alexis (San Diego, CA), respectively. Anti-Oct-1 and anti-Oct-3/4 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids—Serial 5′-deletion constructs corresponding to murine iNOS promoter regions −915/+137, −849/+137, −805/+137, and −752/+137 were amplified by PCR and subcloned into pGL3-Basic vector at Mlu I and BglII sites. The resultant plasmids were designated pNiNOS(−915/+137)-luc, pNiNOS(−849/+137)-luc, pNiNOS(−805/+137)-luc, and pNiNOS(−752/+137)-luc. Site-directed mutation of the −859/−850 iNOS promoter element (5′-AATTATA TT-3′ replaced with 5′-CCCCAGCCCTTT-3′; mutations are underlined) in pNiNOS-luc was accomplished by PCR splicing by overlap extension, using the wild-type iNOS promoter DNA as a template. The resulting construct was designated pNiNOS(−915A−859/−850/−850/+137)-luc. All plasmids were sequenced to verify the presence of the desired mutations and the absence of spurious mutations.

Transient Transfections—Mesangial cells were seeded in 24-well plates, grown to 90–95% confluency in complete medium without antibiotics, and transfected the following day using the Lipofectamine 2000 reagent according to the manufacturer’s protocol and a total of 1 μg/well of plasmid DNAs. Where necessary, the amount of transfected plasmid DNA was kept constant by addition of appropriate amounts of the parental empty expression vector. Transfection efficiencies were normalized by cotransfection with 20 ng/well of the Renilla luciferase expression plasmid pRL-SV40. As indicated in the text and figure legends, vehicle, IL-1β, i-NAME, i-NIL, S-nitroso-gluthathione, SNAP, or C-PTIO was added after transfection for 24 h before cell lysates were prepared. For RNA interference, mesangial cells grown on 24-well plates were cotransfected with 0.5 μg of pNiNOS-luc and 0.5 μg of pKD-NegCon-v1 or pKD-PARP-v2 along with 20 ng of pRL-SV40 using Lipofectamine reagent. The efficiency and specificity of siRNA gene knockdown of PARP-1 was determined by Western blotting for PARP-1 and α-tubulin expression.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared from time-paired vehicle-, IL-1β-treated mesangial cells as detailed in our earlier work (30, 31). A double-stranded oligonucleotide 865S′-GTAAGAAATTAATT-TATTCGTGTT-3′−842S′ from the murine iNOS promoter or a mutated form of the probe 5′-GTAAGACCCAGCCCTATTCGTGTT-3′ (mutations underlined; designated “Δ—865/−842 oligo” in the figures and legends) was generated for use as probes and for competition studies. The −865/−842 probe was end labeled with [γ-32P]ATP (300 Ci/mmol) using T4 polynucleotide kinase. Binding reactions were performed in 20 μg of solution for 30 min at room temperature by incubating 10 μl of nuclear extract protein with duplex DNA probe (2 × 105 cpm) in reaction buffer (13 mM HEPES, pH 7.9, 65 mM NaCl, 0.14 mM EDTA, 1 mM MgCl2, 1 mM dithiothreitol, 8% glycerol, and 50 mg/ml of poly(dI-dC). For supershift assays, antibodies specific for PARP-1, Oct-1, or Oct-3/4 or nonimmune IgG were added to the binding reaction and incubated on ice for 10 min before the addition of labeled probe. Aliquots of the reactions were resolved on 5% native polyacrylamide gels in 0.5× Tris borate-EDTA buffer. The gels were dried and exposed to x-ray film with an enhancing screen at −70 °C to detect the DNA–protein and DNA–protein-antibody complexes. Experiments were replicated a minimum of three times as indicated in the figure legends.

In Vitro DNase I Footprinting—DNase I footprinting analysis was performed with the Core Footprinting System (Promega) according to the manufacturer’s instructions and our published work (32). A PCR fragment corresponding to −966 to −696 of the murine iNOS S′-flanking region was generated using pNiNOS-luc as a template. This fragment was used as a 32P-ATP-labeled DNA template for footprinting and as a 35S-dATP-labeled fragment for DNA sequencing performed with the fmol DNA Cycle Sequencing System (Promega).

Southwestern (DNA-Protein) Blotting—Approximately 100 μg of nuclear protein was resolved on a 12% SDS-PAGE gel, transferred to nitrocellulose, and the filter air dried. The nitrocellulose was then immersed in binding buffer (25 mM HEPES, pH 7.6, 60 mM KCl, 1 mM EDTA, 1 mM DTT) supplemented with 6 μl guanidinium chloride, followed by 8 sequential 2-fold dilutions of guanidinium chloride, with gentle rocking for 10 min at 4 °C each incubation. The nitrocellulose filter was then transferred to binding buffer containing 3 μl guanidinium chloride and gently rocked for 10 min at 4 °C. The final wash step lacked guanidinium chloride. The nitrocellulose was prehybridized in binding buffer containing 5% gelatin and 5 mg/ml of sonicated salmon sperm DNA for 1 h at room temperature. The nitrocellulose filter was then immersed in binding buffer containing 0.25% gelatin and 5 mg/ml of sonicated salmon sperm DNA and incubated for 30 min. 32P-dCTP-labeled −865/−842 iNOS DNA probe (sense strand 5′-GTAAGAAATTAATT-TATTCGTGTT-3′) was added to the binding buffer containing 0.25% gelatin and 5 mg/ml of sonicated salmon sperm DNA and incubated with the nitrocellulose filter overnight at room temperature. The filter was then washed four times with binding buffer for 5 min at room temperature, air dried, and autoradiographed at −80 °C with an intensifying screen.

Purification of DNA-binding Protein—The DNA-binding protein was isolated by reacting the biotinylated DNA-protein complex with streptavidin paramagnetic particles (Dynal Biotech Inc.), using the protocol described by Gao et al. (26). Nuclear proteins from mesangial cells were incubated for 15 min at 25 °C with HPLC-purified biotinylated −865/−842 iNOS DNA probe containing the identified binding site bound to Dynabeads M280 streptavidin in protein binding buffer (50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 20% (v/v) glycerol, 5 mM MgCl2, 250 mM NaCl, 0.25 mg/ml poly(dI-dC), and 2.5 mM DTT). The mag-
nentic beads were washed three times with protein binding buffer in 100 mM NaCl containing excess nonbinding poly(dl-dc) competitor DNA. Serial elutions were performed using elution buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% (v/v) glycerol, 0.01% Triton X-100, 1 mM NaCl, and 1 mM DTT). The fractions were typically stored at −80 °C prior to subsequent use.

**Protein Sequencing**—Proteins were identified in the Proteomics Core Facility at the University of Texas Health Science Center at Houston. Proteins were separated by SDS-PAGE, and the gel was stained with Coomassie Blue and silver. For protein identification, bands were excised from the gel and subjected to in-gel proteolytic digestion with trypsin essentially as described by Simpson and co-workers (33). After extraction of the peptides, an aliquot was separated by HPLC on a C18 75 μ × 10 cm reverse phase capillary column (Vydac 218RS3.07510). The column was developed with a gradient of 2–50% acetonitrile in 0.1% formic acid, 0.005% trifluoroacetic acid over 30 min at a flow rate of 200 nl/min. The nanospray source was fitted with a 30-μm coated tapered fused silica tip (New Objective, Cambridge, MA) used to elute the peptides directly into the mass spectrometer for tandem mass spectrometry analysis, which was performed on an Applied Biosystems QStar XL LC/MS/MS mass spectrometer equipped with an LC Packings HPLC for capillary chromatography. The HPLC was coupled to the mass spectrometer by a nanospray electrospray ionization head for direct analysis of the eluate. The QSTAR was operated in Information-dependent Acquisition mode, using a 1-s survey scan followed by two consecutive 3-s product ion scans of 2+, 3+, and 4+ parent ions (m/z 350–1500). Identification was performed on MASCOT with an MS and MS/MS mass tolerance of 0.15 Da.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP analysis was performed using chromatin from vehicle-, IL-1β-, l-NNAME + IL-1β, or SNAP + l-NNAME + IL-1β-treated mesangial cells. Chromatin was fixed and immunoprecipitated using the ChIP assay kit (Upstate Biotechnology) following the manufacturer’s protocol as described in detail in our previous work (34), except that immunoprecipitation was performed with anti-PARP-1 antibody or rabbit nonimmune IgG. The presence of the selected DNA sequence was assessed by PCR using primers framing the murine iNOS promoter region of interest: forward primer, 5′-TGCTAGGGGGATTTTCCCTCTCTC-3′ (nucleotides −978 to −955), and reverse primer, 5′-AGGCTTTATACCCCGGATTCAGG-3′ (nucleotides −732 to −710). PCR conditions were as follows: 94 °C × 3 min; 30 cycles at 94 °C × 1 min, 52 °C × 1 min, and 72 °C × 1 min; final elongation at 72 °C × 10 min. PCR for the input was performed with genomic DNA. The input fraction corresponded to 1% of the chromatin solution before immunoprecipitation. The PCR products were analyzed on a 2% agarose gel.

**Immunoprecipitation and Western Blotting**—Vehicle or IL-1β-treated mesangial cells were harvested and lysed in radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 8.0, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride). These lysates were then precleared by incubating with 20 μl of protein A/G-agarose beads (Santa Cruz) for 1 h at 4 °C. After brief centrifugation, the supernatant was incubated with the anti-nitrocysteine antibody or control IgG in radioimmune precipitation assay buffer over 30 min at a flow rate of 200 nl/min. The column was developed with a gradient of 2–50% acetonitrile in 0.1% formic acid, 0.005% trifluoroacetic acid over 30 min at a flow rate of 200 nl/min. The nanospray source was fitted with a 30-μm coated tapered fused silica tip (New Objective, Cambridge, MA) used to elute the peptides directly into the mass spectrometer for tandem mass spectrometry analysis, which was performed on an Applied Biosystems QStar XL LC/MS/MS mass spectrometer equipped with an LC Packings HPLC for capillary chromatography. The HPLC was coupled to the mass spectrometer by a nanospray electrospray ionization head for direct analysis of the eluate. The QSTAR was operated in Information-dependent Acquisition mode, using a 1-s survey scan followed by two consecutive 3-s product ion scans of 2+, 3+, and 4+ parent ions (m/z 350–1500). Identification was performed on MASCOT with an MS and MS/MS mass tolerance of 0.15 Da.

**RESULTS**

**NO-sensitive Inhibition of the iNOS Promoter**—To localize potential NO-sensitive cis-acting elements in the murine iNOS promoter, iNOS promoter deletion constructs fused to the firefly luciferase gene, designated piNOS(−915/+137)-luc, piNOS(−849/+137)-luc, piNOS(−805/+137)-luc, and piNOS(−752/+137)-luc, were transiently transfected into mesangial cells. The nitric-oxide synthase inhibitor l-NNAME was used to inhibit endogenous NO production. The cells were subsequently treated with vehicle, IL-1β, or IL-1β + l-NNAME, and the fold induction of iNOS promoter-driven luciferase activity was measured. Using this strategy, the region −915/−849 was determined to be uniquely NO sensitive, because l-NNAME blockade of IL-1β-stimulated endogenous NO production resulted in greatly enhanced luciferase activity compared with cells exposed to endogenously produced NO (IL-1β without l-NNAME) (Fig. 1A). None of the iNOS promoter deletion-luciferase constructs exhibited appreciable luciferase activity in the absence of IL-1β. The piNOS(−915/−137)-luc was studied in further detail, using SNAP as an exogenous NO donor in the presence of IL-1β + l-NNAME (Fig. 1B). The piNOS(−915/−137)-luc exhibited enhanced activity in the IL-1β + l-NNAME-treated cells, but this was reversed by co-administration of SNAP with IL-1β + l-NNAME. In fact, the IL-1β + l-NNAME + SNAP-treated cells exhibited slightly lower levels of luciferase activity compared with cells exposed to IL-1β alone (Fig. 1B). However, co-administration of the NO scavenger C-PTIO significantly limited the ability of SNAP to reverse the induction of piNOS(−915/+137)-luc activity in IL-1β + l-NNAME-treated cells (Fig. 1B). As seen in Fig. 1C, l-NIL, a nitric-oxide synthase inhibitor structurally unrelated to l-NNAME, yielded essentially the same results as observed with l-NNAME (Fig. 1B), and this effect was overcome by excess l-arginine (Fig. 1C). Moreover, S-nitroso-gluthione, a different NO donor, yielded results comparable with those observed with SNAP (Fig. 1C).

**In Vitro DNase I Footprinting Analysis**—To determine whether a trans-activating factor may reside in the region of the iNOS promoter implicated in the promoter-reporter gene studies, in vitro DNase I footprinting analysis was performed using a radiolabeled PCR ampiclon corresponding to nucleotides −966/−696 of the iNOS promoter as template. Nuclear extracts were prepared from control, IL-1β-treated, IL-1β + l-NNAME-treated, and IL-1β + l-NNAME + SNAP-treated cells. Protected sites in the region −865/−842 were detected in the binding reactions that included nuclear extracts from the vehicle- or IL-1β + l-NNAME-treated cells, when compared with the reactions with nuclear extracts from IL-1β- or IL-1β + l-NNAME + SNAP-treated cells (Fig. 2A). The sequence of this protected region reads as −865-GTAA-GAAATTTAATTTATCGTT-3′. In the presence of a 50-fold excess of unlabeled full-length probe, this binding was eliminated (data

blots were washed extensively with a solution containing 50 mM Tris, pH 8.0, 138 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20. The antigen-antibody complexes were detected by the ECL protocol using horse-radish peroxidase-conjugated donkey anti-rabbit IgG as secondary antibody.

**Biotin-Switch Method to Detect S-Nitrosylated Proteins**—S-Nitrosylated proteins in nuclear protein fractions from mesangial cells that had been treated with vehicle or SNAP were selectively biotinylated and purified using our minor modification (35) of the biotin-switch protocol established by Jaffrey et al. (36). The purified proteins were then analyzed by PARP-1 immunoblot analysis.

**Data Analysis**—Quantitative data are presented as mean ± S.E. and were analyzed by analysis of variance. Significance was assigned at p < 0.05.
not shown). Computer-assisted inspection of this sequence with the Transcription Element Search System (TESS, www.cbil.upenn.edu/tess) revealed a potential consensus binding element for Oct-1 (−8595/H11032−850) within it, but no other consensus binding sites elements were detected.

Gel shift studies using the −865/−842 oligomer implicated in the footprinting studies demonstrated sequence-specific DNA-protein complexes under basal conditions in nuclear extracts harvested from mesangial cells (Fig. 2B). Thehigher molecular weight complex, designated complex I, was up-regulated following IL-1β treatment, whereas complex II consistently was slightly down-regulated after IL-1β treatment. Competition experiments revealed sequence specificity of the DNA binding reaction for complex I, because it was virtually abolished when a 50-fold molar excess of unlabeled −865/−842 oligomer was included in the binding reaction but was unchanged when a 50-fold molar excess of unlabeled, mutated −859/−850 region, was present (Fig. 2B). These competition maneuvers did not significantly disrupt com-
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Isolation and Characterization of DNA-binding Protein—To characterize the factor(s) contained within the −865/−842 DNA–protein complex I, nuclear extracts prepared from IL-1β-treated mesangial cells were resolved on a SDS-polyacrylamide gel and then transferred to nitrocellulose membrane and detected by Southwestern blotting using radiolabeled −865/−842 oligonucleotide probe containing the described binding sequence. After autoradiography, a band of ~110 kDa (after subtracting the molecular mass of the DNA probe) was detected that was dependent on addition of nuclear extract (Fig. 3A). Utilizing the biotin–streptavidin DNA affinity technique with the identified DNA binding sequence, the transcription factor was purified and isolated from nuclear extract isolated from IL-1β-treated mesangial cells using radiolabeled −865/−842 oligonucleotide probe containing the described binding sequence. The band was then excised and subjected to protein sequencing (Fig. 3B). Analysis of separate tryptic digests of the band yielded identical matches with PARP-1 (NCBI accession code NP058085).

Confirmation of PARP-1 DNA Binding to the iNOS Promoter in Vitro and in Vivo—To confirm PARP-1 binding to the iNOS promoter, supershift and ChIP assays were performed using an anti-PARP-1 antibody. Supershift/antibody competition assays showed that anti-PARP-1 antibody nearly completely abolished the formation of complex I (Fig. 4A). This result most likely indicates that the antibody disrupted the PARP-1–DNA interaction, resulting in reduction in the amount of the characteristic gel shift but no supershift. Because the region −859/−850 contains a consensus binding element for Oct-1, supershift experiments were also conducted with antibodies against Oct-1 or against Oct-3/4 included in the binding reaction. No supershift or change in the intensity of complex I was noted using the Oct family antibodies.

To determine the effects of NO on PARP-1 binding activity to the −859/−850 region, nuclear extracts were isolated from mesangial cells that had been treated with vehicle or SNAP, and the extracts were treated with vehicle or DTT (1 mM), which inhibits S-NO bond formation. Gel shift assays were then performed. SNAP treatment resulted in significantly diminished PARP-1 DNA binding activity (Fig. 4B). Addition of DTT resulted in PARP-1 DNA binding activity that was unaltered in the presence of SNAP. These data suggest that S-nitrosylation of PARP-1 ex vivo results in diminished DNA binding in gel shift assays.

ChIP assays were performed to confirm the in vivo binding of PARP-1 to this region of the iNOS promoter (Fig. 4C). Little chromatin-associated PARP-1 at the iNOS promoter region −978/−710 was observed under basal conditions and under conditions in which NO was produced endogenously (IL-1β-treated cells) or added exogenously (IL-1β + L-NAME + SNAP-treated cells). In contrast, there was a much greater level of PARP-1 associated with the iNOS promoter in vivo when IL-1β-induced NO production was blocked by L-NAME at a concentration we have previously shown to block completely iNOS-generated NO production (37). These results are in keeping with the in vitro DNase I footprinting results (Fig. 2A) and suggest that endogenously synthesized or exogenously administered NO inhibits the association of PARP-1 with the iNOS promoter in IL-1β-stimulated cells.

Mutation of the −859/−850 Region Inhibits iNOS Promoter Activity and NO Sensitivity—Mutation of the −859/−850 sequence (which prevented competition with the wild-type probe in DNA–protein complex I in the gel shift experiments depicted in Fig. 4A) in piNOS(−915Δ−859/−850 + 137)-luc resulted in a 40% lower levels of iNOS promoter activity compared with the wild-type piNOS(−915/+137)-luc construct (Fig. 5A). This mutation also resulted in a loss of NO sensitivity, because luciferase activity following treatment with IL-1β or IL-1β + L-NAME was equivalent (Fig. 5B). Taken together with the promoter–luciferase and in vitro footprinting data, these results

FIGURE 2. A, in vitro DNase I footprinting analysis of murine iNOS promoter. A 32P-labeled DNA probe (−966 to −696) was incubated with nuclear extracts prepared from vehicle-, IL-1β-, and IL-1β + L-NAME-, or IL-1β + L-NAME + SNAP-treated cells. A representative (n = 4) gel is shown. A sequencing ladder was also generated for definition of footprinted or hypersensitive sites (not shown). The sequence of the footprinted area is indicated. B, gel shift analysis. Nuclear proteins were harvested from vehicle- or IL-1β-treated mesangial cells and subjected to gel shift analysis using radiolabeled oligomer corresponding to −865/−842 of the murine iNOS promoter. Binding specificity of Complex I was verified in competition experiments performed with a 50-fold molar excess of unlabeled −865/−842 oligomer or of a mutant −865/−842 oligomer harboring mutations in the −859/−850 region to prevent protein binding (n = 3).
FIGURE 3. Isolation of PARP-1 as a NO-sensitive DNA-binding protein from IL-1β-stimulated mesangial cells. A, crude nuclear protein and nuclear protein purified using the biotin-streptavidin DNA affinity technique with the identified putative DNA binding sequence were electrophoresed on 8% SDS-PAGE and stained with silver. The Southwestern blot was performed using radiolabeled 865–842 DNA probe containing the described binding sequence and the purified nuclear protein fraction. The blots are representative of four experiments. B, the amino acid sequence of PARP-1 is shown at the top, with shaded regions indicating peptides that were identified by MS/MS analysis. An example MS/MS spectrum of the peptide LQLLDEDDKESR is shown. The spectrum shown in this example was obtained by MS/MS analysis of the doubly charged [M + H2] peptide.
suggestion NO inhibits the binding and/or function of a trans-activator in the -859/-850 region of the iNOS promoter.

Inhibition of PARP-1 Expression by RNA Interference Inhibits Endogenous iNOS Protein Expression and iNOS Promoter Activity—If PARP-1 indeed functions as a trans-activator of the iNOS gene, reduced PARP-1 expression should result in reduced iNOS promoter activity. Accordingly, we tested the functional relevance of PARP-1 in the setting of IL-1β- and NO-dependent regulation of iNOS expression by transfecting mesangial cells with equimolar amounts of plasmids containing either siRNA targeting PARP-1 or a negative control siRNA and then treated the cells with IL-1β. Western blots of nuclear extracts showed that PARP-1 levels in cells treated with vehicle or with IL-1β + negative control siRNA were similar (Fig. 6A). However, cells transfected with PARP-1 siRNA and treated with IL-1β alone revealed substantially less PARP-1 protein compared with vehicle or IL-1β + negative control siRNA (Fig. 6A), whereas levels of α-tubulin were comparable between the two conditions, indicating the specificity of the siRNA for its target. The effects of PARP-1 knockdown on IL-1β-stimulated endogenous iNOS protein expression and on the activity of transfected piNOS-luc were then studied in mesangial cells cotransfected with the PARP-1 siRNA or negative control siRNA. iNOS protein levels (Fig. 6, A and B) and iNOS promoter activity (Fig. 6C) were significantly lower in cells transfected with PARP-1 siRNA than in the negative control siRNA, consistent with a role for PARP-1 as a trans-activator of the iNOS gene. Moreover, L-NAME treatment accentuated IL-1β-stimulated iNOS protein expression and iNOS promoter activity in cells transfected with the negative control siRNA. This effect was abrogated by co-administration of SNAP (as in the cells not exposed to siRNA, Fig. 1B), whereas these manipulations of NO levels had no effect in the cells transfected with PARP-1 siRNA (Fig. 6, B and C).

PARP-1 Is S-Nitrosylated in IL-1β-treated Mesangial Cells—S-Nitrosylation of protein thiols is a well described mechanism for NO-me-
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FIGURE 6. RNA interference knockdown of PARP-1 limits endogenous iNOS protein expression and the activity of an iNOS promoter-luciferase construct. A, mesangial cells were transiently transfected with the negative control siRNA plasmid pKD-NegCon-v1 (V1) or with the PARP-1 siRNA plasmid pKD-PARP-v2 (V2) and treated with vehicle or IL-1β for 24 h. Cytoplasmic and nuclear extracts were then prepared and iNOS and PARP-1 protein levels were analyzed, respectively, on immunoblots of these fractions. The blots were stripped and reprobed with an antibody against α-tubulin as a loading control. B, mesangial cells were cotransfected with pKD-NegCon-v1 or pKD-PARP-v2 and treated with the indicated combinations of reagents for 24 h before cell lysates were isolated and immunoblotted with iNOS or α-tubulin antibodies. The blot is representative of three experiments. C, mesangial cells were cotransfected with pKD-NegCon-v1 or pKD-PARP-v2 together with plNOS (-915/+137)-luc and the Renilla luciferase expression plasmid. The cells were subsequently treated with vehicle or IL-1β for 24 h, cell lysates prepared, and luciferase activity assayed. *p < 0.05 versus pKD-NegCon-v1-transfected cells (n = 4).

diated regulation of protein function. Murine PARP-1 has 15 cysteine residues (at positions 21, 24, 56, 125, 128, 162, 257, 296, 299, 312, 322, 430, 456, 908, and 983) that may serve as targets for S-nitrosylation. To determine whether PARP-1 is S-nitrosylated in the presence of IL-1β-triggered endogenous NO generation, proteins harvested from vehicle- or IL-1β-treated mesangial cells were immunoprecipitated with an anti-nitrocysteine antibody or non-immune IgG as a negative control, and the resulting immunoprecipitates were immunoblotted with an anti-PARP-1 antibody. As shown in Fig. 7A, PARP-1 was found to have significantly increased S-nitrocysteine content in the IL-1β-treated, but not control, cells, and IgG failed to immunoprecipitate PARP-1.

As a second measure of S-nitrosylation, the biotin-switch method was applied to mesangial cell nuclear proteins that had been treated with SNAP or vehicle. This method allowed the purification of protein S-nitrosothiols, which were then immunoblotted with an anti-PARP-1 antibody. As seen in Fig. 7B, PARP-1 was evident in the SNAP-treated, but not the vehicle-treated, samples, consistent with S-nitrosylation of the protein.

DISCUSSION

Because of the potent biological actions of NO in the kidney and other tissues, considerable effort has been directed toward identifying the mechanisms that activate and limit iNOS gene expression. In this report, we present evidence that PARP-1 is a novel trans-activator of the iNOS promoter in mesangial cells and that its binding to a specific cis-element of the iNOS promoter appears to be regulated by the end product NO in a feedback inhibition circuit: 1) PARP-1 binds to the distal region of the iNOS promoter and that its binding to a specific cis-element of the iNOS promoter fails to compete with the wild-type probe in gel shift studies, inhibits IL-1β-induced activity of the iNOS promoter–luciferase construct; 2) mutation of the −859 to −850 binding site of the iNOS promoter fails to compete with the wild-type probe in gel shift studies, inhibits IL-1β-induced activity of the iNOS promoter–luciferase construct, and abolishes the NO-sensitive inhibition of iNOS promoter activity; and 4) siRNA knockdown of endogenous PARP-1 inhibits IL-1β-induced iNOS protein expression and iNOS promoter activity and eliminates NO-dependent effects on these parameters. In addition, co-immunoprecipitation of PARP-1 with an anti-nitrocysteine antibody, immunoblot detection of PARP-1 in a sample of S-nitrosylated proteins that had been isolated by the biotin-switch method, and reversal of the NO inhibitory effect on PARP-1 DNA binding activity with DTT all support the con-
conclusion that PARP-1 is a target of NO-mediated S-nitrosylation and that this modification limits its DNA binding activity and ability to trans-activate the iNOS promoter.

The available data suggest that the activity of PARP-1 in transcriptional regulation occurs by functioning as part of enhancer/promoter binding complexes in conjunction with other DNA binding factors and coactivators and/or by modification of histones to alter chromatin binding complexes in conjunction with other DNA binding factors and transcriptional regulation occurs by functioning as part of enhancer/promoter complexes (40). YY1 (11), AP-1 (10), AP-2 (12), cAMP-response element-binding protein (40), and NF-xB (16) all have been reported to be highly specific substrates for poly(ADP-ribosyl)ation of transcription factors, which impairs their binding to specific promoter sequences (40).

In the present study, the binding of proteins to the −859/−850 region was specific in as much as the formation of the major complex 1 was inhibited by the addition of excess unlabeled oligomer, but not by an excess of unlabeled sequence in which the binding element was mutated (Fig. 28). However, because anti-PARP-1 antibody did not completely disrupt the DNA-protein complex in supershift assays (Fig. 4A), other transcription factors or co-regulatory proteins may contribute, to a quantitatively much lesser degree, to the complex. Further studies will be required to identify such proteins.

PARP-1 is a highly conserved, modular protein of 1014 amino acids comprised of an amino-terminal DNA binding domain that contains two Cys-Cys-His-Cys zinc finger motifs (amino acids 8–92 and 112–202) and a centrally located automodification domain that contains a “WGR” domain (amino acids 547–632) of PARP-1 binding and action at the iNOS promoter represents an endogenous mechanism to limit excessive NO generation in pathological states. Whether S-nitrosylation also alters PARP-1 catalytic function remains unknown but could potentially represent a dual mechanism to limit excessive PARP and iNOS activation.

**REFERENCES**

1. Kone, B. C. (1997) *Am. J. Kidney Dis.* 30, 311–333
2. Szabo, C. (2006) *Nitric Oxide* 14, 169–179
3. Ame, J. C., Speltehauer, C., and de Murcia, G. (2004) *BioEssays* 26, 882–893
4. Kraus, W. L., and Lis, J. T. (2003) *Cell* 113, 677–683
5. Soldatenkov, V. A., Chasovnikhskii, S., Potaman, V. N., Trofimova, I., Smulson, M. E., and Dritscho, A. (2002) *J. Biol. Chem.* 277, 665–670
6. Nagele, A. (1995) *Radiat. Environ. Biophys.* 34, 251–254
7. Mably, J. G., and Soriano, F. G. (2005) *Curr. Vasc. Pharmacol.* 3, 247–252
8. Verses, B., Radnui, B., Gallyas, F., Jr., Varibio, G., Berente, Z., Osz, E., and Sumegi, B. (2006) *J. Pharmacol. Exp. Ther.* 310, 247–255
9. Meder, V. S., Boeglin, M., de Murcia, G., and Schreiber, V. (2005) *J. Cell Sci.* 118, 211–222
10. Andreeone, T. L., O’Connor, M., Denenberg, A., Hake, P. W., and Zingarelli, B. (2003) *J. Immunol.* 170, 2113–2120
11. Oei, S. L., and Shi, Y. (2001) *Biochem. Biophys. Res. Commun.* 285, 27–31
12. Li, M., Naidz, P., Yu, Y., Berger, N. A., and Kannam, P. (2004) *Biochem. J.* 382, 323–329
13. Hassa, P. O., Covic, M., Hasan, S., Imhof, R., and Hottinger, M. O. (2001) *J. Biol. Chem.* 276, 45588–45597
14. Hassa, P. O., Haenni, S. S., Buerki, C., Meier, N. I., Lane, W. S., Owen, H., Gersbach, M., Imhof, R., and Hottinger, M. O. (2005) *J. Biol. Chem.* 280, 40450–40464
15. Hassa, P. O., and Hottinger, M. O. (1999) *Biochim. Biophys. Acta* 1403, 953–959
16. Hassa, P. O., and Hottinger, M. O. (2002) *Cell Mol. Life Sci.* 59, 1533–1553
17. Zhang, X., Hildebrandt, E. F., Simbulan-Rosenthal, C. M., and Anderson, M. G. (2002) *Virology* 296, 107–116
18. Boulouar, A. H., Zoltoski, A. J., Sherif, Z. A., Jolly, P., Massaro, D., and Smulson, M. E. (2003) *Am. J. Respir. Cell Mol. Biol.* 28, 322–329
19. Stamler, J. S., Lamas, S., and Fang, F. C. (2001) *Cell* 106, 675–683
20. Ohn, S. J., and Brune, B. (1996) *J. Biol. Chem.* 271, 4209–4214
21. Xu, X., Li, X. P., Meissner, G., and Stamler, J. S. (1998) *Science* 279, 234–237
22. Choi, Y. B., Treniti, L., Le, D. A., Ortiz, J., Bai, G., Chen, H. S., and Lipton, S. A. (2000) *Nat. Neurosci.* 3, 15–21
23. Wolosker, H., Panizzutti, R., and Engelender, S. (1996) *FEBS Lett.* 392, 274–276
24. Marshall, H. E., and Stamler, J. S. (2001) *Biochemistry* 40, 1688–1693
25. Vossen, C., and Erard, M. (2002) *Med. Sci. Monit.* 8, RA217–220
26. Gao, C., Guo, H., Wei, J., Mi, Z., Wai, P., and Kuo, P. C. (2004) *J. Biol. Chem.* 279, 11236–11243
27. Schonhoff, C. M., Daou, M. C., Jones, S. N., Schiffer, C. A., and Ross, A. H. (2002) *Biochemistry* 41, 13570–13574
28. Palmer, L. A., Gaston, B., and Johns, R. A. (2000) *Mol. Pharmacol.* 58, 1197–1203
29. Gow, A. J., Chen, Q., Hess, D. T., Day, B. J., Ischiropoulos, H., and Stamler, J. S. (2002) *J. Biol. Chem.* 277, 9637–9640
30. Gupta, A. K., and Kone, B. C. (1999) *Am. J. Physiol.* 276, Pt. 2, F599–F605
31. Gupta, A. K., Diaz, R. A., Highnam, S., and Kone, B. C. (2000) *Kidney Int.* 57, 2239–2248
32. Zhang, W., Kuncwicz, T., Highnam, S. C., and Kone, B. C. (2001) *Am. J. Soc. Nephrol.* 12, 2554–2564
33. Moritz, R. L., Eddes, J. S., Reid, G. E., and Simpson, R. J. (1996) *Electrophoresis* 17, 907–917
34. Yu, Z., and Kone, B. C. (2006) *Am. J. Physiol. Renal Physiol.* 290, F496–F502
35. Kuncwicz, T., Sheta, E. A., Goldknoedl, I. L., and Kone, B. C. (2003) *Mol. Cell Proteomics* 2, 156–163
36. Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P., and Snyder, S. H. (2001) *Nat. Cell Biol.* 3, 193–197
37. Yu, Z., Zhang, W., and Kone, B. C. (2002) *J. Am. Soc. Nephrol.* 13, 2009–2017
38. Butler, A. J., and Ordahl, C. P. (1999) *Mol. Cell. Biol.* 19, 296–306
39. Plaza, A., Aumerier, M., Bailly, M., Dozier, C., and Saule, S. (1999) *Oncogene* 18, 1041–1051
40. Oei, S. L., Griesenbeck, J., Schweiger, M., and Ziegler, M. (1998) *J. Biol. Chem.* 273, 31644–31647
41. Nguewa, P. A., Fuertes, M. A., Valladares, B., Alonso, C., and Perez, J. M. (2005) *Prog. Biol. Mol. Biol.* 88, 143–172
42. D’Amours, D., Desnoyers, S., D’Silva, I., and Poirier, G. G. (1999) *Biochem. J.* 342, 249–268
43. Hausladen, A., Privalle, C. T., Keng, T., DeAngelo, J., and Stamler, J. S. (1996) *Cell* 86, 719–729
44. Everson, O. V., and Liaudet, L. (2005) *Carr. Vasc. Pharmacol.* 3, 293–299
45. van Wijk, S. J., and Hageman, G. J. (2005) *Free Radic. Biol. Med.* 39, 81–90