Heme Oxygenase-1 Induction Attenuates Inducible Nitric Oxide Synthase Expression and Proteinuria in Glomerulonephritis

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Abstract. In glomerulonephritis, there is intraglomerular activation of inducible nitric oxide synthase (iNOS) leading to high output production of nitric oxide (NO). This can result in supraphysiologic amounts of NO and cause oxidative injury. It is unknown whether mechanisms of cellular defense against NO-mediated injury exist. Induction of the heme catabolizing enzyme heme oxygenase-1 (HO-1), which generates biliverdin, carbon monoxide (CO), and iron (Fe), may provide such a mechanism, as CO and Fe are two negative modulators of iNOS activity and expression. This study assessed whether upregulation of HO-1 by a specific inducer, hemin, in nephritic animals was upregulated by treatment with hemin (30 μmol/kg body wt). iNOS and HO-1 mRNA expression were assessed by reverse transcription-PCR of glomerular total RNA from nephritic animals or nephritic animals pretreated with hemin. iNOS activity in glomeruli was measured by assessing conversion of [14C] L-arginine to [14C] L-citrulline. HO-1 protein levels in glomeruli were assessed by Western blot analysis. The effect of hemin treatment on monocyte/macrophage infiltration was assessed by enumeration of ED-1-positive cells in nephritic glomeruli.

Glomerular HO-1 expression in nephritic animals was upregulated by treatment with hemin (30 μmol/kg body wt). iNOS and HO-1 mRNA expression were assessed by reverse transcription-PCR of glomerular total RNA from nephritic animals or nephritic animals pretreated with hemin. iNOS activity in glomeruli was measured by assessing conversion of [14C] L-arginine to [14C] L-citrulline. HO-1 protein levels in glomeruli were assessed by Western blot analysis. The effect of hemin treatment on monocyte/macrophage infiltration was assessed by enumeration of ED-1-positive cells in nephritic glomeruli. Hemin treatment of nephritic animals resulted in upregulation of glomerular HO-1 levels and a two- to threefold reduction in glomerular iNOS mRNA levels. iNOS activity in glomeruli was significantly reduced in hemin-treated nephritic animals in which proteinuria was also attenuated without a change in monocyte/macrophage infiltration. Hemin (100 to 200 μM) also reduced iNOS protein levels and enzyme activity in cultured mesangial cells stimulated with cytokines. These studies demonstrate that in glomerular immune injury, hemin treatment upregulates glomerular HO-1 with an attendant downregulation of iNOS expression, and thus points to regulatory interaction between the two systems. The beneficial effect of hemin treatment on proteinuria could be linked to downregulation of iNOS.

The role of nitric oxide (NO) in renal immune injury has recently become the focus of intense investigation. In various forms of glomerulonephritis, there is increased production of NO in isolated glomeruli and enhanced expression and activation of the inducible isoform of nitric oxide synthase (iNOS) (1–8). iNOS-derived NO release is sustained and of high output (9) and can reach supraphysiologic levels within nephritic glomeruli thereby causing oxidative injury. This form of injury can occur by NO itself (9) or by the potent oxidant peroxynitrite (ONOO−), which is formed by the interaction of NO with superoxide (10). Whether glomerular cells possess systems that can be rapidly recruited to defend against NO-mediated oxidative injury is unknown. One putative candidate is heme oxygenase (HO), whose glomerular expression becomes enhanced after immune injury (11). HO is the rate-limiting enzyme in the catabolism of heme-generating biliverdin, carbon monoxide (CO), and iron (12). Three distinct isoforms of HO have been identified: HO-1, HO-2 (13), and HO-3 (14). HO-1 is an inducible isoform activated by most oxidative stress inducers and cytokines (13). HO-2 is constitutively expressed and is regulated by glucocorticoids and adrenocorticoids (13). The recently described isoform HO-3 has 90% homology with HO-2 at the amino acid sequence level and is a poor heme catalyst (14). In glomerular immune injury, activation of HO-1 may defend against NO-mediated toxicity by negatively modulating iNOS expression or activity. Thus, catabolism of cellular heme by HO-1 would limit iNOS synthesis as iNOS contains a heme iron moiety (15). Moreover, CO released in the process of heme catabolism can inactivate existing iNOS by interacting with its heme iron moiety (16). Finally, the released iron can downregulate iNOS transcription (17).

Whether upregulation of HO-1 negatively modulates iNOS...
zyme activity of iNOS 24 h after administration of anti-GBM anti-

previous observations that demonstrated a peak expression and en-

previously (18). This time point was chosen on the basis of our

were isolated by differential sieving of minced cortex as described

7.4 with 0.1N HCl, and further diluted in saline for subcutaneous

immediately before use by dissolving in 0.1N NaOH, adjusted to pH

rabbit nonimmune serum. A solution of hemin (10 mM) was prepared

24 h before the first and second injection of anti-GBM serum or of

administration to rats not preimmunized with rabbit IgG (subnephri-

HO-1/iNOS Interactions in Nephritis 2541


Materials and Methods

Development of Experimental Glomerulonephritis

Male Lewis rats weighing 175 to 200 g were immunized intraperi-
toneally with 1 mg of rabbit IgG emulsified with Freund’s adjuvant
and given as a total volume of 0.5 ml. Five days after this immuni-

animals were injected in the tail vein with a subnephriotogenic

(0.3 ml/100 g body wt) dose of rabbit immune serum raised against rat

particulate GBM as described previously (18). This dose of anti-rat

GBM serum does not cause significant proteinuria when administered

to rats not preimmunized with rabbit IgG (subnephriotogenic). The

intravenous injection of anti-rat GBM serum was repeated 24 h after

this first injection. This protocol results in heavy proteinuria and

accelerated onset of glomerular cell proliferation, crescent formation, and

scarring. Control rats were preimmunized with rabbit IgG emul-

sified with Freund’s adjuvant and subsequently given two intravenous

injections of nonimmune rabbit serum. Before sacrifice, animals were

placed in metabolic cages for urine collection to assess urinary protein

and creatinine excretion.

Animals were nephrectomized on days 2, 7, and 14 after the second

injection of anti-rat GBM serum or of nonimmune rabbit serum to

isolate glomeruli by differential sieving of minced cortex as described

previously (18). This procedure typically yields glomerular prepara-

tions of 95% to 98% purity. Isolated glomeruli were used for prepa-

ration of protein lysates and total RNA.

Administration of Hemin

Hemin (Ferrirprotoporphyrin IX chloride; Sigma, St. Louis, MO), a

specific inducer of HO-1, was used to study the effect of HO-1 in-
duction on iNOS expression. Animals were injected subcutaneously

with hemin at a dose of 30 μmol/kg body wt, or with saline vehicle

24 h before the first and second injection of anti-GBM serum or of

rabbit nonimmune serum. A solution of hemin (10 mM) was prepared

immediately before use by dissolving in 0.1N NaOH, adjusted to pH

7.4 with 0.1N HCl, and further diluted in saline for subcutaneous

injections. Animals were nephrectomized 24 h after the second in-

jection of anti-rat GBM serum or of nonimmune rabbit serum. Glomeruli

were isolated by differential sieving of minced cortex as described

previously (18). This time point was chosen on the basis of our

previous observations that demonstrated a peak expression and en-

zyme activity of iNOS 24 h after administration of anti-GBM anti-
body (6).

Immunofluorescence Microscopy

Renal cortical sections were snap-frozen in OCT embedding me-
dium and processed for immunofluorescence studies. These studies

defined deposition of anti-GBM antibody and also assessed the

extent of glomerular infiltration by monocytes/macrophages identified

as cells positive for the rat monocyte-macrophage marker ED-1.

Evaluation of ED-1-Positive Cells

Renal cortical sections were stained with an FITC-conjugated

anti-ED-1 antibody (Serotec, Westbury, NY) at a dilution of 1:50 in

phosphate-buffered saline. Sections of at least 25 glomeruli with a

cross-sectional profile of 103 μm were examined. Results were ex-

pressed as ED-1 (+) cells per glomerulus.

Estimation of Urinary Protein and Creatinine

Urinary protein concentrations were estimated by a colorimetric

protein assay. Urinary creatinine concentrations were determined us-

ing a creatinine assay kit (Sigma).

Isolation of Total Glomerular RNA

Total RNA was prepared from glomeruli isolated by differential sieving using Tri-Reagent (Sigma) as described earlier (19). RNA

concentration and quality were assessed spectrophotometrically at

wavelengths of 260 and 280 nm.

Quantification of Glomerular iNOS and HO-1 mRNA Levels by Reverse Transcription-PCR

mRNA for iNOS and HO-1 was detected and quantified by reverse

transcription (RT)-PCR. Aliquots of total glomerular RNA (200 or

400 ng) were used in a RT reaction in the presence of 2.5 μM of

random hexamer primers, 1 mM each of dATP, dCTP, dGTP, and
dTTP, 1 μM RNase inhibitor, 5 mM MgCl2, 1× PCR buffer II (50

mM KCl, 10 mM Tris-HCl, pH 8.3), and 2.5 U/μl murine leukemia

virus reverse transcriptase in a total volume of 10 μl according to the

manufacturer’s instructions (Perkin Elmer, Foster City, CA). After

heating at 95°C for 5 min, the entire reaction mixture was used for

PCR amplification. Primers (1 μM) for the iNOS, HO-1, and GAPDH

were synthesized by Operon (Alameda, CA) and used in a final

reaction volume of 50 μl containing 1× PCR buffer II and 0.05 U/μl of

AmpliTaq DNA polymerase (Perkin Elmer).

The iNOS primers were as follows: forward, 5’-GATGGAAC-

CAGTATAAGGCAAACA-3’; reverse, 5’-TTTCTGGTGCAT-

AGCAA-3’. These primers yield a PCR product of 222 bp (20).

The HO-1 primers were as follows: forward, 5’-CAGTGGAA-

CAGTATAAGGCAAACA-3’; reverse, 5’-GTTTCTGGTGCAT-

AGCAA-3’. These primers yield a PCR product of 284 bp (21).

The GAPDH primers were as follows: forward, 5’-GATGGAAC-

CAGTATAAGGCAAACA-3’; reverse, 5’-CAGTATAAGGCAAACA-

GCAAACA-3’. These primers yield a PCR product of 298 bp (22).

The PCR cycling profile was 1 min at 95°C, 1 min at 55°C, and 3

min at 72°C. Thirty cycles were carried out and followed by a final

extension at 72°C for 10 min. Ten microliters of PCR products was

then separated on a 2% agarose gel, stained with ethidium bromide,

and photographed. In pilot experiments, the identities of the PCR

products were verified by dyeoxy sequencing.

Competitive RT-PCR

Design of Primers for Rat iNOS Competitor Construct. The

forward and reverse primers for rat iNOS were edited to the 5’ end

of the respective forward and reverse neutral primer, the sequence of

which was obtained from Clontech (Palo Alto, CA).

The sequences of the edited composite primers were: forward, 5’-

TATACCGA ACTATAGGGGCGATGGTGAAGCAG- GCAAACAGCAATCCG-3’ (the sequence in boldface represents

the respective forward and reverse neutral primer, the sequence of

which was obtained from Clontech (Palo Alto, CA)).

The GAPDH primers were as follows: forward, 5’-GTTTCTGGTGCAT-

AGCAAACA-3’; reverse, 5’-CAGTATAAGGCAAACA-3’.

These primers yield a PCR product of 298 bp (22).

The PCR cycling profile was 1 min at 95°C, 1 min at 55°C, and 3

min at 72°C. Thirty cycles were carried out and followed by a final

extension at 72°C for 10 min. Ten microliters of PCR products was

then separated on a 2% agarose gel, stained with ethidium bromide,

and photographed. In pilot experiments, the identities of the PCR

products were verified by dyeoxy sequencing.

PCR Amplification of Rat iNOS cDNA Construct. To gener-

ate a DNA template for the rat iNOS cDNA construct, the DNA of

known sequence (PCR mimic construct kit K 1700-1, Clontech) was

amplified by PCR using gene-specific composite primers to generate

a 364-bp product.
Synthesis of Competitor RNA. The 364-bp PCR product was transcribed in vitro using a T7 RNA polymerase-based transcription kit (Invitrogen, San Diego, CA) to generate a 348-nucleotide-long competitor RNA. The template was then digested with DNase I, and the reaction products were extracted with phenol:chloroform. After removal of unincorporated nucleotides and precipitation of the RNA, the amount of the competitor RNA synthesized was quantified by spectrophotometry. The RNA was diluted to 1000 attomoles and stored at $-70^\circ$C.

The Competitive Reaction. Serial dilutions of competitor (mimic) RNA of known concentration were added to RT-PCR reactions containing a constant amount of total RNA (200 ng). The RT-PCR and PCR mixture was the same as that for PCR described above. The PCR cycling profile was 1 min at 94°C followed by 40 s at 92°C, 40 s at 60°C, and 1.5 min at 75°C for 35 cycles with a final extension at 75°C for 5 min.

Quantification of Target and Mimic Amounts

Twenty microliters of PCR product was separated on 1.5% agarose gels and visualized by staining with ethidium bromide. The gels were directly scanned by an image analysis system (FluorImager SI; Molecular Dynamics, Sunnyvale, CA), and the optical density of the PCR products was determined by an image quantification system (ImagQuant Software; Molecular Dynamics). The log of the ratio of the target to that of the mimic was plotted against the log of the concentration of the mimic. The point of equivalence was defined as the point on the line at which the log of the ratio of target to mimic equals zero. The concentration of the competitor (mimic) at equivalence can thus be derived and is identical to the amount of iNOS cDNA (target).

Mesangial Cell Culture

SV40-transformed mouse mesangial cells (MES13) obtained from American Type Culture Collection (Rockville, MD) were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Life Technologies, Gaithersburg, MD) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 14 mM Heps, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Cells were grown at 37°C under 5% CO$_2$/95% air. For experiments (see below), subcultured cells were seeded into 6-well plates and grown to confluence.

Effect of Hemin on iNOS Expression/Activity in Mesangial Cells

These experiments were performed to strengthen the evidence that the HO-1 inducer hemin downregulates iNOS expression/activity. To examine the effect of hemin on mesangial cell iNOS expression and activity, cells were incubated for 1 h in medium containing 0.1% FBS (control) or in medium containing 0.1% FBS and hemin (100 or 200 μM). After this pretreatment (HO-1 induction), the media were removed, fresh media were added, and cells were allowed to recover for 3 h. Cells were then treated with a combination of lipopolysaccharide (Sigma) and interferon-γ (IFN-γ) (Genzyme, Boston, MA) at 10 μg/ml and 10 ng/ml, respectively, for 24 h to activate iNOS expression and activity.

Effect of HO-1 Inhibition on iNOS Activity in Mesangial Cells

These experiments were performed to explore whether HO-1 inhibition upregulates iNOS activity. To assess the effect of HO-1 inhibition on iNOS activity, MES 13 cells were treated with a Heme oxygenase inhibitor. Two inhibitors were considered, tin protoporphyrin (SnPP-9) and zinc protoporphyrin (ZnPp-9). We used SnPP-9 because it has better solubility in physiologic buffers (23). Cells were treated with a combination of lipopolysaccharide (Sigma) and IFN-γ (Genzyme) at 10 μg/ml and 10 ng/ml, respectively, for 24 h to activate iNOS expression and activity. This was performed in the presence and absence of the HO inhibitor SnPP-9 at concentrations of 100 and 200 μM.

Protein lysates prepared from cells subjected to the various treatments described above were processed for Western blot analysis to assess changes in iNOS protein levels.

Preparation of Protein Lysates

Mesangial cells or glomeruli were sonicated in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl$_2$, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetra-acetic acid, 10% glycerol, 1% Triton X-100, 200 μM sodium orthovanadate, the protease inhibitors aprotinin and leupeptin [1 μg/ml], and 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 14,000 rpm for 30 min, and aliquots were stored frozen at $-70^\circ$C. Protein concentrations were estimated by the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

Glomerular or mesangial cell protein lysates (100 μg) were mixed with sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH 7.4, 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol and dye), boiled for 5 min, and separated by SDS-polyacrylamide gel electrophoresis using 4 to 20% Tris-glycine gel (Bio-Rad Laboratories). The separated proteins were transferred electrophoretically (Bio-Rad Laboratories) to Hybond-ECL membranes (Amersham, Arlington Heights, IL) at 25 V at 4°C as described previously (24). The membranes were blocked for 1 h in TBS-T (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat milk. Membranes were then incubated with rabbit polyclonal antibody against rat HO-1 (1:1000 dilution) obtained from StressGen Biotechnologies (Vancouver, British Columbia, Canada), subsequently washed thoroughly in TBS-T and incubated with anti-rabbit horseradish peroxidase-conjugated antibody (1:7500 dilution) for 2 h. Detection of signal used an enhanced chemiluminescence detection kit (Amersham).

iNOS Enzyme Activity

Activity of iNOS in glomerular lysates was determined by monitoring conversion of l-$^{[14]}$CJarginine to l-$^{[14]}$Cцитrulline (25). Reaction mixtures contained in a final volume of 50 μl, 50 mM Na$^+$ Heps buffer, pH 7.4, 100 μM ethylenediaminetetra-acetic acid, 50 μM BH$_4$, 2.5 μM flavin adenine dinucleotide, 2.5 μM flavin mononucleotide, 500 μM NADPH, 100 μM glutathione, and 20 μM l-$^{[14]}$CJarginine. Reactions were initiated by addition of protein lysate (260 μg) and allowed to proceed at 25°C for 6 min. Reaction mixtures were then quenched by addition of 200 μl of stop buffer (100 mM Na$^+$ Heps buffer, pH 5.5, and 5 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetra-acetic acid) and heated in a boiling water bath for 1 min. Samples were then chilled and centrifuged, and the supernatant was applied to a Dowex 50 column (1 ml resin, Na$^+$ form). The product, l-$^{[14]}$Ccitrulline, was eluted with 2 ml of water and quantified by liquid scintillation counting. Results were expressed as pmol citrulline/min.

Nitrite Production Assay

Nitrite levels were measured in mesangial cell culture medium after 24 h incubation of cells with lipopolysaccharide (LPS)/IFN-γ mixture or LPS/IFN-γ mixture containing SnPP-9 (100 to 200 μM) using the
Griess reaction method (Promega, Madison, WI). A total of 50 μl of culture medium was reacted with an equal volume of 0.5% sulfanilamide for 5 min and then with 50 μl of 0.05% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% H₃PO₄ for 5 min in 96-well plates at room temperature. The resulting azodye product was spectrophotometrically quantified at 540 nm using a microplate reader (SLT-Labinstruments, Salzburg, Austria), and nitrite levels were determined by comparison with standard curves made from a solution of sodium nitrite.

**Statistical Analyses**

Data are expressed as mean ± SEM. Comparisons used t test statistics for unpaired observations. P < 0.05 was considered significant.

**Results**

*In Anti-GBM Antibody-Induced Injury, Glomerular HO-1 and iNOS Are Co-Induced*

Using RT-PCR, we assessed changes in HO-1 and iNOS expression (mRNA) in total RNA of glomeruli isolated from rats with anti-GBM antibody-induced injury and controls. Detectable levels of iNOS and HO-1 mRNA were found in glomeruli isolated from control animals on days 2, 7, and 14 after injection of nonimmune rabbit serum (Figure 1, A and B, lanes 1, 3, and 5). In glomeruli isolated from nephritic animals (days 2, 7, and 14), there was a marked increase in iNOS and HO-1 mRNA levels (Figure 1, A and B, lanes 2, 4, and 6). There was no change in the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a marker of equal RNA amounts, in glomeruli of control compared to nephritic animals (Figure 1C, lanes 1 to 6).

**Hemin Treatment Enhances Glomerular HO-1 Expression**

Hemin injected subcutaneously at a dose of 30 μmol/kg body wt upregulated glomerular HO-1 (mRNA) expression as assessed by RT-PCR. This was observed in glomeruli of both non-nephritic (Figure 2A panel A, lane 1 compared to lane 2) and nephritic animals (Figure 2A panel A, lanes 5 and 6 compared to lanes 3 and 4).

We also explored whether pretreatment of nephritic animals with hemin increases glomerular HO-1 protein levels. We assessed HO-1 protein levels by Western blot analysis in glomerular lysates prepared either from nephritic animals that were not treated with hemin or from nephritic animals pretreated with hemin. HO-1 protein levels were markedly increased in nephritic glomeruli of hemin-pretreated animals (Figure 2B, lane 2) compared to nephritic animals not pretreated with hemin (Figure 2B, lane 1). The equal representation of a nonspecific band (n.s.) that cross-reacts with HO-1 antibodies serves as an internal control to demonstrate equal loading of proteins.

**Hemin Treatment Attenuates Glomerular iNOS Enzyme Activity**

We assessed whether hemin treatment reduced iNOS enzyme activity in nephritic glomeruli. iNOS activity was determined by the conversion of [¹⁴C]arginine to L-[¹⁴C]citrulline in the absence of Ca²⁺ as described in Materials and Methods. iNOS activity measured in glomerular lysates obtained from nephritic animals pretreated with hemin was significantly reduced compared to that in lysates from nephritic animals that did not receive hemin (0.846 ± 0.26 pmol citrulline/min versus 2.158 ± 0.4 pmol citrulline/min, n = 3) (Figure 3). Levels of iNOS activity in glomeruli isolated from control animals that received nonimmune serum alone or from animals that received nonimmune serum and hemin were no different from assay background.

**Hemin Attenuates Glomerular iNOS (mRNA) Expression**

Using RT-PCR, we first assessed the effect of hemin on levels of iNOS mRNA in glomeruli of non-nephritic animals.
Levels of iNOS mRNA in glomeruli of non-nephritic rats pretreated with hemin and in glomeruli of non-nephritic rats that were not are shown in Figure 4A. In rats that were not pretreated with hemin, detectable levels of iNOS mRNA were found in glomeruli (lane 1). In glomeruli of non-nephritic rats that were pretreated with hemin, iNOS mRNA levels were barely detectable (lane 2). Levels of GAPDH were similar in glomeruli of either group of animals (Figure 4A, lane 3 compared to lane 4).

We subsequently assessed the effect of hemin treatment on iNOS mRNA levels in glomeruli of nephritic animals to de-
termine whether the reduction in activity of iNOS enzyme activity observed in nephritic animals pretreated with hemin was associated with a reduced iNOS expression. In rats with anti-GBM antibody-induced injury that were pretreated with hemin, glomerular iNOS mRNA levels were lower compared to those in nephritic animals that were not pretreated with hemin (Figure 4B, lane 1 compared to lane 2). Hemin treatment had no effect on levels of GAPDH (Figure 4B, lane 4 compared to lane 5).

**Quantitative Assessment of the Reduction in iNOS Expression in Glomeruli of Hemin-Treated Nephritic Animals**

Quantification of the reduction in glomerular iNOS mRNA levels in hemin-treated nephritic animals and in untreated nephritic controls was performed by competitive RT-PCR. Figures 5 and 6 show two independent experiments.

Panel A in both figures demonstrates agarose gel electrophoresis profiles of PCR products obtained using a constant amount of total RNA and varying amounts of competitor iNOS mimic RNA. The total RNA used in the competitive RT-PCR reactions was isolated from glomeruli of nephritic animals that were not pretreated with hemin (lanes 1 to 7), and from glomeruli of nephritic animals pretreated with hemin (lanes 8 to 14).

The left plot in Panel B of Figure 5 is a regression analysis of the effect of decreasing amounts of competitor RNA (mimic) added in the competitive reaction on the ratio of amplified products shown in lanes 1 to 7 of the gel above the plot. Equivalence, as defined in Materials and Methods and as derived from this plot, was reached when 0.036 attomoles of competitor (mimic) were present in the reaction. The right plot in panel B of Figure 5 is a regression analysis of the effect of decreasing amounts of competitor RNA (mimic) added in the competitive reaction on the ratio of amplified products shown in lanes 8 to 14 of the gel above the plot. Equivalence, as derived from this plot, was reached when 0.012 attomoles of competitor (mimic) was present in the reaction. Thus, in this particular experiment, there was a threefold decrement in iNOS mRNA levels in glomeruli of nephritic animals pretreated with hemin.

A similar analysis was performed in the experiment shown in Figure 6. Equivalence, as derived from the left plot in panel B of Figure 6, was reached when 0.026 attomoles of competitor (mimic) were present in the competitive reaction. Equivalence, as derived from the right plot in panel B of Figure 6, was reached when 0.012 attomoles of competitor (mimic) were present in the competitive reaction. Thus, in this particular experiment, there was a twofold decrement in iNOS mRNA levels in glomeruli of nephritic animals pretreated with hemin.

**Hemin Treatment Attenuates Proteinuria without an Effect on Glomerular Monocyte/Macrophage Infiltration**

A significant reduction of urine protein excretion was observed in nephritic animals pretreated with hemin compared to nephritic animals that were not (Figure 7). Because the anti-GBM antibody model of injury used in our studies is characterized by prominent macrophage monocyte infiltration in glomeruli (26), we assessed whether hemin treatment attenuated macrophage monocyte infiltration thereby accounting for the reduction in proteinuria. In glomeruli from nephritic animals that were not pretreated with hemin, there were 17.8 ± 0.78 ED-1-positive cells per glomerulus. In glomeruli from nephritic animals that were pretreated with hemin, there were 16.3 ± 0.36 ED-1-positive cells per glomerulus. These values were not statistically different (P < 0.1).

**Hemin Attenuates iNOS Activity and Expression in Mesangial Cells Stimulated with Cytokines**

To strengthen the evidence that hemin specifically down-regulates iNOS, we assessed the effect of hemin on changes in iNOS activity and iNOS protein levels in cultured mesangial cells stimulated with LPS and IFN-γ. iNOS activity, as assessed by nitrite levels in the media, and iNOS protein levels, as assessed by Western blot analysis, were significantly increased in cells stimulated with LPS and IFN-γ (Figure 8, A and B, lane 2). In cells pretreated with hemin and then stimulated with LPS and IFN-γ, nitrite levels in the media and iNOS protein levels were reduced compared to those obtained in cytokine-stimulated cells that were not pretreated with hemin (Figure 8, A and B, lanes 3 and 4).

**Effect of Inhibition of the HO-1 Inhibitor SnPP-9 on Mesangial Cells’ iNOS Activity**

To explore whether inhibition of HO-1 activity upregulates iNOS, we assessed the effect of SnPP-9, a potent inhibitor of heme oxygenase, on iNOS activity. In mesangial cells stimulated with LPS and IFN-γ, nitrite production was greatly enhanced (Figure 9). In cells stimulated with LPS and IFN-γ in the presence of SnPP-9 (concentrations of 100 or 200 μM), nitrite levels in media were attenuated (Figure 9).

**Discussion**

In the present study, we demonstrate that in anti-GBM antibody-mediated glomerular injury there is coinduction of iNOS and HO-1 and that upregulation of HO-1 by hemin treatment of nephritic animals attenuates glomerular iNOS expression and activity. These observations point to a regulatory interaction between the HO-1 and iNOS systems within the nephritic glomerulus. The cellular origin of HO-1 in anti-GBM antibody-mediated nephritis was addressed in a recent study in which HO-1 was immunohistochemically localized in infiltrating macrophages, although the contribution of the resident glomerular cells could not be ruled out (11). The mechanism whereby upregulation of HO-1 attenuates iNOS expression requires exploration. There are a number of potential mechanisms that can mediate this effect. Activation of HO-1 by hemin can promote catabolism of cellular heme to biliverdin, carbon monoxide (CO), and iron (Figure 10). Catabolism of cellular heme by HO-1 would limit new iNOS synthesis as iNOS contains heme-iron moiety (15). CO released in the process of heme degradation can inactivate existing iNOS by...
interacting with its heme iron moiety (16). Moreover, the released iron can downregulate iNOS transcription (17). Thus, HO-1 activation can negatively modulate iNOS by three different mechanisms: (1) reduction of cellular heme levels; (2) CO-mediated iNOS inactivation; and (3) iron-mediated inhibition of iNOS transcription. Our results indicate that the negative modulation of iNOS occurs possibly at the transcription (Figures 5 and 6) and enzyme activity (Figure 3) levels.

Recent studies have established that in various forms of glomerular immune injury, there is enhanced expression and activation of iNOS in nephritic glomeruli associated with increased generation of NO (1–8). iNOS-derived NO release can be sustained and of high output thereby reaching cytotoxic levels (9). Cytotoxicity can be mediated by NO itself (9) or by peroxynitrite (ONOO⁻), a relatively stable oxidant formed as a product of NO and superoxide interaction (10). In inflammatory forms of glomerular injury, cells could defend themselves against NO-mediated toxicity by recruiting systems to inactivate iNOS. In this context, HO-1 may emerge as an effective and versatile system. We have recently shown that NO is a direct activator of HO-1; specifically, in glomerular mesangial cells, exogenous NO or NO derived from iNOS activation upregulates HO-1 expression and synthesis (24). This points to a cross-talk between the iNOS and HO-1 systems whereby iNOS-derived NO can upregulate HO-1. The present studies suggest that a cross-talk in the opposite direction may also exist; that is, HO-1 activation may downregulate iNOS. Thus, a bidirectional regulatory interaction between HO-1 and iNOS may be operative whereby iNOS-derived NO upregulates HO-1, which can negatively modulate iNOS (Figure 10). To strengthen the evidence for this interaction, we used cultured mesangial cells in which iNOS was upregulated by cytokine treatment (LPS and IFN-γ mixture). Pretreatment with hemin attenuated iNOS expression and enzyme activity in response to these cytokines (Figure 9, A and B). Thus, these in vitro experiments strengthen the evidence that the HO-1 inducer hemin downregulates iNOS as we observed in glomeruli of normal and nephritic animals treated with hemin. We also explored the strategy of assessing the effect of HO-1 inhibition on iNOS expression, the expected results being an upregulation of iNOS expression/activity. This approach requires use of HO-1 inhibitors. Known inhibitors of HO activity are SnPP-9 and ZnPP-9 (12,13). However, the use of these metalloporphyrins to assess the effect of HO-1 inhibition on iNOS expression/activity is problematic because both metalloporphyrins directly inhibit nitric oxide synthase (23,27), guanylate cyclase (23,28,29), and interleukin-1 activity (30,31). Indeed, our experiments (Figure 9) confirm earlier reports that metalloporphyrins inhibit iNOS activity (23,27), and this makes SnPP-9 an inappropriate tool to inhibit HO-1 to assess effect of this inhibition on iNOS expression/activity in nephritic animals.

The beneficial effect of HO-1 activation in glomerular immune injury becomes apparent from the effect of hemin treatment of nephritic animals on urine protein excretion (Figure 7). In these animals, proteinuria was reduced without a change in the extent of glomerular infiltration by monocytes/macrophages. The attenuation of proteinuria in hemin-treated nephritic animals confirms a recent study by Mosley and coworkers (11). Whether the reduction of proteinuria in hemin-treated nephritic animals is specifically due to the observed downregulation of iNOS or occurs independently of this effect on iNOS is unknown. Upregulation of HO-1 by hemin may reduce the

**Figures 5 and 6.** Two independent analyses of changes in glomerular iNOS mRNA levels determined by quantitative RT-PCR in nephritic animals treated with the HO-1 inducer hemin. In both figures, Panel A is the agarose gel electrophoresis profile of PCR products obtained from competitive RT-PCR reactions. Lanes 1 to 7 in each Panel A show PCR products obtained using RNA of glomeruli from nephritic animals not treated with hemin. In the competitive reaction, 200 ng of total glomerular RNA and varying amounts of mimic RNA were added. These were (attomoles): 2.0 (lane 1); 0.4 (lane 2); 0.08 (lane 3); 0.016 (lane 4); 0.0032 (lane 5); 0.00064 (lane 6); and 0.000126 (lane 7). Lanes 8 to 14 in each Panel A show PCR products obtained using RNA of glomeruli from nephritic animals treated with hemin. In the competitive reaction, 200 ng of total glomerular RNA and varying amounts of mimic RNA were added. These were (attomoles): 2.0 (lane 8); 0.4 (lane 9); 0.08 (lane 10); 0.016 (lane 11); 0.0032 (lane 12); 0.00064 (lane 13); and 0.000126 (lane 14). In both figures, Panel B, left plot is a regression analysis of the effect of decreasing amounts of competitor mimic RNA used in the RT-PCR reactions (lanes 1 to 7, in the gel above the plot) on the log of the ratio of amplified products (iNOS target cDNA/competitor mimic cDNA). The log of the amount of competitor used is indicated in the abscissa (attomoles mimic). In both figures, Panel B, right plot is a regression analysis of the effect of decreasing amounts of competitor mimic RNA used in the RT-PCR reactions (lanes 8 to 14, in the gel above the plot) on the log of the ratio of amplified products (iNOS target cDNA/competitor mimic cDNA). The log of the amount of competitor used is indicated in the abscissa (attomoles mimic).
extent of glomerular inflammation thereby attenuating proteinuria. An anti-inflammatory effect as a result of upregulation of HO-1 can occur by a number of mechanisms: In addition to release of CO and iron, which can downregulate iNOS as reviewed above, catabolism of heme by HO-1 also generates biliverdin, which is converted to bilirubin by biliverdin reductase (Figure 10). Biliverdin and bilirubin can scavenge reactive oxygen species (ROS) and inhibit lipid peroxidation (32–34).

Moreover, biliverdin inhibits complement cascade at the level of C1 activation (35). Thus, HO-1 activation within nephritic glomeruli may provide these two ROS scavengers that could protect against supraphysiologic levels of ROS generated in the course of inflammatory injury (36–38). The HO-1 catalyzed production of CO from heme is a stimulator of guanylate cyclase (39). This could increase intraglomerular levels of cGMP thereby preserving GFR in a manner similar to nitric oxide. Finally, the HO-1 catalyzed release of free iron can induce the cytoprotective protein ferritin (40). This protein sequesters free cytosolic iron, the main catalyst of oxygen radical formation. In this regard, it has been demonstrated that HO-1 induced by mildly oxidized LDL in cocultures of human aortic endothelial cells and smooth muscle cells may protect these cells through production of the antioxidants biliverdin and bilirubin (41). Studies in HO-1 knockout mice have also
demonstrated a beneficial role of HO-1 against oxidative injury of embryonic fibroblasts by hydrogen peroxide, paraquat, or cadmium chloride (42).

In summary, our studies demonstrate that in glomerular immune injury, hemin treatment upregulates glomerular HO-1 with an attendant downregulation of iNOS expression. This raises the possibility for a regulatory interaction between the two systems. Whether the beneficial effect of hemin treatment on proteinuria is specifically linked to downregulation of iNOS requires further investigation.

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