GEC-targeted HO-1 expression reduces proteinuria in glomerular immune injury

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Submitted 17 April 2009; accepted in final form 2 July 2009

Duann P, Lianos EA. GEC-targeted HO-1 expression reduces proteinuria in glomerular immune injury. Am J Physiol Renal Physiol 297: F629–F638, 2009. First published July 8, 2009; doi:10.1152/ajprenal.00213.2009.—Induction of heme oxygenase (HO)-1 is a key defense mechanism against oxidative stress. Compared with tubules, glomeruli are refractory to HO-1 upregulation in response to injury. This can be a disadvantage as it may be associated with insufficient production of cytoprotective heme-degradation metabolites. We, therefore, explored whether 1) targeted HO-1 expression can be achieved in glomeruli without altering their physiological integrity and 2) this expression reduces proteinuria in immune injury induced by an anti-glomerular basement membrane (GBM) antibody (Ab). We employed a 4.125-kb fragment of a mouse nephrin promoter downstream to which a FLAG-tagged hHO-1 cDNA sequence was inserted and subsequently generated transgenic mice from the FVB/N parental strain. There was a 16-fold higher transgene expression in the kidney than nonspecific background (liver) while the transprotein immunolocalized in glomerular epithelial cells (GEC). There was no change in urinary protein excretion, indicating that GEC-targeted HO-1 expression had no effect on glomerular protein permeability. Urinary protein excretion in transgenic mice with anti-GBM Ab injury (days 3 and 6) was significantly lower compared with wild-type controls. There was no significant change in renal expression levels of profibrotic (TGF-β1) or anti-inflammatory (IL-10) cytokines in transgenic mice with anti-GBM Ab injury. These observations indicate that GEC-targeted HO-1 expression does not alter glomerular physiological integrity and reduces proteinuria in glomerular immune injury.

glomerular epithelial cells; anti-GBM disease; heme oxygenase 1; glomerulonephritis

IN VARIOUS FORMS OF GLOMERULAR immune injury, there is excessive production of reactive oxygen species (ROS) by intrinsic glomerular cells or infiltrating leukocytes (20, 21). ROS induce expression of heme oxygenase (HO), which is the rate-limiting enzyme in heme catabolism. It catalyzes the NADPH-, O2-, and cytochrome P-450 reductase-dependent oxidation of heme to carbon monoxide (CO), reactive iron (Fe2+), and biliverdin, which is reduced to bilirubin by biliverdin reductase (25). Three HO isoforms have been identified, HO-1, HO-2, and HO-3, of which HO-1 is the inducible heme oxygenase (25). Three HO isoforms have been identified, HO-1, HO-2, and HO-3, of which HO-1 is the inducible heme oxygenase (25).

induction can be achieved in glomeruli without altering their physiological integrity; and 2) this expression reduces proteinuria in immune injury induced by an anti-glomerular basement membrane (GBM) antibody (Ab). We employed a 4.125-kb fragment of a mouse nephrin promoter downstream to which a FLAG-tagged hHO-1 cDNA sequence was inserted and subsequently generated transgenic mice from the FVB/N parental strain. There was a 16-fold higher transgene expression in the kidney than nonspecific background (liver) while the transprotein immunolocalized in glomerular epithelial cells (GEC). There was no change in urinary protein excretion, indicating that GEC-targeted HO-1 expression had no effect on glomerular protein permeability. Urinary protein excretion in transgenic mice with anti-GBM Ab injury (days 3 and 6) was significantly lower compared with wild-type controls. There was no significant change in renal expression levels of profibrotic (TGF-β1) or anti-inflammatory (IL-10) cytokines in transgenic mice with anti-GBM Ab injury. These observations indicate that GEC-targeted HO-1 expression does not alter glomerular physiological integrity and reduces proteinuria in glomerular immune injury.

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MATERIALS AND METHODS

Generation of pNeph-FLAG-hHO1 Expression Vector

A 4.125-kb fragment of the mouse nephrin promoter (accession no. AF296764, source: Dr. S. Quaggin, University of Toronto) (10) was used to construct an expression vector (pNeph-FLAG-hHO1), which contained a tandem-arrayed nephrin promoter, a FLAG-tagged hHO-1 cDNA sequence, a β-globin intron, and a polyadenylation signal sequence (Fig. 1). The strategy of preparing this vector is shown in Fig. 2. We chose to add the FLAG tag sequence at the NH2 rather than the COOH terminal of the hHO-1 gene for two reasons: 1) both the hHO-1 catalytic center and the transmembrane domain localize near its COOH terminal (15); therefore, addition of the FLAG sequence to this terminal could interfere with catalytic activity; and 2) it was previously shown that tagging sequences larger than FLAG, for example a 19-amino acid (aa) transgene (6×Histidine + TAT), to the hHO-1 NH2 terminal has no effect on HO catalytic activity, measured as biliverdin generation from heme (27).

Briefly, a two-step PCR amplification of pTOB7-HO plasmid (Invitrogen), which contains the entire coding region of the hHO-1 gene, was performed. In the first PCR step, the forward primer (P1 in Fig. 2A) included a hanging NotI sequence (underlined) upstream of...
the ATG initiation site of the hHO-1 coding region, while the reverse primer (P2 in Fig. 2A) included a XbaI site (underlined) 158 bp downstream to the hHO-1 stop codon. The 1-kb PCR product was inserted between the NotI and XbaI cloning sites of the pFLAG-CMV2 vector (Sigma-Aldrich) to generate pFLAG-HO-1. In the second PCR step, a new forward primer (P3 in Fig. 2A) with a 5'-overhanging XhoI restriction sequence was annealed to a target sequence of 51 bp upstream to the FLAG translation start site and the Kozak Consensus Sequence. The reverse primer (P2 in Fig. 2A) remained identical to that used in the first PCR step. The product of the second PCR step was a 1.1-kb fragment containing a fusion sequence composed of the coding sequence for the NH2-terminal 10 aa of FLAG, the 3-aa linker, and the 288 aa of hHO-1. Subsequently, this sequence was inserted between the 4.125-kb nephrin promoter and a 782-bp fragment of a generic β-globin intron and the polyadenylation signal sequence (Fig. 2C). The final construct, pNeph-FLAG-HO (Fig. 2E), was sequence confirmed. A 7.02-kb fragment of the tandem-arrayed nephrin promoter, FLAG-tagged hHO-1 transgene, β-globin intron, and polyadenylation signal sequence were subsequently excised with MluI and EcoRV (Fig. 2E) from the cloning vector and used for microinjection into pronuclei of fertilized mouse oocytes to generate transgenic mice expressing hHO-1 in GEC (see below).

**Generation of mNephrin-hHO1 Transgenic Mice**

mNephrin-hHO1+/-, mNephrin-hHO1-/-, and wild-type mice were generated from a FVB/N parental strain obtained from Jackson Laboratories (Bar Harbor, ME) and handled according to protocols approved by the Institutional Animal Care and Use Committee. Briefly, a MluI-EcoRV fragment was excised from the pNeph-FLAG-HO1, shown in Fig. 2, and used for microinjection into oocytes obtained from superovulating FVB/N female mice. The FVB/N strain was chosen because of its superior reproductive performance and prominent pronuclei, which facilitate microinjection of DNA (32). Following microinjection of the DNA, oocytes were transferred to the oviduct of pseudopregnant females. Pups were born 19 days later. Genomic DNA was extracted from mouse tail and isolated by proteinase K digestion, phenol/chloroform (24:1) extraction, and precipitation with isopropyl alcohol. It was used for Southern blot-based or PCR-based genotyping.

To perform Southern blot-based genotyping, genomic DNA (~15 μg) was subjected to EcoR I digestion, electrophoresed through 0.9% agarose gels, and transferred to an Ny/nylon membrane (Amersham Biosciences). Blots were hybridized at 42°C for at least 16 h with...
DNA probes labeled with [32P]dCTP using a DNA labeling kit (Amersham Biosciences). A 1,428-bp EcoRI-EcoRI fragment, incorporated within the FLAG-hHO-1 sequence of the expression vector (Fig. 1) and spanning a 3′ segment of the nephrin promoter, the FLAG sequence, and the 5′-segment of hHO-1 (indicated by the bold bar in Fig. 1), was labeled with [32P] and used as a screening probe.

To perform PCR-based genotyping, PCR primers were designed to amplify three distinct loci (amplicons) spanning the FLAG-tagged hHO-1 junction (Fig. 1). These were amplicon 1: (forward: 5′-AGA AGA GCT CTT TTA GTG AAC CGT-3′; reverse: 5′-CTT GA AAT CTT GGT GGC ACT GGC AAT-3′; product size: 615 bp); amplicon 2: (forward: 5′-TCC TGA GCT GGA GAT GAC CCT GCT CAA AAA-3′; reverse: 5′-ATG AAC TCA GCA TTC TCT GCC TGG GTG TGC-3′; product size: 737 bp); and amplicon 3: (forward: 5′-CTG GGG CCT CTG ACA AAT CAA GCT T-3′; reverse: 5′-ATG AAC TCA GCA TTC TCT GCC TGG GTG TGC-3′; product size: 537 bp). The PCR amplification protocol was denaturing (94°C) for 45 s, annealing (65°C) for 1 min, and extension (72°C) for 40 s (40 thermo-cycles). Genomic DNA samples were considered as positive for the presence of the FLAG-hHO1 transgene if PCR reactions on all three amplicons yielded the anticipated products.

**Assessment of Organ Specificity of FLAG-hHO1 Expression**

**Organ distribution of transgene.** Total RNA was isolated from the brain, heart, lung, stomach, liver, muscle, pancreas, intestine, and kidney by using a TRIzol reagent kit (Invitrogen) and the manufacturer’s instructions. cDNA was synthesized from total RNA with reverse transcriptase (Roche, Indianapolis, IN) using oligo (dT) primers (Qiagen, Valencia, CA). Organ mRNA expression levels were quantified by real-time PCR using an ABI PRISM 7100 sequence detection system (SDS; Applied Biosystems, Foster City, CA) and the TaqMan PCR Master Mix (Qiagen) in a one-step reaction according to the manufacturer’s instructions. Primers were designed using the Primer Express V2.0 software program (Applied Biosystems). The following primers and probes were used:

- **Murine HO-1:** forward primer: 5′-GAA AGA CGA CCA GCA CAA GCT T3′-3′; reverse primer: 5′-CTGA CCT CGT ACA AAT CC-3′; double-dye oligonucleotide probe: FAM-CCG CGG TCG AGC GT-3′/BHQ1; product size: 70 bp.
- **FLAG-hHO1:** forward primer: 5′-CCAA AGA CGA TCA CGG AGC GT-3′; reverse primer: 5′-TCCA GCT CCTG ACA AAT CC-3′; double-dye oligonucleotide probe: FAM-CCG CGG TCG AGC GT-BHQ1; product size: 78 bp.
- **Murine glucagon (GLCOE4F, endogenous control gene):** forward primer: 5′-AAC ATT GCC AAA CGT CAT GAT G-3′; reverse primer: 5′-GCC TTC TCT GCC GGC CTT TCA-3′; double-dye oligonucleotide probe: FAM-ACA TGA TCA GGAC C-BHQ1; product size: 129 bp.

The following PCR protocol was used: denaturation (95°C for 10 min) followed by an amplification and quantification program (95°C for 15 s, 55°C for 30 s, 72°C for 35 s), repeated for 40 cycles.

**Detection of FLAG-hHO1 protein in GEC.** The accelerated variant of this model was induced as previously described (19). Briefly, kidneys from control and mNephrin-FLAG-hHO1 mice were minced into 1-mm2 pieces and fixed in 2.5% glutaraldehyde/4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h followed by post fixation in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. Subsequently, the tissues were dehydrated in a graded series of acetone and embedded in Embed 812 Resin (Electron Microscopy Science, Hatfield, PA). Tissue sections were cut at 90-nm thickness using a Leica UC6 Ultramicrotome (Leica Microsystems, Vienna, Austria) and stained with uranyl acetate and lead citrate. Images were captured at 80 K√ using a Phillips CM-12 electron microscope equipped with an AMT XR41 CCD camera.
was measured colorimetrically using a protein-assay kit (Bio-Rad DC-Protein Assay, Bio-Rad, San Diego, CA) according to the manufacturer’s instructions. Urine and plasma creatinine were measured by the Jaffé colorimetric method using a creatinine assay kit (BioQuant, San Diego, CA).

Assessment of inflammatory cell infiltration. In the accelerated anti-GBM glomerulonephritis model, there is macrophage infiltration in glomeruli (22). We assessed changes in glomerular macrophage infiltration by immunohistochemistry and Western blotting using an anti-mouse macrophage (F4/80) antibody. Immunohistochemical analysis was performed on renal cortical cryosections. Five micrometer-thick sections were fixed with methanol for 5 min followed by 4% paraformaldehyde for 5 min and then stained with FITC-conjugated rat monoclonal antibody to the mouse macrophage marker F4/80 (1:200; Abcam, Cambridge, MA). Nonspecific florescence was blocked by preincubating sections with nonimmune rat serum (1:200; Pierce, Rockford, IL) in TBS. For comparison purposes, kidney sections from control and transgenic mice littermates were fixed on the same slide to permit immunostaining under identical conditions, thus avoiding differences in staining intensity.

Western blot analysis was performed in protein lysates prepared from kidney cortices obtained from transgenic mice 4 days before injection of the anti-GBM antibody and on days 1, 2, and 3 thereafter. Levels of the murine macrophage marker F4/80 in each lysate were assessed using a polyclonal rat anti-F4/80 antibody (1:1,000; Abcam). Following protein transfer to polyvinylidene difluoride membranes, nonspecific binding of this antibody was blocked by incubating the membranes for 1 h with 1% nonimmune rat serum in TBS.

Changes in IL-10 and TGF-β1 expression. As glomerular inflammation and proteinuria in this model is cytokine regulated, expression of two cytokines known to regulate the inflammatory response to an anti-GBM antibody-mediated injury was assessed. These were IL-10, whose anti-inflammatory effects were shown to be mediated by HO-1 (16), and TGF-β1, a profibrotic cytokine (2).

Total kidney RNA was isolated using an RNaseasy kit (Qiagen, Valencia, CA), and cDNA was reverse transcribed and synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to manufacturers’ instructions. Real-time PCR was performed using the SYBR Green Master Mix Kit (Bio-Rad) and the ABI 7000 thermal cycler (Applied Biosystems). Product size was validated by routine agarose gel electrophoresis. The GAPDH gene was used as an internal standard. The primers used in this study were TGF-β1: forward 5'–CAA CAA TTC TTG CGC GEC GTT ACC TTG G and reverse 5'–GAA AGC CCT GTA TTG CTT CTC CT; IL-10: forward 5'–GGC CCT TTG CCA TGG TGT CC and reverse 5'–AAG CCG CTC GGG GAT GAC; and GAPDH: forward 5'–GCC AAA AGG GTC ATC ATC TC and reverse 5'–GGC CAT CCA CAG TCT TCT.

Statistical Analysis

All data are expressed as means ± SE. Differences across multiple conditions were tested by one-way ANOVA for repeated measures. Comparisons between conditions were tested by Student’s unpaired t-test using the Bonferroni correction for multiple comparisons. P values <0.05 were taken to indicate statistically significant differences.

RESULTS

NH2-Terminal Tagging of hHO-1 Has No Effect on Its Antigenicity

The 4.125-kb murine nephrin promoter was previously used to achieve GEC-specific expression of Cre-recombinase (10). We used this promoter to achieve GEC-targeted expression of hHO-1. The human HO-1 gene (Hmox1, Homo sapiens, Gene ID 3162) is a very conserved gene sharing a 89.62% amino acid sequence homology to mouse or rat HO-1 (Hmox1, Mus musculus, gene ID: 15368; Hmox 1, Rattus norvegicus, gene ID 24451). hHO-1 (288 aa), mouse HO-1 (289 aa), and rat HO-1 (289 aa) share an identical 259-aa sequence and a conserved 29-aa sequence. Therefore, distinguishing endogenous mouse HO-1 from hHO-1 in transgenic mice expressing hHO-1 in GEC could be problematic. To circumvent this problem, we employed an epitope (FLAG)-tagging approach to generate a FLAG-hHO-1 chimera. We constructed a pNeph-FLAG-hHO1 plasmid (Fig. 1) consisting of a tandem-arrayed nephrin promoter, a FLAG-tagged hHO-1 cDNA sequence, a β-globin intron, and a polyadenylation signal sequence. We then examined whether the FLAG-hHO-1 sequence can encode a chimeric protein which retains the immunogenicity of both FLAG and HO-1. Achieving this is important as the highly specific anti-FLAG antibodies available would allow distinguishing between FLAG-hHO-1 transprotein and endogenous HO-1 protein in transgenic mice.

To examine whether FLAG tagging alters antigenicity of the hHO-1 protein, HeLa cells, chosen in these experiments due to their high transfection efficiency, were mock transfected or transfected with either 1 μg pCMV-FLAG-hHO1 construct or with pCMV-FLAG-bacterial alkaline phosphatase (BAP) used as a positive control. Protein lysates were separated on 4–12% NuPAGE gels (Invitrogen), and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was first probed with an anti-FLAG monoclonal antibody (1:1,000, Sigma-Aldrich) (Fig. 3, top). The membrane was then stripped and reprobed with an anti-HO-1 polyclonal antibody (1:500, AJP-Renal Physiol • VOL 297 • SEPTEMBER 2009 • www.ajprenal.org • Downloaded from http://ajprenal.physiology.org/ by 10.220.33.4 on June 15, 2017
Zygosity was determined using real-time quantitative PCR (Q-PCR) and Southern blot analysis and PCR (designated as Fig. 4 summarizes gender, weight, and genotype assessed by as 3 separate litter groups based on date of birth. The table in ( generates 23 F3 pups shown in the table in Fig. 4 founder 6 from one of the founders. F2 hemizygotes from this founder (founder 6) generated 23 F3 pups shown in the table in Fig. 4 as 3 separate litter groups based on date of birth. The table in Fig. 4 summarizes gender, weight, and genotype assessed by Southern blot analysis and PCR (designated as + or − to indicate the presence or absence of the FLAG-hHO1 transgene). Zygosity in these mice was determined using real-time Q-PCR and the comparative $2^{-\Delta\Delta C_{\text{t}}}$ method (17) (see MATERIALS AND METHODS for details).

Organ Distribution of Transgene and Validation of GEC-Targeted hHO-1 Expression

To confirm kidney-specific expression of FLAG-hHO1, we screened RNAs extracted from the brain, heart, intestine, kidney, liver, lung, pancreas, muscle, and stomach by Q-PCR. Levels of FLAG-hHO1 and GAPDH cDNA were quantified by measuring increases in fluorescence occurring due to the cleavage of the reporter dye as the PCR reaction proceeded relative to the starting values of normalized reporter fluorescence. Normalized reporter fluorescence (Rn) values (Rn is the cycle-by-cycle ratio of fluorescence of the reporter dye and the fluorescence of the internal passive reference dye in any given well) was determined and plotted against cycle number. In Fig. 5A, an amplification plot for FLAG-hHO1 in total RNA obtained from the kidney and brain is shown. It was derived by plotting the Rn values against the PCR cycle number. Figure 5B demonstrates the abundance of FLAG-hHO1 mRNA level in kidney tissue relative to that in the brain, heart, intestine, liver, lung, pancreas, muscle, and stomach based on Ct values derived from plots similar to that shown in Fig. 5A {Ct is the cycle number (C) at which detected fluorescence [originating from the double-dye oligonucleotide probe TaqMan crosses a threshold (t)]. There was a 15.6-fold higher transcript expression in kidney extract compared with that of the brain. FLAG-hHO1 transprotein level (Western blot) and immunolocalization in glomeruli using an anti-FLAG antibody are shown in Fig. 6, A and B. The transprotein was detectable in lysates of whole kidney but not brain obtained from transgenic mice (Fig. 6A, top, lanes 2 and 3). Furthermore, it was undetectable in kidneys obtained from wild-type mice (Fig. 6A, top, lane 1). The FLAG-hHO1 transprotein immunolocalized in glomerular epithelial cells (Fig. 6B).

To further validate GEC specific expression of the FLAG-hHO1 protein in transgenic mice, we examined whether this protein colocalized with the GEC marker WT-1 using dual-color immunohistochemistry. Figure 7A shows localization of FLAG, localization of WT-1 is in Fig. 7B, and merging of the two images is in Fig 7C.

Effect of GEC-Targeted hHO-1 Expression on Glomerular Morphology

GEC morphology in transgenic mice was assessed by electron microscopy with particular attention paid to the structure of foot processes. These were well preserved without apparent changes such as flattening or effacement (Fig. 8A). Also, there was no microvillus transformation of GEC. The presence of “outpockets” or “humps” (arrows in Fig. 8B) was apparent on the podocyte side of the GBM.

GEC-Targeted hHO-1 Expression Reduces Proteinuria in Glomerular Immune Injury

Urine protein excretion (urine protein/urine creatinine; Up/Uc; mg protein/mg creatinine) values in individual wild-type and

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* Determined by real-time PCR and the comparative Ct ($2^{-\Delta\Delta C_{\text{t}}}$) method

Wild type: □ male, ○ female; Hemizygote: □ male, ○ female; Homozygote: ■ male, ● female

Fig. 4. Summary of characteristics of mice that originated from one of the founders (founder 6). In the table, F2 hemizygotes from founder 6 generated 23 F3 pups, shown as 3 separate litter groups based on their dates of birth (DOB). The table summarizes gender, weight, genotype assessed by Southern blot analysis and PCR (+ or − indicates the presence or absence of the FLAG-hHO1 transgene), and urine albumin (Ualb) excretion factored by that of urine creatinine (Uc). Zygosity was determined using real-time quantitative PCR (Q-PCR) and $2^{-\Delta\Delta C_{\text{t}}}$ method (described in MATERIALS AND METHODS).
transgenic mice at 10 wk of age are shown in Fig. 9. There was no statistically significant difference in Up/Uc values in transgenic compared with wild-type mice. Values (means ± SD) were 1.86 ± 0.93 (transgenic) and 1.95 ± 1.06 (wild-type). In Fig. 10, urine protein Up/Uc values in individual transgenic (originated from founder 6) and wild-type mice as well as the mean ± SD are shown on days 3 and 6 following administration of anti-GBM antibody. At both time points, Up/Uc in transgenic mice was significantly lower than in wild-type controls. Values (means ± SD) in wild-type mice were 5.48 ± 2.56 on day 3 and 8.34 ± 4.76 on day 6 Values in transgenic mice were 2.62 ± 1.41 on day 3 and 3.43 ± 1.94 on day 6; P values were 0.017 and 0.019 for days 3 and 6, respectively. There was no difference in serum creatinine levels in transgenic mice (0.164 ± 0.008 mg/dl) compared with the levels in the wild-type control mice (0.183 ± 0.008 mg/dl) following onset of anti-GBM injury. Attenuation of proteinuria was also observed in mice with anti-GBM disease originating from a different founder (founder 16) and studied on days 9 and 12 following administration of anti-GBM antibody. [Up/Uc values (means ± SD) in transgenic mice on days 9 and 12 were 10.42 ± 3.77 and 12.44 ± 3.49, respectively. Up/Uc values in wild-type mice on days 9 and 12 were 12.44 ± 2.74 and 27.13 ± 9.41, respectively. P values were 0.035 and 0.00024 for days 9 and 12, respectively]. The reproducibility of this observation (attenuation of proteinuria) in mice originating from two different founders (founders 6 and 16) argues against the possibility that the antiproteinuric effect observed was due to random gene targeting following insertion of the FLAG-hHO1 transgene to achieve GEC targeted HO-1 expression.

Effect of GEC-Targeted hHO-1 Expression on IL-10, TGF-β1, and Macrophage Infiltration

Levels of both IL-10 and TGF-β1 mRNA (assessed by real-time PCR) were increased following anti-GBM antibody-mediated injury. However, differences in these levels did not reach statistical significance in transgenic mice compared with wild-type controls (Fig. 11, top and bottom). Assessment of glomerular infiltration by macrophages (changes in levels of the mouse macrophage marker F4/80 measured by immunohistochemistry and Western blotting) revealed no apparent difference in transgenic vs. wild-type mice with anti-GBM injury (data not shown).
DISCUSSION

Studies on compartmentalization of HO-1 induction in the nephron in response to potent HO-1 inducers or in various forms of glomerular injury indicate that robust induction of this enzyme occurs at sites that are located primarily “downstream” of the glomerular tuft rather than within resident glomerular cells. Thus systemic administration of HO-1 inducers, including hemin, stannous chloride, and cobalt chloride, causes a robust HO-1 induction in tubular epithelial cells but not in resident glomerular cells (30). Similarly, in glomerular immune injury developing following administration of anti-GBM antibody or in nonimmune injury developing following administration of the GEC toxin, aminonucleoside of puromycinc (PAN), induction of HO-1 immunolocalized primarily in infiltrating leukocytes (5), in parietal glomerular epithelial cells, and in proximal tubules (8). In marked contrast, HO-1 induction in resident glomerular cells was weak or absent (5, 8) despite the robust prooxidant environment generated within glomeruli in both of these models of injury (28). These observations prompted us to ask two questions: 1) can HO-1 induction in glomerular cells be tolerated so far as their functional integrity is concerned? and 2) can targeting of HO-1 expression to glomerular cells attenuate the severity of injury?

To address the first question, we employed the 4.125-kb mouse nephrin promoter to achieve constitutive hHO-1 expres-

Fig. 7. Colocalization of transprotein FLAG-hHO1 with the podocyte marker WT1. A renal cortical section of a mNephrin-hHO1 transgenic mouse was double immuno labeled with FITC-conjugated sheep anti-FLAG antibody (A) and Cy3-conjugated anti-WT1 antibody (B). Superimposed FITC and Cy3 signals are shown in C.

Fig. 8. Electron micrograph of glomeruli from a mNephrin-hHO1 transgenic mouse. Glomerular epithelial cell foot processes are clearly visible with no apparent effacement or microvillus transformation (A). Occasional “pockets” or “humps” (white arrow with asterisk) were present on the podocyte side of the glomerular basement membrane (GBM; B).
tion in GEC. FLAG tagging the hHO-1 sequence provided a useful marker of transprotein detection in GEC (Fig. 3), as distinguishing mHO-1 from hHO-1 in transgenic mice using available antibodies could be problematic given their 89.6% amino acid sequence homology. In cultured cells, constitutive HO-1 overexpression can have beneficial or detrimental effects depending on levels of ferrous (Fe$^{2+}$) iron released from the HO-1 substrate (free cellular heme) (31). In the presence of reactive oxygen species, Fe$^{2+}$ can promote Fenton-type prooxidant reactions and cell injury. In normal glomeruli, there is constitutive production of superoxide and superoxide-derived highly reactive species, i.e., H$_2$O$_2$ (28), which, if unopposed, can increase permeability of the glomerular capillary barrier to protein (29). It is, therefore, possible that targeting HO-1 to GEC may result in Fe$^{2+}$ production sufficient to promote prooxidant reactions that could perturb integrity of this barrier. Our results argue against this possibility as urine protein excretion in transgenic mice was not different than that in wild-type controls (Fig. 9). These observations indicate that HO-1 targeting to GEC did not perturb functional integrity of these cells. Expression of hHO-1, a protein that is not natively expressed in GEC, did not cause morphological changes such as microvillus formation or effacement of foot processes (Fig. 8A). The apparent formation of “outpockets” or “humps,” as shown in Fig. 8B, has previously been described in knockout mice in which GEC lack the Ext1 gene, thereby being unable to polymerize heparin sulfate glycosaminoglycans (4). It was proposed that this phenomenon is indicative of regions of the GBM where newly synthesized matrix is being spliced into existing GBM (1).

We next examined whether GEC-targeted HO-1 expression attenuates proteinuria in a model of glomerular immune injury known as the accelerated variant of experimental anti-GBM glomerulonephritis. In this model, GEC injury is a prominent feature and its extent plays a major role in preserving integrity of the glomerular capillary permeability barrier to protein (24). We chose to examine the effect of GEC-targeted HO-1 expression on proteinuria at early stages of anti-GBM antibody-mediated injury (days 3 and 6) because, at these stages, a robust prooxidant environment develops within glomeruli due to the accumulation of superoxide and other reactive oxygen species.

![Fig. 9. Up/Uc values (mg protein/mg creatinine) in individual wild-type (Wt, •) and transgenic (Tg; ◦) mice. Mean values are also shown.](image9)

![Fig. 10. Proteinuria in mNephrin-hHO1 transgenic mice and their wild-type littersates in accelerated anti-GBM antibody-mediated injury. Shown are individual Up/Uc values (mg protein/mg creatinine) in Wt (•) and Tg (◦) mice 4 days before (-4) and on days 3 and 6 after administration of anti-GBM antibody. *P = 0.017 Tg vs. Wt on day 3. **P = 0.019 Tg vs. Wt on day 6.](image10)

![Fig. 11. IL-10 and transforming growth factor (TGF)-β1 mRNA levels in Tg or control mice with anti-GBM nephritis assessed by RT-PCR. Total RNA was extracted from kidneys of Tg (grey bars) or Wt (white bars) mice 4 days before and on days 1, 2, and 3 after injection of anti-GBM antibody. Values are means ± SE of 5 mice. *P = 0.336, **P = 0.367 compared with Wt control mice on day 3.](image11)
to production of reactive oxygen and nitrogen species, including superoxide (9) and peroxynitrite (13), which are potent HO-1 inducers. As shown in Fig. 10, proteinuria was significantly attenuated in transgenic mice with anti-GBM antibody-induced injury compared with wild-type controls with the same form of injury.

Previous studies attempted to increase glomerular HO-1 expression using systemic administration of the HO-1 inducer hemin before administration of an anti-GBM antibody. In these studies, proteinuria at early stages of immune injury was also reduced (7). However, induction of HO-1 in hemin-treated animals occurred mainly in leukocytes (macrophages) infiltrating nephritic glomeruli and in tubular epithelial cells (5). Induction of HO-1 in the former could reduce their ability to cause glomerular injury (23) while induction in the tubular epithelial cells could alter their capacity to reabsorb filtered protein. Both effects could account for the reduction of proteinuria. Thus the question of whether HO-1 expression in the latter stages of anti-GBM antibody-induced injury via a mechanism apparently independent of TGF-β or IL-10.

GRANTS

This work was supported by a Paul Teschan Research Fund Grant and an American Heart Association Scientist Development Grant to P. Duann.

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