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Induction of heme oxygenase-1 protects against podocyte apoptosis under diabetic conditions

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Heme oxygenase-1 (HO-1) is an anti-oxidant enzyme normally upregulated in response to oxidant injury. Here we determined the role of HO-1 in podocyte apoptosis in glomeruli of streptozotocin-treated rats and in immortalized mouse podocytes cultured in media containing normal or high glucose. HO-1 expression, its activity, the ratio of Bax/Bcl-2 protein, and active caspase-3 fragments were all significantly higher in isolated glomeruli of diabetic rats and in high glucose–treated podocytes. These increases were inhibited by zinc protoporphyrin treatment of the rats or by HO-1 siRNA treatment of the podocytes in culture. The number of apoptotic cells was also significantly increased in the glomeruli of diabetic rats and in high glucose–treated podocytes. Inhibition of HO-1 accentuated the increase in apoptotic cells both *in vivo* and *in vitro*. Our findings suggest that HO-1 expression protects against podocyte apoptosis under diabetic conditions.

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KEYWORDS: apoptosis; diabetic nephropathy; heme oxygenase-1; high glucose; podocyte

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Heme oxygenase (HO) is a microsomal rate-limiting enzyme involved in the degradation of heme to biliverdin, which is rapidly converted into bilirubin by biliverdin reductase.[1,2] During this step, iron is released from the heme ring and carbon monoxide is generated.[1] To date, three distinct isoforms of HO have been identified: HO-1, an inducible form; HO-2, a constitutive form; and HO-3, probably a pseudogene.[3–5] Among these isoforms, HO-1 has been the most extensively studied HO isoenzyme and is known to be upregulated in the kidney under various conditions characterized by oxidative stress, including toxic nephropathy,[6,7] ischemia–reperfusion injury,[8,9] contrast nephropathy,[10] and acute transplant rejection.[11] Oxidants generating molecules such as angiotensin II,[12,13] heme,[14] proinflammatory cytokine,[15] and transforming growth factor-β1[16] are also reported to induce HO-1. In addition, pharmacological inhibition of HO activity or deletion of the HO-1 gene worsened renal injury induced by toxic substances and ischemia–reperfusion,[7,17–19] whereas earlier induction of HO-1 protected against renal injury.[18,20–23] On the basis of these findings, HO-1 is thought to function as an antioxidant, which is upregulated to alleviate the deleterious consequences of oxidant injury.

The number of podocytes is decreased in the glomeruli of diabetic patients and animal models of diabetes (DM),[24–26] and apoptosis is known to be involved in podocyte loss under DM conditions.[26,27] Previous studies have demonstrated that high glucose (HG) *per se* and transforming growth factor-β1, two major mediators in the development and progression of DM nephropathy, increase reactive oxygen species production and induce apoptosis in cultured podocytes.[26,28,29] Moreover, podocyte apoptosis under these conditions is prevented by the administration of antioxidants, suggesting that oxidative stress with increased reactive oxygen species is responsible for apoptosis of podocytes under DM conditions.[26,30]

On the basis of these findings, an increase in HO-1 expression has been expected and been demonstrated in experimental DM glomeruli, mainly in mesangial cells and

podocytes,[31,32] but the functional significance of this increase has not yet been explored. In this study, we investigated the expression of HO-1 as well as podocyte apoptosis in experimental DM glomeruli and in HG-stimulated cells before and after inhibiting HO activity to elucidate the role of HO-1 in podocyte apoptosis under DM conditions.

RESULTS

Animal studies

Animal data. Body weight and the ratio of kidney weight to body weight were significantly different between DM (275 ± 8 g, $1.12 \pm 0.11\%$) and control (C) rats (398 ± 8 g, $0.69 \pm 0.04\%$) ($P < 0.05$). However, the administration of zinc protoporphyrin (ZnPP) had no effect on either body weight or the ratio of kidney weight to body weight in C and DM rats. The mean blood glucose levels of C, C + ZnPP, DM, and DM + ZnPP were 97.2 ± 7.7, 94.1 ± 5.4, 497.2 ± 14.8, and 488.7 ± 17.2 mg per 100 ml, respectively ($P < 0.01$). Compared with the C group (0.32 ± 0.04 mg/day), 24-h urinary albumin excretion was significantly higher in the DM group (1.18 ± 0.11 mg/day, $P < 0.05$), and was further increased in DM rats by ZnPP treatment (1.59 ± 0.19 mg/day, $P < 0.05$) (Table 1). The administration of ZnPP also significantly aggravated the increase in 24-h urine albumin excretion in female DM rats (1.37 ± 0.15 vs 0.85 ± 0.11 mg/day, $P < 0.05$), suggesting that the effect of ZnPP was not gender-specific.

In contrast, the blood urea nitrogen and serum creatinine levels and systolic blood pressure were comparable among the four groups (Table 1).

Localization of glomerular HO-1 protein. Double immunofluorescence staining for HO-1 and synaptopodin revealed that podocytes were the main cells responsible for the increase in HO-1 protein under DM conditions (Figure 1).

Glomerular HO-1 and HIF-1 protein expression. Glomerular HO-1 protein expression assessed by western blot was significantly higher in DM compared with C rats ($P < 0.05$), whereas ZnPP treatment significantly inhibited the increase in HO-1 protein expression in DM rats ($P < 0.05$) (Figure 2). In contrast, ZnPP had little effect on glomerular HO-1 protein expression in C rats. To explore the possible mechanism how ZnPP inhibited HO-1 protein expression, we investigated the effect of ZnPP treatment on the expression of hypoxia-inducible factor-1 (HIF-1). As HO-1 is a downstream factor of HIF-1, we assumed that ZnPP

Table 1 | Animal data

	Control (n=8)	Control+ZnPP (n=8)	DM (N=8)	DM+ZnPP (n=8)
Body weight (g)	398 ± 8	405 ± 7	275 ± 8*	266 ± 2*
Kidney weight/body weight (%)	0.69 ± 0.04	0.75 ± 0.05	1.12 ± 0.11*	1.20 ± 0.09*
Blood glucose (mg per 100 ml)	97.2 ± 7.7	94.1 ± 5.4	497.2 ± 14.8$^{\#}$	488.7 ± 17.2$^{\#}$
24-h UAE (mg/day)	0.32 ± 0.04	0.29 ± 0.08	1.18 ± 0.11*	1.59 ± 0.19$^{\#,\dagger}$
BUN (mg per 100 ml)	19.8 ± 1.3	20.3 ± 2.1	23.9 ± 2.9	24.2 ± 3.3
Serum creatinine (mg per 100 ml)	0.83 ± 0.05	0.80 ± 0.07	0.85 ± 0.10	0.87 ± 0.13
Systolic blood pressure (mm Hg)	111.1 ± 9.8	112.5 ± 8.5	115.3 ± 11.8	116.7 ± 10.5

BUN, blood urea nitrogen; DM, diabetes; UAE, urinary albumin excretion; ZnPP, zinc protoporphyrin.
*$P < 0.05$ vs control group, $^{\#}P < 0.01$ vs control group, $^{\dagger}P < 0.05$ vs DM group.

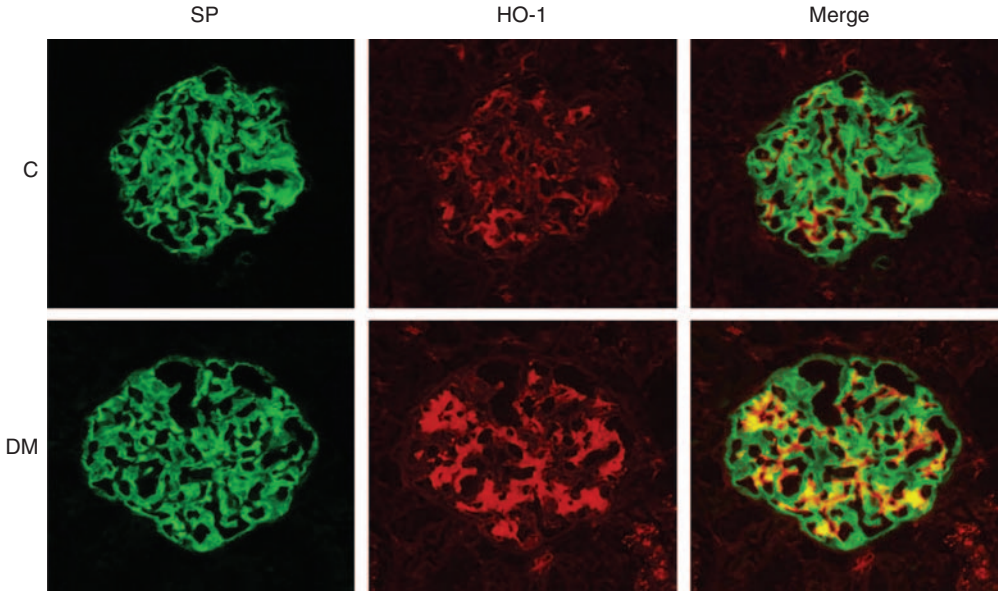

Figure 1 | Double immunofluorescence staining for heme oxygenase (HO)-1 (red) and synaptopodin (green) in control (C) and diabetic (DM) rats. Compared with C, immunofluorescence staining for HO-1 was increased in DM glomeruli, and double immunofluorescence staining revealed that the increase in HO-1 protein expression was mainly attributed to its increase in podocytes. (×400).

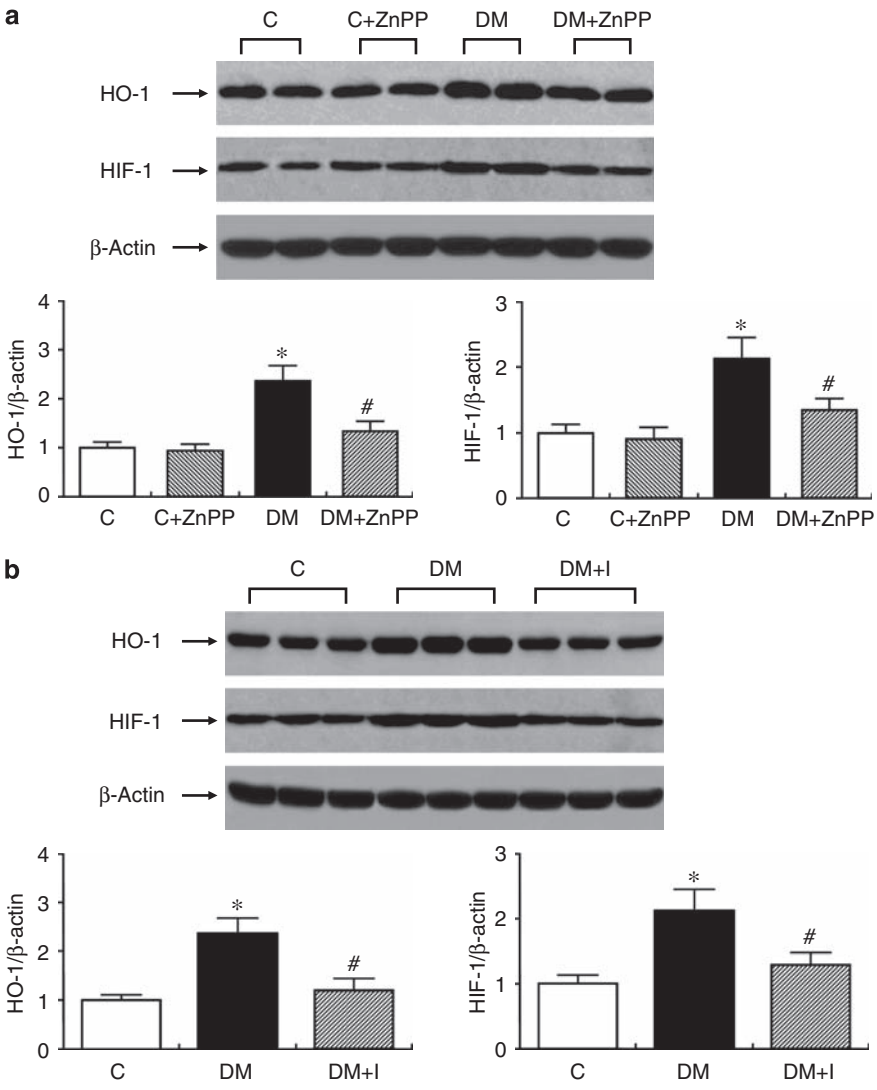

Figure 2 | Western blot analysis of glomerular heme oxygenase (HO)-1 and hypoxia-inducible factor-1 (HIF-1) protein expression. (**a**) A representative western blot of HO-1 and HIF-1 in control (C), C + ZnPP, diabetic (DM), and DM + ZnPP rats (representative of four blots). HO-1 and HIF-1 protein expression was 2.4- and 2.1-fold higher in DM compared with C glomeruli, respectively, and these increases were significantly ameliorated in DM rats by ZnPP treatment. (**b**) A representative western blot of glomerular HO-1 and HIF-1 in C, DM, and DM + insulin (I) rats (representative of four blots). The increases in HO-1 and HIF-1 expression in 6-week DM glomeruli were significantly attenuated by insulin treatment, suggesting that these changes in STZ-induced DM rats were not due to STZ *per se*. $^{*}P<0.05$ vs C and C + ZnPP groups, $^{\#}P<0.05$ vs DM group. STZ, streptozotocin; ZnPP, zinc protoporphyrin.

may inhibit HIF-1 expression, leading to the decrease in HO-1 expression. Western blot for HIF-1 revealed that the increase in glomerular HIF-1 protein expression in DM rats was significantly abrogated by ZnPP treatment ($P<0.05$) (Figure 2a).

In the experiments using DM rats treated with insulin, the increases in HO-1 and HIF-1 expression in 6-week DM glomeruli were significantly ameliorated by insulin treatment ($P<0.05$), suggesting that these changes in STZ-induced DM rats were not because of STZ *per se* (Figure 2b).

Immunohistochemical staining for glomerular HO-1 confirmed the western blot findings, as glomerular HO-1 staining was significantly stronger in DM than in C rats, and ZnPP treatment attenuated the increase in HO-1 staining in DM glomeruli (Figure 3). The mean semiquantitative staining score for glomerular HO-1 was significantly higher in DM (75.7 ± 9.0) compared with C rats (22.4 ± 2.4) ($P<0.01$), and this increase was inhibited in DM glomeruli by the administration of ZnPP (37.8 ± 5.4) ($P<0.05$).

Glomerular HO activity. In addition to HO-1 protein expression, HO activity was also significantly increased in DM compared with C glomeruli ($P<0.01$), and ZnPP treatment significantly abrogated the increase in glomerular HO activity in DM rats ($P<0.05$) (Figure 4).

Bax, Bcl-2, and active fragments of caspase-3 protein expression. Bax and active fragments of caspase-3 protein expression were significantly increased, whereas Bcl-2 protein expression was significantly decreased in DM compared with C glomeruli ($P<0.05$). The administration of ZnPP significantly aggravated the increases in the ratios of

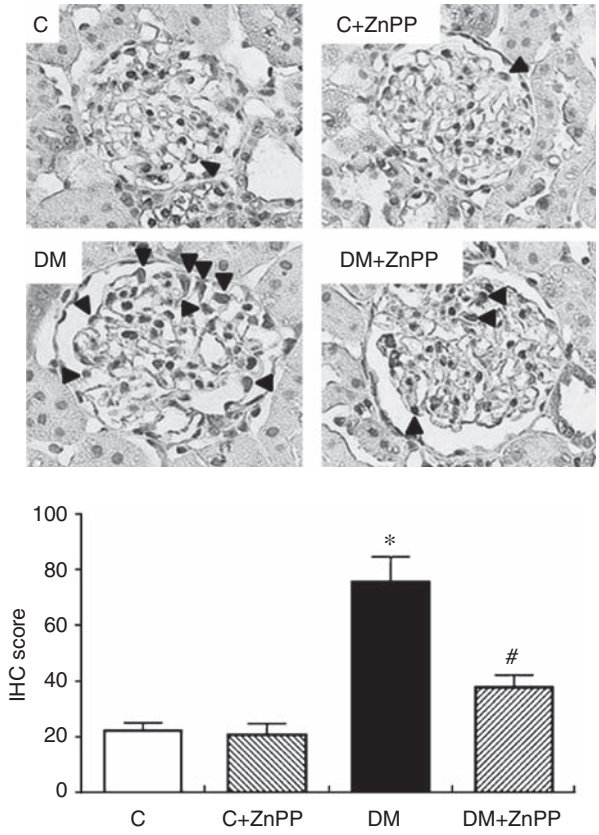

Figure 3 | Immunohistochemical staining for heme oxygenase (HO)-1 in control (C), C + ZnPP, diabetic (DM), and DM + ZnPP rats. Glomerular HO-1 staining (arrowheads) was significantly stronger in DM compared with C and C + ZnPP rats, and this increase was inhibited in DM glomeruli by the administration of ZnPP. (×400). *$P<0.01$ vs C and C + ZnPP groups, #$P<0.05$ vs DM group. ZnPP, zinc protoporphyrin.

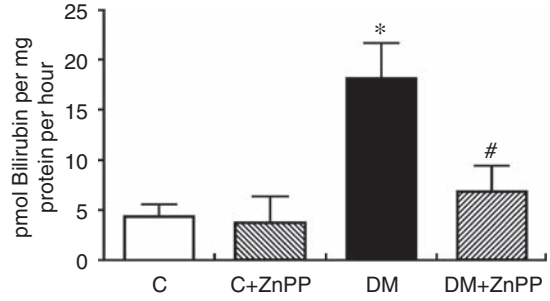

Figure 4 | Glomerular heme oxygenase (HO) activity (pmol bilirubin/mg protein per h) in control (C), C + ZnPP, diabetic (DM), and DM + ZnPP rats. HO activity was significantly increased in DM compared with C glomeruli, and ZnPP treatment significantly abrogated the increase in glomerular HO activity in DM rats. *$P<0.01$ vs C and C + ZnPP groups, #$P<0.05$ vs DM group. ZnPP, zinc protoporphyrin.

Bax/Bcl-2 ($P<0.01$) and active fragments of caspase-3 protein expression in DM glomeruli ($P<0.05$) (Figure 5).

TUNEL assay and double immunofluorescence staining. In addition to Bax, Bcl-2, and active fragments of caspase-3 protein expression, apoptosis in glomeruli was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay. The number of glomerular apoptotic cells was significantly increased in DM compared with C and C + ZnPP rats ($P<0.01$), and was further increased in DM rats by ZnPP treatment ($P<0.05$) (Figure 6a). To identify podocyte apoptosis in glomeruli, double immunofluorescence staining with antibodies to synaptopodin, a podocyte marker, and active fragments of caspase-3 was performed. As seen in Figure 6b and c, C + ZnPP rats showed a faint staining of active fragments of caspase-3 with little colocalization with synaptopodin, while the colocalization signal was increased in DM glomeruli and was highest in the glomeruli of DM + ZnPP rats.

Podocyte numbers. Compared with C (170.0 ± 4.2) and C + ZnPP rats (168.3 ± 5.9), the number of podocyte tended to be decreased in DM rats (159.1 ± 3.7), and the reduction in podocyte numbers was aggravated in DM rats treated with ZnPP (142.9 ± 3.2) ($P<0.05$).

Cultured podocytes studies

HO-1 and HIF-1 protein expression. Western blot analysis revealed that HO-1 small interfering RNA (siRNA) inhibited HO-1 protein expression in a dose-dependent manner (Figure 7). In contrast, HG significantly increased HO-1 and HIF-1 protein expression in cultured podocytes by 131 and 103%, respectively, compared with normal glucose (NG) cells ($P<0.05$), and the increase in HO-1 expression in HG-stimulated podocytes was significantly ameliorated by HO-1 siRNA treatment (Figure 8a).

Podocyte HO activity. In addition to HO-1 protein expression, HO activity was also significantly increased in HG-stimulated podocytes compared with podocytes exposed to NG medium (4.4 ± 0.6 vs 21.8 ± 3.1 pmol bilirubin per mg protein per h, $P<0.01$), and HO-1 siRNA treatment significantly attenuated the increase in HO activity in HG cells (3.1 ± 0.5 pmol of bilirubin per mg protein per h) ($P<0.005$).

Bax, Bcl-2, and active fragments of caspase-3 expression. The ratios of Bax/Bcl-2 and active fragments of caspase-3 protein expression were significantly increased in HG-stimulated podocytes compared with podocytes exposed to NG and NG + M media ($P<0.05$), and these increases were accentuated in HG cells by HO-1 siRNA ($P<0.05$). In contrast, there were no changes in Bax/Bcl-2 protein ratios and active fragments of caspase-3 protein expression in HO-1 siRNA-treated NG cells (Figure 8b).

HO-1, Bax, Bcl-2, and active fragments of caspase-3 expression in hemin-pretreated podocytes. Hemin pretreatment significantly induced HO-1 protein expression in cultured podocytes exposed to NG and HG media ($P<0.005$). In addition, HO-1 induction by hemin before HG stimulation significantly attenuated the increases in Bax/Bcl-2 ratios and active fragment of caspase-3 protein expression in HG-stimulated podocytes ($P<0.05$) (Figure 9).

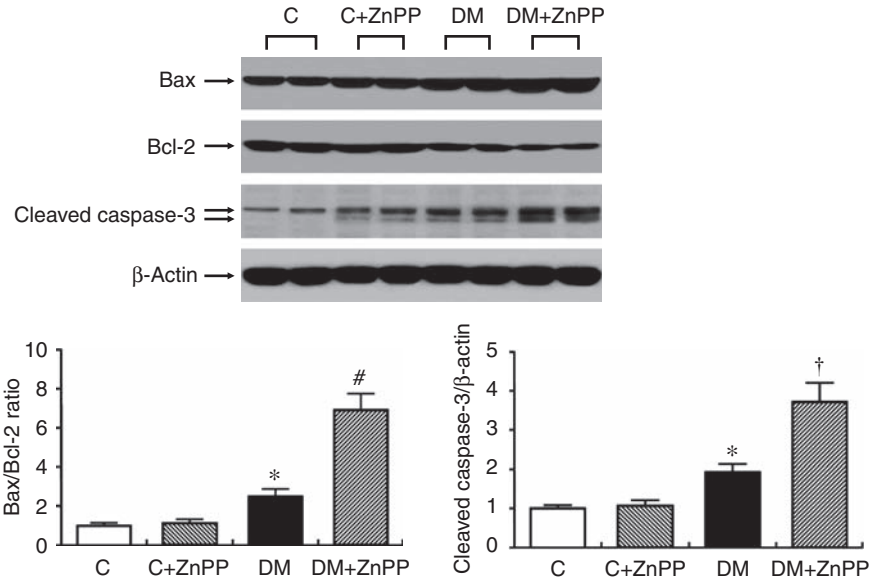

Figure 5 | A representative western blot of Bax, Bcl-2, and active fragments of caspase-3 protein expression in control (C), C + ZnPP, diabetic (DM), and DM + ZnPP glomeruli (representative of four blots). The ratios of Bax/Bcl-2 and active fragments of caspase-3 protein expression were significantly increased in DM compared with C glomeruli, and the administration of ZnPP significantly aggravated these increases in DM glomeruli. *$P<0.05$ vs C and C + ZnPP groups, $^{\#}P<0.01$ vs DM group, $^{\dagger}P<0.05$ vs DM group. ZnPP, zinc protoporphyrin.

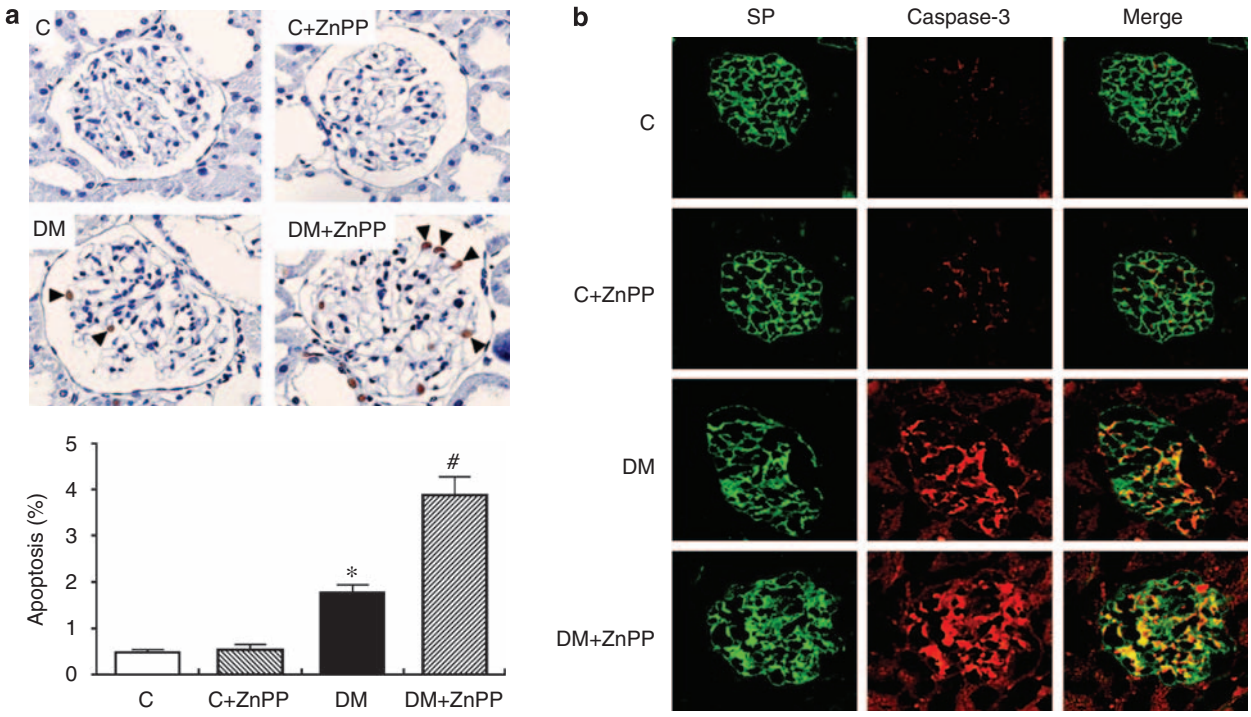

Figure 6 | Assessment of apoptosis in glomeruli. (**a**) Apoptosis assessed by TUNEL assay in control (C), C + ZnPP, diabetic (DM), and DM + ZnPP rats. There was a significant increase in apoptotic cells (arrow heads) in DM compared with C and C + ZnPP glomeruli, and this increase in apoptotic cells was accentuated with ZnPP treatment. (**b**) Double immunofluorescence staining for active fragments of caspase-3 (red) and synaptopodin (SP) (green). Compared with C and C + ZnPP rats, the colocalization signal (yellow) was increased in DM glomeruli, and was highest in the glomeruli of DM + ZnPP rats. (×400). *$P<0.01$ vs C and C + ZnPP groups, $^{\#}P<0.05$ vs DM group. ZnPP, zinc protoporphyrin.

Hoechst 33342 staining. Apoptotic cells assessed by Hoechst 33342 staining were significantly increased in HG-stimulated podocytes compared with NG cells ($P<0.05$), and apoptosis in HG podocytes was further increased by HO-1 siRNA ($P<0.05$). In contrast, hemin pretreatment significantly protected HG podocytes against apoptosis ($P<0.05$) (Figure 10).

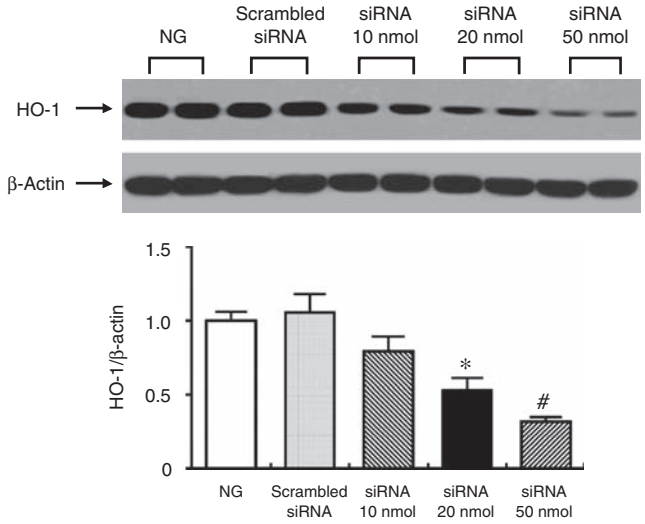

Figure 7 | A representative western blot of heme oxygenase (HO)-1 in cultured podocytes treated with three different concentrations of HO-1 small interfering RNA (siRNA) (representative of four blots). HO-1 siRNA inhibited HO-1 protein expression in a dose-dependent manner. $^{*}P<0.05$ vs NG group, $^{\#}P<0.01$ vs NG group. NG, normal glucose.

DISCUSSION

Earlier studies have demonstrated that HO-1 has a protective role in various kidney diseases in which oxidative stress is thought to have a role.[6–11] However, the functional significance of HO-1 in terms of podocyte apoptosis in DM nephropathy has not been elucidated. In this study, we demonstrate that HO-1 expression is increased in experimental DM glomeruli and in HG-stimulated podocytes. In addition, to our knowledge, this is the first study showing that podocyte apoptosis under DM conditions is partly protected by this increase in HO-1 expression.

Heme oxygenase is an enzyme that cleaves the heme ring, thereby converting heme into biliverdin and releasing iron and carbon monoxide.[1,2] By this process, HO regulates the cellular levels of heme, which is known to act as a pro-oxidant and to induce cell dysfunction. Therefore, when cellular heme levels are increased by denaturation or destabilization of heme protein during cell injury, HO increases to remove heme and replace it with bilirubin, a potent antioxidant.[1,2] The cytoprotective role of HO was first suggested by Keyse and Tyrrell[33] who identified HO as a sodium arsenite-induced 32-kDa protein in cultured human skin fibroblasts and found that the HO gene is also induced by ultraviolet A radiation, hydrogen peroxide, and heavy metal salts. On the basis of these findings, they suggested that the induction of HO may be a general response to oxidant stress and that HO constitutes an important cellular defense mechanism against oxidative damage. Following this report, numerous studies have investigated the expression and the functional role of HO, especially of HO-1, the isoform of HO induced under stressed conditions. In the kidney, HO-1 is weakly expressed in proximal and distal tubules, in Henle's loop, and in medullary collecting ducts under normal state, but its expression is increased under various conditions.[1] The functional role of increased HO-1 expression has been elucidated in toxic nephropathy and in renal ischemia–reperfusion injury,[7,17–19,23,34–36] in which oxidative stress has a role in the pathogenesis. Upregulation of HO-1 reduced renal injury induced by cyclosporine[23] and potassium dichromate,[34] and protected tubular cell injury induced by cisplatin,[18] whereas inhibition of HO-1 expression exacerbated renal injury in maleate nephropathy[19] and worsened cisplatin-induced cell injury.[7] In addition, induction of HO-1 by hemolysate,[35] tin chloride,[21] or cobalt[36] ameliorated ischemic acute renal injury in rats, whereas mild renal ischemia exerted severe renal dysfunction and increased mortality in HO-1 knockout mice compared with control mice.[37] Taken together, HO-1 seems to be induced to protect cells against oxidative stress-induced renal injury.

On the basis of these earlier reports, HO-1 expression was surmized to be increased in DM nephropathy in response to oxidative stress. Recently, Hayashi *et al.*[31] and Koya *et al.*[32] demonstrated that HO-1 mRNA and protein expression were induced in DM glomeruli and antioxidant treatment nearly normalized the increase in HO-1 expression, suggesting that glomerular HO-1 expression was increased secondary to DM-induced oxidative stress. The former investigators also showed that the glomerular cells with increased HO-1 protein expression were mesangial cells and podocytes. However, these two studies did not examine the biological significance of increased glomerular HO-1 expression under DM conditions. This study also demonstrates that HO-1 expression is increased not only in experimental DM glomeruli but also in HG-stimulated podocytes, which is in accordance with the earlier studies.[31,32]

The cytoprotective effect of HO-1 seems to be attributed to the antiapoptotic role of the HO system. Shiraishi *et al.*[18] showed that renal failure and renal injury characterized by tubular apoptosis were more severe in transgenic mice deficient in HO-1 compared with wild-type mice treated with cisplatin. Cisplatin-induced apoptosis in cultured proximal tubular epithelial cells was significantly attenuated by inducing HO-1 expression, whereas inhibition of HO-1 enzyme activity reversed the antiapoptotic effect. On the basis of these findings, the researchers postulated that HO-1 had a cytoprotective effect by preventing apoptosis induced by oxidative stimuli. The antiapoptotic effect of HO-1 seems to be mediated by upregulating the expression of p21, an antiapoptotic cell-cycle protein, in renal tubular epithelial cells.[38] In some cells, carbon monoxide, a metabolite of the HO system, is responsible for the antiapoptotic effect of HO-1,[39,40] while augmentation of iron efflux by HO-1 contributes to its antiapoptotic property in cultured fibroblasts.[41] This study demonstrates for the first time that apoptosis in experimental DM glomeruli and in HG-stimulated podocytes is further accentuated in an HO-1-suppressed state both *in vivo* and *in vitro*, suggesting that podocyte HO-1 expression is induced to protect against

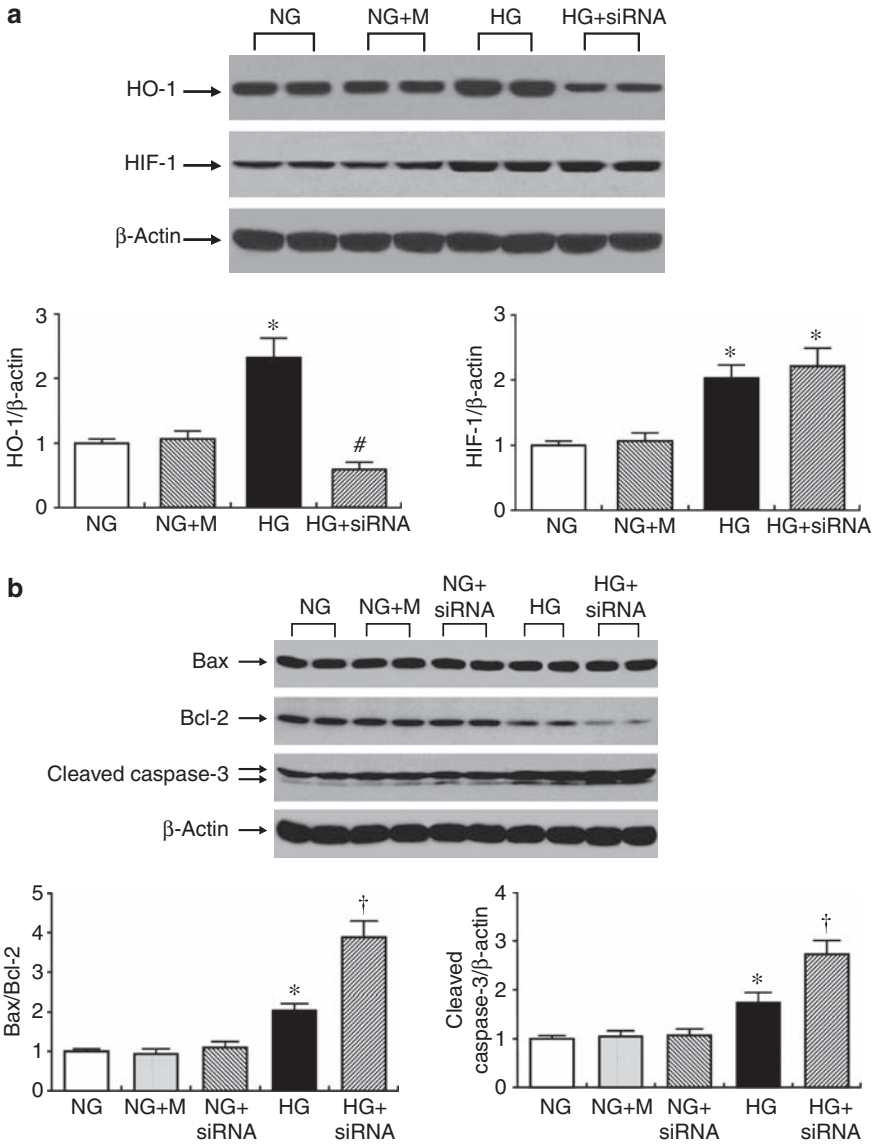

Figure 8 | A representative western blot of heme oxygenase (HO)-1, hypoxia-inducible factor-1 (HIF-1), and apoptosis-related molecules in cultured podocytes (representative of four blots). (**a**) There were significant increases in HO-1 and HIF-1 expression in HG-stimulated podocytes compared with NG and NG + M cells, and the increase in HO-1 protein expression in HG podocytes was significantly ameliorated by HO-1 siRNA treatment (20 nmol). (**b**) The ratios of Bax/Bcl-2 and active fragments of caspase-3 protein expression were significantly increased in HG-stimulated podocytes compared with normal glucose (NG) and NG + M groups, and these increases were accentuated in high glucose (HG) cells by HO-1 siRNA (20 nmol). $^{*}P<0.05$ vs NG and NG + M groups, $^{\#}P<0.01$ vs HG group, $^{\dagger}P<0.05$ vs DM group. HG, high glucose; Ng, normal glucose; NG+M, normal glucose plus mannitol.

podocyte apoptosis under DM conditions. In addition, double immunofluorescence staining with active fragments of caspase-3 and synaptopodin in ZnPP-treated DM rats revealed that podocytes were the glomerular cells with augmented apoptosis. On the basis of these findings, the further increase in albuminuria in DM rats treated with ZnPP may be attributed to exacerbated apoptosis of podocytes under an HO-1-inhibited state.

To clarify the functional role of HO-1 in DM nephropathy, ZnPP and HO-1 siRNA were used in this study to inhibit HO-1 *in vivo* and *in vitro*, respectively, and hemin to induce HO-1 *in vtiro*. Accumulating evidence have shown that both HO-1 expression and activity were inhibited by ZnPP treatment.[12,42,43] Moreover, earlier studies have shown that HIF-1 is an upstream factor of HO-1 and that the administration of ZnPP inhibited the mRNA expression and activity of HIF-1, leading to a decrease in HO-1 expression.[44] In this study, we demonstrated that the expression of HIF-1 was increased in DM glomeruli and in HG-stimulated podocytes and these increases were abrogated by ZnPP treatment, suggesting that the inhibitory effect of ZnPP on HO-1 expression under DM conditions was attributed to its effect on HIF-1 expression.

Although the majority of earlier *in vivo* studies used ZnPP to suppress HO activity, ZnPP is known to inhibit not only HO-1 but also HO-2.[45] For this reason, the results of this

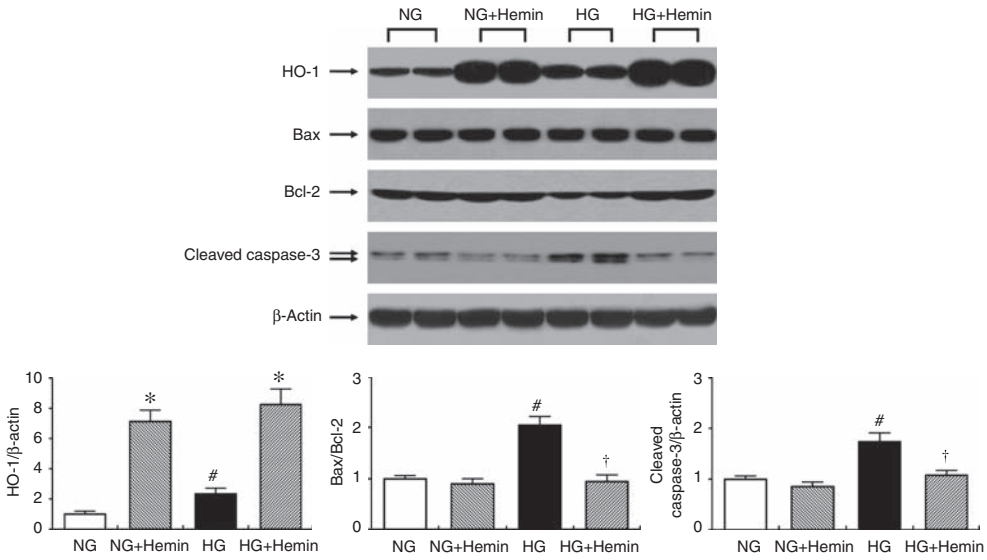

Figure 9 | A representative western blot of heme oxygenase (HO)-1 and apoptosis-related molecules in cultured podocytes (representative of four blots). Hemin pretreatment (20 μmol) significantly induced HO-1 protein expression in cultured podocytes exposed to normal glucose (NG) and high glucose (HG). In addition, HO-1 induction by hemin before HG stimulation significantly attenuated the increases in Bax/Bcl-2 ratio and active fragment of caspase-3 protein expression in HG-stimulated podocytes. $^{*}P<0.005$ vs NG and HG groups, $^{\#}P<0.05$ vs NG group, $^{\dagger}P<0.05$ vs HG group.

in vivo study could not definitively determine whether the increase in podocyte apoptosis was due to the blockade of HO-1 and/or HO-2 activity. For the current *in vitro* experiments, HO-1 siRNA was therefore introduced to selectively inhibit HO-1, which led to an increase in apoptosis of podocytes under DM conditions, consistent with the animal study. In contrast, we also demonstrated that HO-1 induction by hemin pretreatment significantly ameliorated apoptosis in HG-stimulated podocytes. All these findings imply that HO-1 has an important function in cytoprotection in DM nephropathy.

In conclusion, HO-1 expression was increased in both experimental DM glomeruli and HG-stimulated podocytes. Moreover, inhibition of HO-1 expression further increased albuminuria, reduced podocyte numbers in DM rats, and accentuated podocyte apoptosis in DM glomeruli and in podocytes exposed to HG, whereas induction of HO-1 protected HG-stimulated podocytes from apoptosis. Taken together, these results suggest that podocyte HO-1 expression is induced to protect podocytes against apoptosis under DM conditions.

MATERIALS AND METHODS

Animals

All animal studies were conducted under an approved protocol. Male Sprague–Dawley rats weighing 250–280 g were injected either with diluent ($n=16$, C) or with 65 mg/kg streptozotocin intraperitoneally ($n=16$, DM). Diabetes was confirmed by tail vein blood glucose levels above 300 mg per 100 ml on the third day after injection. After confirming DM, eight rats from each group were treated with intraperitoneally ZnPP (50 μmol/kg/day) (Sigma-Aldrich, Saint Louis, MO, USA) (C + ZnPP, DM + ZnPP) for 6 weeks. To determine whether there is a gender-specific effect of ZnPP on DM nephropathy, the same experiments were performed with female Sprague–Dawley rats ($n=24$). In addition, experiments with DM rats ($n=8$) treated with 2 U/day of insulin (Ultralente, Eli Lilly, Indianapolis, IN, USA) were also conducted to control the effect of streptozotocin. Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the 6-week study period.

Body weights were checked biweekly, and the kidney weights were measured at the time of killing. Systolic blood pressure was measured by tail-cuff phlethysmography at 6 weeks. Serum glucose, blood urea nitrogen and serum creatinine levels, and 24-h urinary albumin were also measured at the time of killing. Blood glucose was measured by a glucometer and 24-h urinary albumin excretion by enzyme-linked immunosorbent assay (Nephrat II, Exocell, Philadelphia, PA, USA). Blood urea nitrogen and serum creatinine levels were determined by an automatic biochemical analyzer.

Glomerular isolation

Glomeruli were isolated by sieving. Purity of the glomerular preparation was greater than 98% as determined by light microscopy.

Podocyte culture

Conditionally immortalized mouse podocytes were kindly provided by Dr Peter Mundel (Albert Einstein College of Medicine, Bronx, NY, USA) and were cultured as described earlier.[46] Briefly, frozen podocytes were first grown under permissive conditions at 33 °C in RPMI 1640 media containing 10% fetal bovine serum, 50 U/ml γ-interferon, and 100 U/ml of penicillin/streptomycin in collagen-coated flasks, and the γ-interferon was tapered down to 10 U/ml in successive passages. Cells were then trypsinized and subcultured without γ-interferon (non-permissive conditions) and allowed to differentiate at 37 °C with media changed on alternate days. Differentiation of podocytes grown for 10 days at 37 °C was confirmed by the identification of synaptopodin, a podocyte differentiation marker, by reverse transcription-PCR and western blotting (data not shown).

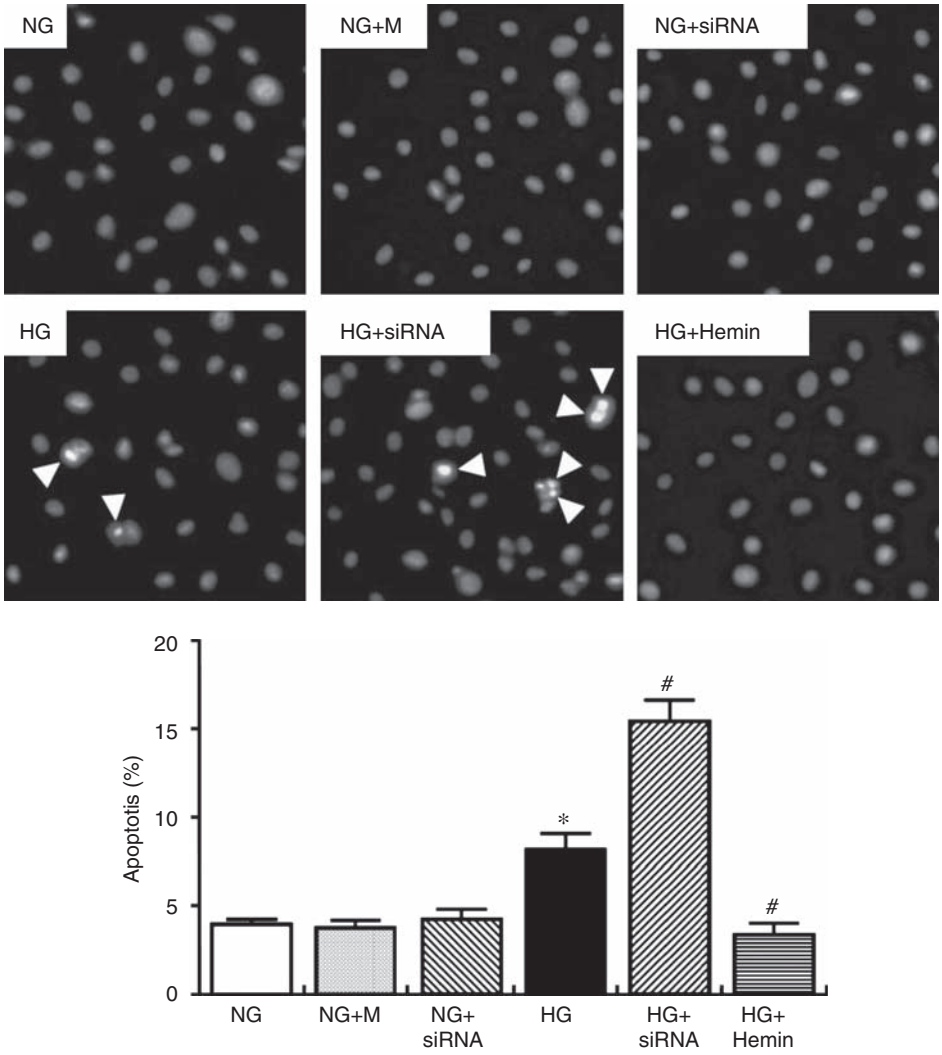

Figure 10 | Apoptosis assessed by Hoechst 33342 staining. There was a significant increase in apoptotic cells (arrowheads) in high glucose (HG)-stimulated podocytes compared with normal glucose (NG) cells, and apoptosis in HG podocytes was further increased by heme oxygenase (HO)-1 small interfering RNA (siRNA) (20 nmol). In contrast, HO-1 induction by hemin (20 μmol) before HG stimulation significantly protected cultured podocytes against apoptosis. $^{*}P<0.05$ vs NG, NG + M, and NG + siRNA groups, $^{\#}P<0.05$ vs HG group.

In this study, siRNA for mouse HO-1 was used to inhibit HO-1 expression. The sense and antisense sequences of mouse HO-1 siRNA were as follows: sense, 5′-GGCUUUAAGCUGGUGAUGGTT-3′; and antisense, 5′-CCAUCACCAGCUUAAAGCCTT-3′. The concentrations of siRNA for this study were determined based on preliminary experiments with three different concentrations of 10, 20 and 50 nmol siRNA. The HO-1 siRNA and a negative control siRNA, scrambled siRNA, were transiently transfected into differentiated podocytes with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 24 h.

Transfected and nontransfected podocytes were serum-restricted for 24 h, after which the medium was changed to RPMI medium containing NG (5.6 mmol glucose), NG + 24.4 mmol mannitol (NG + M), NG + 20 nmol HO-1 siRNA (Ambion, Austin, TX, USA) (NG + siRNA), HG (30 mmol glucose), or HG + 20 nmol HO-1 siRNA (HG + siRNA). To explore the effect of HO-1 induction on HG-induced changes, podocytes were also pretreated with 20 μmol hemin (Fluka, Buchs SG, Switzerland) for 6 h and were exposed to HG medium. After 24 h, cells were harvested.

Western blot analysis

Sieved glomeruli and podocytes harvested from plates were lysed in SDS sample buffer (2% SDS, 10 mmol Tris-HCl, pH6.8, 10% (vol/vol) glycerol). Lysate was centrifuged at 10,000 *g* for 10 min at 4 °C and the supernatant was stored at −70 °C until all rats were killed. Protein concentrations were determined with a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of 50 μg proteins were treated with Laemmli sample buffer, then heated at 100 °C for 5 min, and electrophoresed (50 μg/lane) in 8 or 12% acrylamide denaturing SDS-polyacrylamide gel. Proteins were transferred to a Hybond-ECL membrane (Amersham Life Science, Arlington Heights, IL, USA) using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA), and the membrane was then incubated in blocking buffer A (1 × phosphate-buffered saline, 0.1% Tween-20, and 8% non-fat milk) for 1 h at room temperature, followed by an overnight incubation at 4 °C in a 1:1000 dilution of monoclonal antibody to HO-1 (EMD Biosciences, Darmstadt, Germany), polyclonal antibodies to HIF-1 (ABR-Affinity Bioreagents, Rockford, IL, USA), active fragments of caspase-3 (Cell Signaling, Beverly, MA, USA), Bax, Bcl-2, or β-actin (Santa Cruz

Biotechnology, Santa Cruz, CA, USA). The membrane was washed three times and then incubated with a 1:1000 dilution of horseradish peroxidase-linked goat anti-rabbit IgG (Amersham Life Science). The washes were repeated, and the membrane was developed with chemiluminescent agent (ECL; Amersham Life Science). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany), and the changes in the optical densities relative to C glomeruli and NG cells were used for analysis.

Immunohistochemistry

Slices of kidney for immunohistochemical staining were fixed in 10% neutral-buffered formalin, processed in the standard manner, and 4 μm sections of paraffin-embedded tissues were used. Slides were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in 10 mmol sodium citrate buffer for 20 min using a Black and Decker vegetable steamer (Towson, MD, USA). For HO-1, the primary monoclonal anti-HO-1 antibody (EMD Biosciences) was diluted in 1:200 with 2% casein in bovine serum albumin and was applied for overnight incubation at room temperature. After washing, a secondary goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added for 20 min, and the slides were then washed and incubated with a tertiary rabbit-PAP complex for 20 min. Diaminobenzidine was added for 2 min and the slides were counterstained with hematoxylin. A semiquantitative score for measuring the intensity of HO-1 staining within glomeruli was determined by examining 30 glomeruli in each section and by digital image analysis (MetaMorph version 4.6r5, Universal Imaging, Downingtown, PA, USA) as described earlier.[47] Briefly, the degree of staining was semiquantitated on a scale of 0 to 4+ and a staining score was obtained by multiplying the intensity of staining by the percentage of glomeruli staining for that intensity; these numbers were then added for each experimental animal to give the staining score.

$$\sum(\text{intensity of staining})\times(\%\text{ of glomeruli with that intensity}) = \text{staining score}$$

Immunohistochemical staining for WT-1 was also performed to determine the number of podocytes as described earlier.[48] Briefly, two adjacent 3 μm sections stained with WT-1 were observed in pairs at a magnification of ×400, and the WT-1-positive stained nuclei present in the top but not in the bottom section were counted and summed. Ten glomeruli in five rats from each group and 13–15 sections from the midglomerular area were examined.

Immunofluorescence

Slices of kidney for immunofluorescence staining were snap-frozen in optimal cutting temperature solution and 4 μm sections of tissues were used. Slides were fixed in acetone for 10 min at 4 °C, air-dried for 10 min at room temperature, and blocked with 10% donkey serum for 20 min at room temperature. For double immunofluorescence staining, primary polyclonal antibodies to HO-1 (EMD Biosciences) or active fragments of caspase-3 (Cell Signaling) were diluted in 1:100 with antibody diluent (DAKO, Glostrup, Denmark) and was applied for 3 h at room temperature. After washing, Cy3 (red)-conjugated anti-rabbit IgG antibody (Research Diagnostics, Flanders, NJ, USA) was added for 60 min. A 1:200 dilution of polyclonal anti-synaptopodin antibody (Santa Cruz Biotechnology) was then applied, followed by Cy2 (green)-conjugated anti-goat IgG antibody. A semiquantitative score for measuring the intensity of active fragments of caspase-3 was determined as aforementioned.

Measurement of HO activity

Heme oxygenase activity was determined in rat glomeruli microsomes by bilirubin generation.[7] Microsomes, which were prepared by centrifugation at 105,000 *g* for 60 min at 4 °C, were resuspended in 0.5 ml of 100 mmol potassium phosphate buffer (pH 7.4) containing 2 mmol $MgCl_2$, frozen to −70 °C, and thawed three times and sonicated on ice. The resulting supernatant (400 μl) was added to 200 μl of reaction mixture (2 mg of rat liver cytosol, 10 μmol hemin, 0.2 mmol glucose-6-phosphate, 0.2 U of glucose-6-phosphate dehydrogenase, and 0.8 mmol NADPH) and left at 37 °C for 1 h in the dark. The formed bilirubin was extracted with same volume of chloroform and was calculated by the difference in absorbance between 464 and 530 nm (extinction coefficient 40 mmol/cm for bilirubin). HO activity was expressed as picomoles of bilirubin formed per milligram of protein per hour. The protein content of the microsomal fraction was determined by the method of Bradford.[49]

TUNEL assay and Hoechst 33342 staining

In addition to the changes in the expression of apoptosis-related molecules, apoptosis was also identified within glomeruli by TUNEL using a commercially available kit (Chemicon International, Temecula, CA, USA) and in cultured podocytes seeded on coverslips by Hoechst 33342 (Molecular Probes, Eugene, OR, USA) staining. Apoptosis was defined as TUNEL-positive cells within glomeruli and the presence of nuclear condensation on Hoechst staining. TUNEL-positive glomerular cells in formalin-fixed renal tissue and the percentage of podocytes with nuclear condensation were determined by examining at least 30 glomeruli and 300 cells per condition, respectively, at ×400 magnification.

Statistical analysis

All values are expressed as the mean ± s.e.m. Statistical analysis was performed using the statistical package SPSS for Windows Ver. 11.0 (SPSS, Chicago, IL, USA). Results were analyzed using the Kruskal–Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal–Wallis test were confirmed by the Mann–Whitney *U*-test. *P*-values less than 0.05 were considered to be statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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