

Original Article

HO-1 induction ameliorates experimental murine membranous nephropathy: anti-oxidative, anti-apoptotic and immunomodulatory effects

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Abstract

Background. Therapeutic agents for membranous nephropathy (MN) remain ill-defined. Haeme oxygenase (HO)-1 is considered to play a protective role in various disorders. Here, we assessed the efficacy of HO-1 induction therapy for MN.

Methods. MN was induced in BALB/c mice with intravenous injections of cationic bovine serum albumin. Three groups of mice were administered 100 µmol/kg Cobalt protoporphyrin (CoPP, a potent HO-1 inducer), Tin protoporphyrin (SnPP, a potent HO-1 inhibitor) or phosphate-buffered saline via intra-peritoneal injections once a week starting from the induction of MN. Disease severity was verified by serum and urine metabolic profiles and by renal histopathology. Cytokine profiles, immunoglobulin production, the expression of oxidative stress markers (thio-barbituric acid reactive substances, TBARS) and apoptosis, as measured by TUNEL, were also determined.

Results. Mice treated with CoPP displayed a significant reduction in proteinuria and a marked amelioration of glomerular lesions, accompanied by attenuated immune-complex deposition. The production of immunoglobulins in MN mice treated with CoPP was significantly reduced compared with that of mice in the other two groups. TBARS in the serum and kidneys, as well as apoptosis, were also significantly reduced in CoPP-treated mice. Cytokine mRNA expression in the renal cortex indicated that CoPP not only decreased the expression of proinflammatory cytokines, but also increased the expression of anti-inflammatory cytokines (interleukin-10).

Conclusions. HO-1 induction therapy may ameliorate experimental MN via multiple pathways, including anti-oxidative, anti-apoptotic and immunomodulatory effects.

HO-1 inducing regimens should be considered as a potential therapeutic intervention in MN in the future.

Keywords: apoptosis; haeme oxygenase-1; immunomodulatory; membranous nephropathy; oxidative stress

Introduction

Idiopathic membranous nephropathy (MN), an autoimmune-mediated glomerulonephritis characterized by *in situ* immune-complex deposition over the subepithelial space, is one of the most common causes of nephrotic syndrome in adults [1]. The deposited immune complexes sequentially induce the inflammatory response, complement activation and oxidative injury, all of which participate in the pathogenesis of MN [2,3]. Approximately 30%–40% of patients with MN develop progressive renal impairment, which results in end-stage renal failure after 10–15 years [4]. Presently available immunosuppressive therapies are not always effective and often have many persistent side effects [5]. Therefore, the appropriate treatment of patients with MN is still open to debate.

Haeme oxygenase (HO) is the rate-limiting enzyme that degrades haeme into carbon monoxide (CO), ferritin and biliverdin [6]. The inducible form, HO-1, is expressed in response to various stimuli, such as hydrogen peroxide, heat, heavy metal ions, hyperoxide, endotoxin and inflammatory cytokines, whereas HO-2, another isoenzyme of HO, is constitutively expressed. Recently, HO-1 has been shown to have cytoprotective properties, as well as anti-inflammatory, anti-oxidant, anti-apoptotic and possible immunomodulatory functions [7–9]. Using chemical inducers or genes, HO-1 has been shown to be expressed in various diseases, including respiratory diseases, cardiovascular diseases, renal disease, ocular diseases, liver injury and organ transplantation in animal models [10,11].

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In this study, to investigate the effects of HO-1 induction on murine MN, we monitored renal and immunological parameters in mice with MN that were administered weekly intraperitoneal doses of cobalt protoporphyrin (CoPP), a HO-1 inducer, or tin protoporphyrin (SnPP), an HO-1 inhibitor. The induction of endogenous HO-1 successfully suppressed the pathological injury of the glomeruli and inhibited the deposition of immune complexes. A significant reduction in inflammatory cytokines and circulating levels of serum immunoglobulin, as well as reduced oxidative stress and apoptosis, was also noted.

Materials and methods

Mice

All animal studies were approved by the Animal Care and Use Committee of the National Defence Medical Center (Taipei, Taiwan) and all experiments were conducted according to the National Institutes of Health guidelines. BALB/c female mice (4–6 weeks old, ~20 g body weight) were initially purchased from the National Laboratory Animal Center (Taipei, Taiwan) and were maintained under specific-pathogen-free conditions in the Laboratory Animal Center of the National Defense Medical Center.

Experimental design

The mice were divided randomly into two groups: an experimental group and a control group. Animals in both groups were immunized with 0.2 mg of cationic bovine serum albumin (cBSA) emulsified in an equal volume of complete Freund's adjuvant [12,13]. Two weeks later, the experimental group (MN) received cBSA (13 mg/kg) intravenously three times per week every other day for 6 weeks, and the control group (NC) received pure saline according to the same schedule. Both experimental and control mice were then divided randomly into three subgroups, each of which received one of three treatments administered by intraperitoneal injections: 100 μ mol/kg CoPP, SnPP or saline once a week starting from MN induction (abbreviated as MN-CoPP, MN-SnPP or MN; and NC-CoPP, NC-SnPP or NC, respectively). $N = 8$ in each subgroup. Cobalt (III) and tin (IV) protoporphyrin IX chloride (CoPP and SnPP, respectively) were purchased from Frontier Scientific, Logan, UT, USA. Homogenous cBSA was prepared as previously described [12]. Disease severity was verified by serum and urine metabolic profiles and by renal histopathology.

Serum and urine measurements

Urine samples were collected every week for protein measurements, which were made with a urine dipstick (Albustix, Bayer, Tarrytown, NY, USA). Grades of 0–4 were ascribed to urine protein concentrations corresponding to 0–30, 30–100, 100–300, 300–2000 and over 2000 mg/dL, respectively. Blood samples were collected, microcentrifuged and stored at -70°C until assayed. Concentrations of blood urea nitrogen, creatinine, albumin and total cholesterol were determined by Fuji DRI-CHEM 3030

(Fuji Photo Film Co. Ltd., Tokyo, Japan). All assays were performed in duplicate according to the manufacturer's instructions.

Histological studies of renal tissues

Mice were anaesthetized and the kidneys harvested after perfusion with phosphate-buffered saline (PBS) to remove blood from the tissues via abdominal aorta. Formalin-fixed and paraffin-embedded sections of mouse kidney tissues were cut and stained with haematoxylin and eosin (H&E) and silver stain. Frozen sections were dried in the air, fixed in acetone and washed with PBS before incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig) G, and C₃ (Cappel, Durham, NC, USA) in an immunofluorescence study.

Pathologic findings of MN were classified into four stages: stage I, subepithelial deposits; stage II, spike formation; stage III incorporation of the deposits into the basement membrane and stage IV, disappearance of deposits [14,15]. Fluorescence was observed and evaluated semiquantitatively as described previously [12].

Immunohistochemistry

Immunohistochemical staining was performed on formaldehyde-fixed and paraffin-embedded tissues. The endogenous peroxidase activity was quenched and the sections were blocked with 1% (w/v) BSA in PBS for 1 h. The sections were then incubated with a 1:300 dilution of rabbit polyclonal anti-haeme-oxygenase-1 antibody (Stressgen Biotechnologies, Victoria, BC, Canada) in PBS, followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Novus Biologicals, Littleton, CO, USA). The reaction products were visualized with a colour solution consisting of AEC (Dako, Carpinteria, CA, USA) and the slides were counterstained with haematoxylin.

Western blot analysis of HO-1

Proteins were extracted from the kidney cortex and separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (12%). The gel was electroblotted onto a nitrocellulose membrane, incubated for 1 h in 20 mL of the blocking buffer [Tris-buffered saline (TBS), 5% skimmed milk], washed three times in TBS with 0.1% Tween-20 and incubated overnight at 4°C with rabbit anti-HO-1 antibody (Stressgen). The blots were washed three times and incubated for 1 h with HRP-conjugated goat anti-rabbit antibody (Novus) at room temperature. The membranes were washed three times, and the membrane-bound antibody was detected with Western Lightning Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA) and visualized on X-ray film.

Production of immunoglobulins

Serum concentrations of anti-cBSA immunoglobulins (Igs) were measured as described previously [12]. Briefly, microtitration plates were coated with cBSA, blocked with 1% goat serum albumin, and incubated with the serum

samples at various dilutions. HPR-conjugated goat anti-mouse Ig (DAKO) was added and incubated for 1 h. The substrate solution (Chemicon International Inc., Temecula, CA, USA) was then added and the reaction was stopped with H₂SO₄. The optical density (OD) was read at 450 nm on an automated microplate reader (MRX; Dynex Technology, Chantilly, VA, USA). Data were expressed as OD at 450 nm.

Oxidative stress markers

We chose the lipid peroxidation products, TBARS, as the oxidation markers. Serum TBARS were analysed by the method described by Yagi [16]. Briefly, 20% trichloroacetic acid, 0.67% thiobarbituric acid:glacial acetic acid solution (1:1, v/v) and 150 mM NaCl were added to the serum sample. The mixture was boiled at 95°C for 1 h. The TBARS were extracted with *n*-butanol, and the fluorescence of the extracted solution was analysed by spectrofluorometry (excitation, 515 nm; emission, 553 nm).

Reactive oxygen species (ROS) detection in the kidney

In situ superoxide anion production was determined by dihydroethidium (DHE) labelling as described [17]. Briefly, 15 µm thick frozen sections were incubated with 10 µmol/L DHE (Molecular Probes, Eugene, OR, USA) at 37°C for 30 min in a humidified chamber protected from light. Fluorescent images were quantified by counting the percentage of positive nuclei from total nuclei per kidney cross section.

Terminal deoxynucleotidyl transferase-mediated nick end-labelling assay

Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated nick end-labelling (TUNEL) assay using the *in situ* Cell Death Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, mouse kidney sections were fixed with 4% paraformaldehyde and washed with PBS. The cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate solution, rinsed with PBS and incubated for 1 h in the TUNEL reaction mixture (terminal deoxynucleotidyl transferase with FITC-conjugated dUTP). After the slides were washed in PBS, they were examined with a fluorescence photomicroscope (Olympus, Tokyo, Japan).

RNA extraction and real-time quantitative PCR

Total RNA was extracted from the renal cortex with TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). First-strand complementary DNA (cDNA) was synthesized using the SuperScript III Reverse Transcriptase kit (Life Technologies) following the standard protocol. Real-time polymerase chain reaction (PCR) analyses were performed using the SYBR Green Master Mix Kit (Bio-Rad) and the Opticon PCR thermal cycler (MJ Research, Waltham, MA, USA). The relative expression of each cytokine mRNA was determined and normalized to the expression of the internal housekeeping gene GAPDH. All samples measured in

Table 1. Primer sequences for real-time PCR of cytokine mRNAs

Gene	Forward primer	Reverse primer
HO-1	gccaccaaggaggtagacat	gcttgtgcgctctatctcc
IL-1β	tcatgggatgatgataacctgct	cccatacttagaagaacacggatt
TNF-α	gggccaccacgctcttctgct	gccactccagctgctctccac
IFN-γ	ggccatcagcaacaacataagc	ccccgaatcagcagcgactc
IL-2	gcggcatgttctggattgac	gggctgttgagatgatgcttt
IL-4	gccaacgtcctcacgcaa	gcatgggtgctcagtactaga
IL-10	ggccctttgctatggtgtcc	aagcgctgggggatgac
TGF-β	ccgcaacaacccatcatga	gggggtcagcagccggttac
GAPDH	gccaagaagggtcatctc	ggccatccacagctctt

triplicate for three times. Primer and probe sequences are listed in Table 1.

Statistical analyses

Data are expressed as means ± standard deviations (SD). Statistical analysis with one-way ANOVA was performed for multiple comparisons and Bonferoni test was applied for correcting between group differences. *P* < 0.05 was regarded as statistically significant.

Results

Effects of CoPP and SnPP treatments on general characteristics

Mice with experimental MN developed the characteristic symptoms of nephrotic syndromes, such as proteinuria, hypoalbuminaemia and hypercholesterolaemia (Figure 1). In MN mice that had received CoPP treatment, a marked attenuation of proteinuria, hypoalbuminaemia and hypercholesterolaemia was observed. However, no therapeutic effect was observed in MN mice that had received the SnPP treatment. There was no significant difference between the control and experimental groups in serum creatinine levels. We had also checked the blood pressure in each group during the course of the disease. We did not find any significant differences of blood pressure in each group. The general characteristics of the control groups that had received CoPP or SnPP alone (NC-CoPP and NC-SnPP, respectively) displayed no significant changes when compared with those of the normal control group (data not shown).

Effects of CoPP and SnPP treatments on histopathological findings

Pathological findings in MN mice included a diffuse thickening of the glomerular basement membrane compared with that of the normal control group. Compared with the MN and MN-SnPP mice, the pathological severity of the MN-CoPP mice appeared to be milder (Figure 2A–E). All three experimental groups showed positive immunofluorescent staining for IgG, with a discrete beaded appearance, along the glomerular capillary wall, but the immunofluorescence intensity in the MN-CoPP mice was low compared with that in the MN-SnPP and MN mice (Figure 2F–J). Immunofluorescent staining for C3 also presented as intense

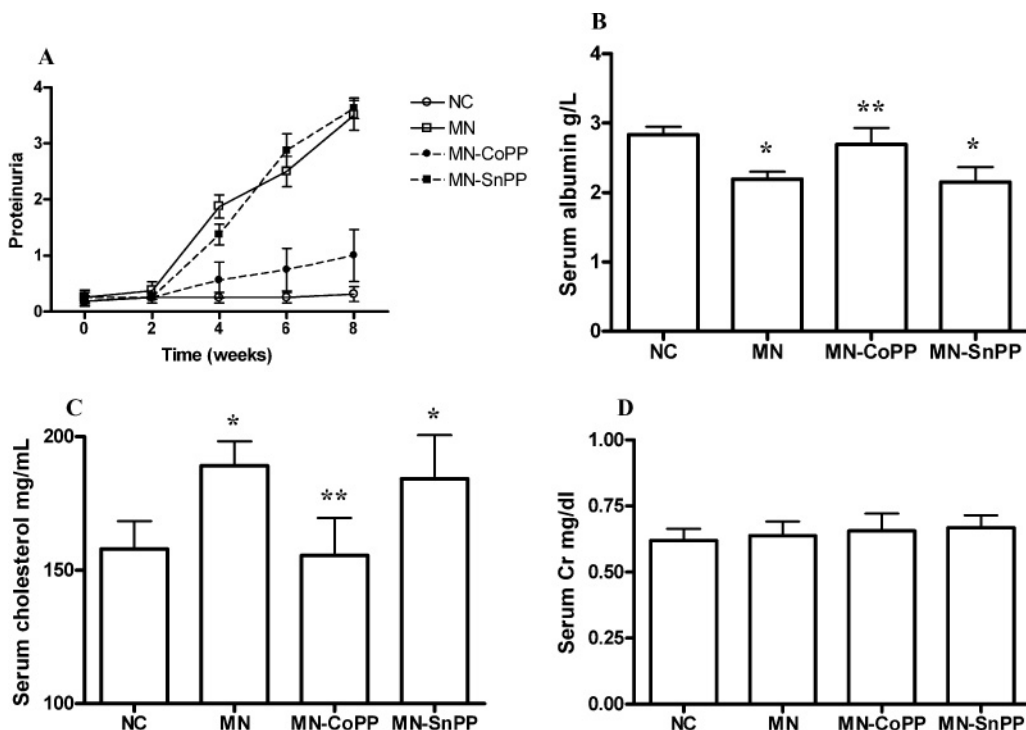


Fig. 1. Effects of HO-1 induction/inhibition on laboratory characteristics of mice with experimental MN. Urinary proteinuria (A), serum albumin (B), serum cholesterol (C) and serum creatinine levels (D) of the normal control group (NC), membranous nephropathy group (MN), mice with membranous nephropathy receiving weekly CoPP treatments (MN-CoPP) and mice with membranous nephropathy receiving SnPP treatments (MN-SnPP). * $P < 0.05$ versus the control group. ** $P < 0.05$ versus the MN group.

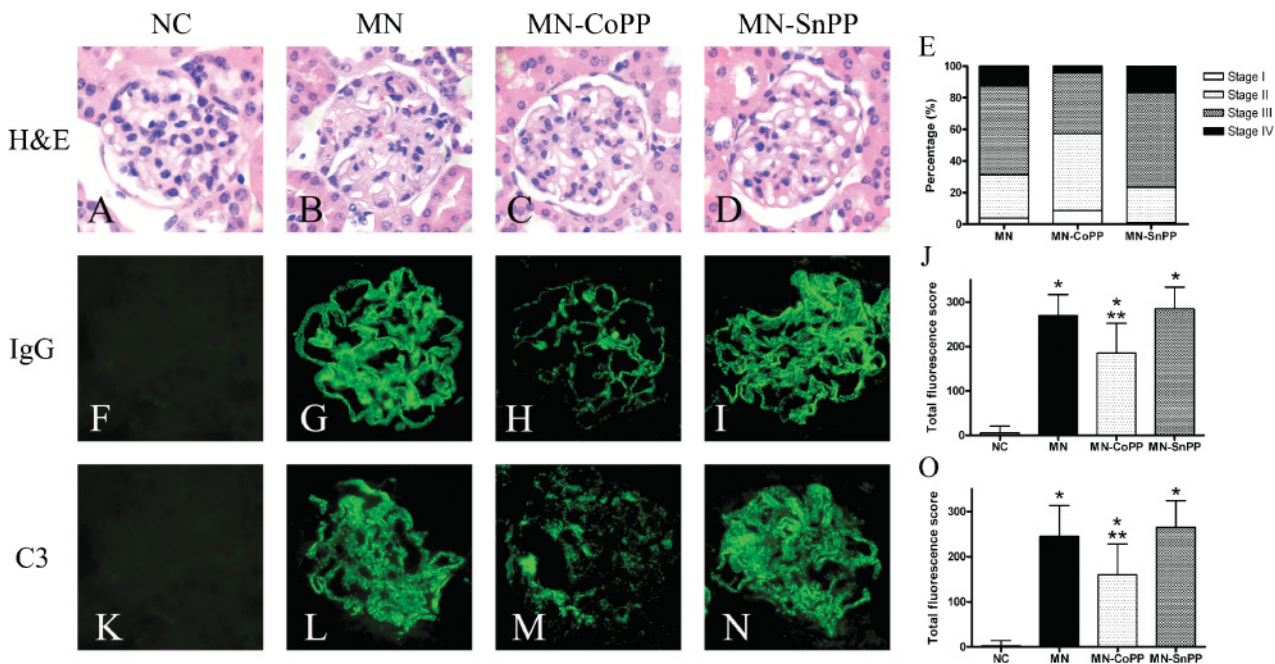


Fig. 2. Changes in the histopathology of mice with experimental MN. Kidneys from mice of the NC group (A, F and K), MN group (B, G and L), MN group receiving CoPP treatment (C, H and M) and MN group receiving SnPP treatments (D, I and N) are shown with H&E staining (A–E), immunofluorescent staining of IgG (F–J) and C3 (K–O). All images are at 400 \times magnification. * $P < 0.05$ versus control group. ** $P < 0.05$ versus the MN group.

granular fluorescence along the glomerular capillary wall, with a similar pattern to that of IgG (Figure 2K–O). The histopathological and immunofluorescent features of the NC-CoPP and NC-SnPP controls did not appear to differ from those of the NC group (data not shown).

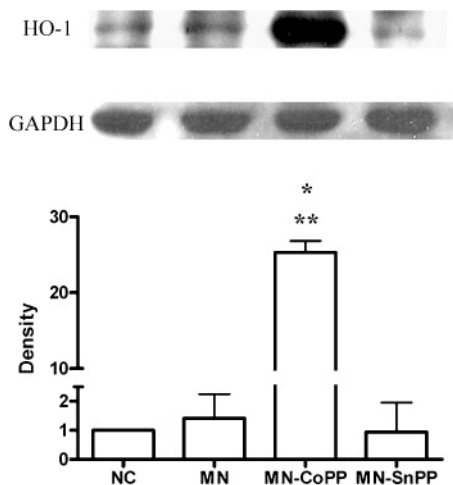


Fig. 3. HO-1 expression in the kidney. Kidneys from mice of the normal control group, membranous nephropathy group, membranous nephropathy group receiving weekly CoPP treatments and membranous nephropathy group receiving SnPP treatments. All samples were obtained from mice at 14 weeks of age. Representative results of more than three individual experiments are shown. * $P < 0.05$ versus the control group. ** $P < 0.05$ versus the MN group ($n = 3$)

Effects of CoPP and SnPP treatments on HO-1 expression in the kidney

To determine whether CoPP and SnPP affect the expression of HO-1, we examined protein expression in homogenates of the kidney cortex from mice of the control and experimental groups by western blot analysis. HO-1 expression was not significantly higher in MN mice than in the normal control group. The administration of CoPP strongly augmented HO-1 protein expression, whereas SnPP caused a reduction in the expression of this protein compared with its expression in the MN group, although the difference was not statistically significant (Figure 3). HO-1 expression assessed by immunohistochemistry was very weak or absent in the glomeruli and tubules of the cortical sections obtained from NC mice (Figure 4A). In the MN group (Figure 4B), HO-1 expression was centralized in the tubules, but was also weak. In MN-CoPP mice, HO-1 expression was much stronger and was localized not only in the glomeruli but also in the renal tubules (Figure 4C). SnPP treatment attenuated HO-1 expression relative to that in the MN mice (Figure 4D). We also measured serum bilirubin level to directly link the results of serum-TBARS to HO-1 induction. However, no significant differences of serum bilirubin levels in each group were noted. It may be due to that there were multiple factors interfering the production of oxidative stress and the homeostasis of bilirubin in the serum.

Effects of CoPP and SnPP treatments on the production of serum immunoglobulins

To investigate whether the induction of HO-1 modulates the production of the immunoglobulins, causing the subsequent decrease in the immunofluorescence intensity of IgG and

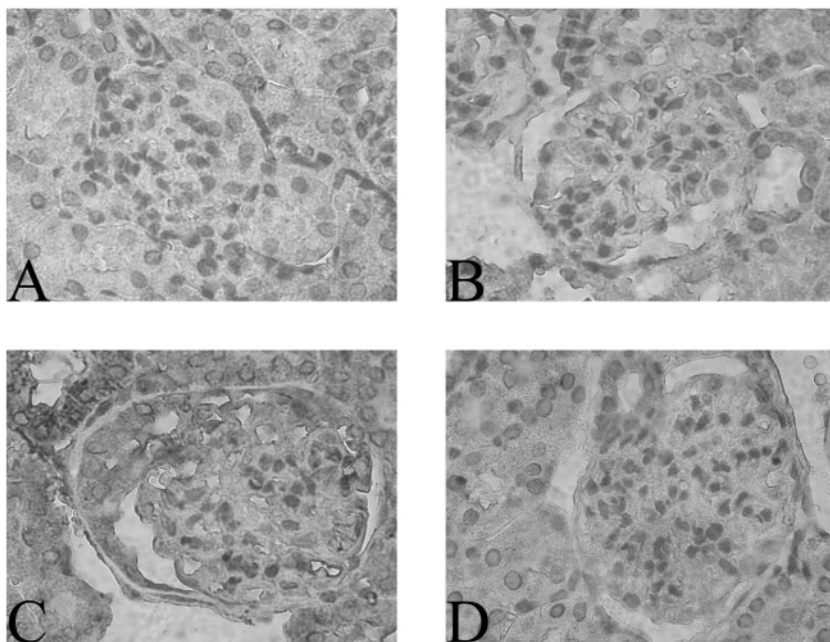


Fig. 4. Immunohistochemistry of HO-1. Kidneys from mice of the normal control group (A), membranous nephropathy group (B), membranous nephropathy group receiving weekly CoPP treatments (C) and membranous nephropathy group receiving SnPP treatments (D) were stained for HO-1. All images are at 400 \times magnification.

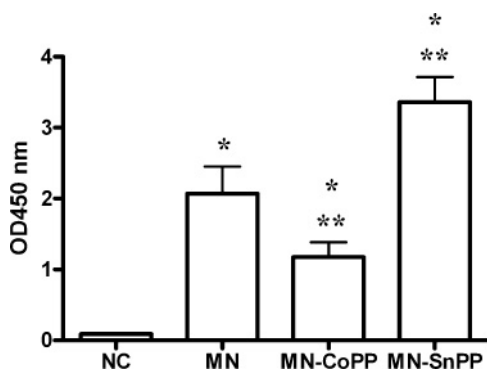


Fig. 5. Effects of HO-1 induction/inhibition treatments on antibody production in mice with experimental MN. Serum levels of total anti-cBSA immunoglobulins in mice from the normal control group, membranous nephropathy group, membranous nephropathy group receiving weekly CoPP treatments and membranous nephropathy group receiving SnPP treatments. Total serum immunoglobulin was evaluated by enzyme-linked immunosorbent assay (ELISA) and the data are expressed as optical densities at 450 nm. * $P < 0.05$ versus the control group. ** $P < 0.05$ versus the MN group.

C3 during the course of experimental MN, we checked the serum levels of anti-cBSA antibodies in mice. Significant elevation of anti-cBSA antibodies was observed in the MN, MN-CoPP and MN-SnPP groups compared with those of the control groups. CoPP inhibited and SnPP enhanced the production of antibodies in the experimental MN mice compared with that of the control MN mice (Figure 5).

Effects of CoPP and SnPP treatments on lipid peroxidation products

Oxidative stress plays an important pathogenic role in MN. We assessed whether CoPP or SnPP treatment modulates the production of oxidative stress in the serum and kidneys. We measured the lipid peroxidation products, thiobarbituric acid reactive substances (TBARS), as markers of oxidative stress. The TBARS levels in the sera of MN mice were significantly increased compared with those of NC mice. CoPP treatment effectively attenuated the levels of TBARS to a level similar to that observed in NC mice. However, SnPP, which inhibits HO-1, did not reduce the level of oxidative stress, as indicated by the higher levels of serum TBARS observed in MN mice treated with SnPP (Figure 6). TBARS in the kidney displayed a similar pattern to that observed in serum. These findings suggest that HO-1 reciprocally affects both local and systemic oxidative stress.

Effects of CoPP and SnPP treatments on ROS production in kidney cells

To more specifically and locally detect the ROS production in the kidney, we analyse *in situ* superoxide anion radical production by using DHE assay in fresh-frozen sections of renal tissue. DHE fluorescence levels were at low levels in normal mouse kidneys (Figure 7A). We obtained a significantly increased DHE fluorescence in MN kidney implicating *in situ* superoxide anion production (Figure 7B). Compared with MN mice, little DHE fluorescence levels

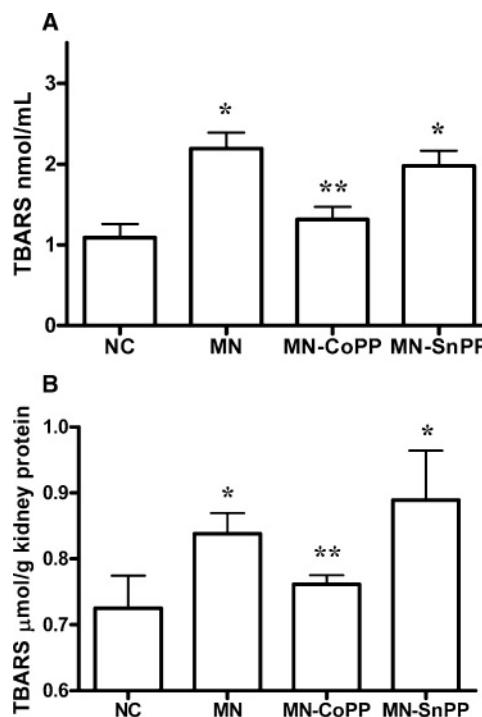


Fig. 6. Effects of HO-1 induction/inhibition treatments on the production of thiobarbituric acid reactive substances (TBARS) in mice with experimental MN. Levels of TBARS in the serum (A) and kidneys (B) were checked in mice from the normal control group, membranous nephropathy group, membranous nephropathy group receiving weekly CoPP treatments and membranous nephropathy group receiving SnPP treatments. * $P < 0.05$ versus the control group. ** $P < 0.05$ versus the MN group.

were observed after MN mice were treated with CoPP (Figure 7C). The administration of SnPP failed to reduce the DHE fluorescence (Figure 7D). Therefore, these results further demonstrated the anti-oxidative effect of HO-1 induction by CoPP.

Effects of CoPP and SnPP treatments on apoptosis in kidney cells

One of the major cytoprotective functions of HO-1 is its anti-apoptosis effect. In this study, we investigated whether HO-1 induction alleviates apoptosis in MN. TUNEL-positive cells, as an index of cell apoptosis, were nearly undetectable in normal mouse kidneys (Figure 8A). Increased numbers of apoptotic cells were detected in the glomeruli and surrounding tubules of MN mice (Figure 8B). Compared with MN mice little apoptosis was observed after MN mice, little apoptosis were treated with CoPP, as indicated by a decrease in the number of TUNEL-positive nuclei (Figure 8C). The administration of SnPP failed to reduce the apoptosis in kidney cells (Figure 8D). Therefore, the anti-apoptotic effect induced by CoPP may contribute to the therapeutic effect of HO-1 in MN mice.

Effects of CoPP and SnPP treatments on cytokine mRNA expression in the kidney

In addition to its anti-oxidative and anti-apoptotic properties, HO-1 also displays well-documented

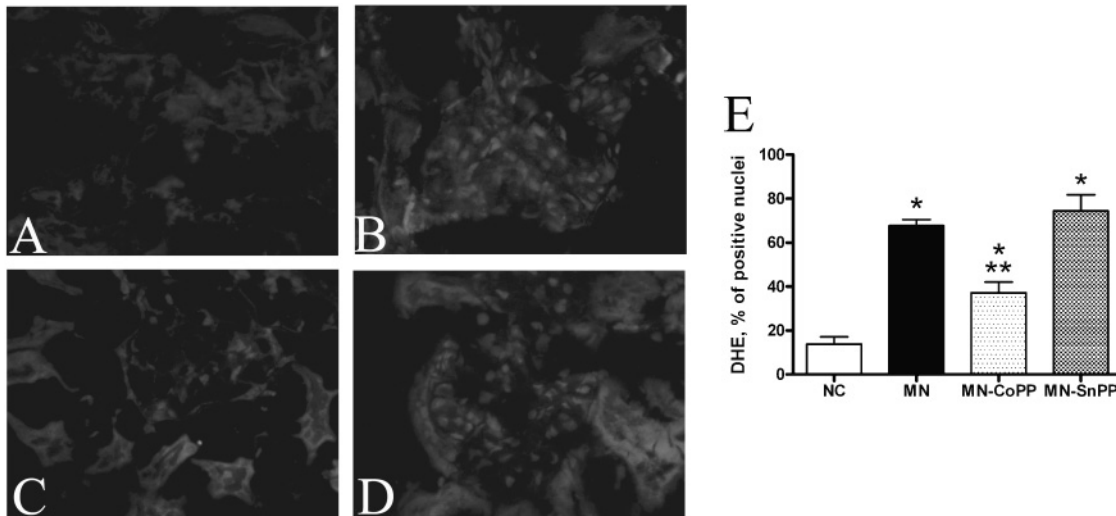


Fig. 7. Superoxide anion production in kidney cell. Fluorescence micrographs of DHE-positive cells in the kidneys of mice from the normal control group (A), membranous nephropathy group (B), membranous nephropathy group receiving weekly CoPP treatments (C), membranous nephropathy group receiving SnPP treatments (D) and quantification (E) are shown. All images are at 400 × magnification. * $P < 0.05$ versus control group. ** $P < 0.05$ versus the MN group.

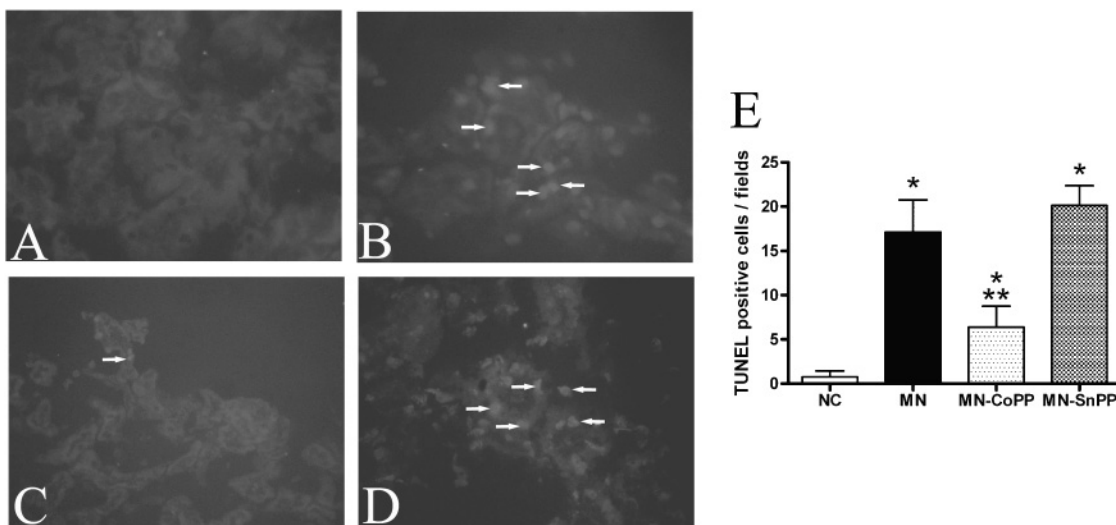


Fig. 8. Kidney cell apoptosis in mice with experimental MN. Fluorescence micrographs of TUNEL-positive cells in the kidneys of mice from the normal control group (A), membranous nephropathy group (B), membranous nephropathy group receiving weekly CoPP treatments (C), membranous nephropathy group receiving SnPP treatments (D) and quantification (E) are shown. All images are at 400× magnification. * $P < 0.05$ versus control group. ** $P < 0.05$ versus the MN group.

anti-inflammatory activity. To address the question of whether the induction of HO-1 modulates the inflammatory state, we examined the mRNA expression of inflammation-associated cytokines in the renal cortex. We checked the expression of pro-inflammatory cytokines (interleukin [IL]-1 β and tumour necrosis factor [TNF]- α), Th1 cytokines (interferon [IFN]- γ and IL-2), Th2 cytokines (IL-4 and IL-10), the fibrogenic cytokine transforming growth factor (TGF) β and HO-1. Quantitative real-time PCR of the renal cortex demonstrated a consistent change in MN, with an increased expression of pro-inflammatory, Th1 cytokines and Th2 cytokines. CoPP treatment dramatically induced HO-1 expression in the kidneys, decreased the level of pro-inflammatory cytokines (IL-1 β and TNF- α)

and extremely increased the level of anti-inflammatory cytokine IL-10. SnPP also decreased the pro-inflammatory cytokines, but did not induce the anti-inflammatory cytokine IL-10. The expression of the fibrogenic cytokine TGF- β was elevated in the MN, MN-CoPP and MN-SnPP groups. The induction/inhibition of HO-1 did not appear to affect the expression of TGF- β (Figure 9).

Discussion

This is the first study to demonstrate that the endogenous induction of HO-1 significantly ameliorates proteinuria and the severity of pathology in MN mice. The induction of

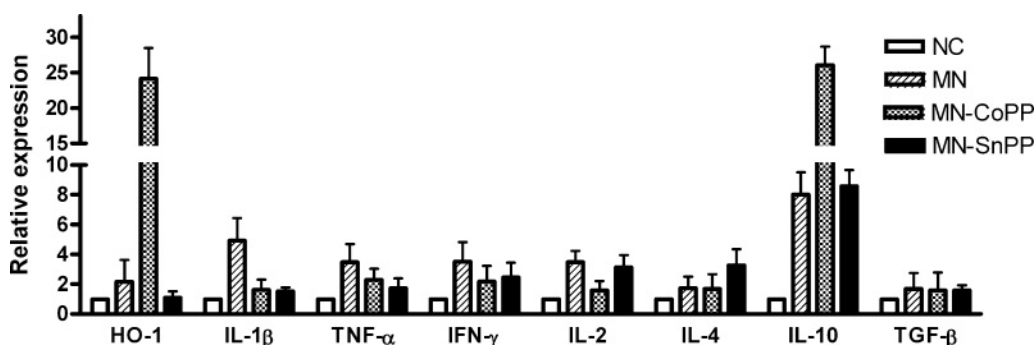


Fig. 9. Cytokine mRNA expression levels. Cytoplasmic RNA was prepared from the kidney cortices of mice with experimental MN, and the levels of cytokine mRNA expression were determined with real-time PCR ($n = 3$).

HO-1 suppressed the production and deposition of immune complexes in the kidney. Both systemic and local oxidative stresses were reduced in the sera and kidneys of CoPP-treated MN mice compared with those of MN mice or SnPP-treated MN mice. Apoptosis in the kidney cells was also reduced after treatment with CoPP. Conversely, decreased pro-inflammatory cytokine expression and increased anti-inflammatory cytokine expression were observed in the kidneys after treatment with CoPP.

The central pathogenesis of MN involves the formation of subepithelial immune deposits and the subsequent production of glomerular injury through complement-dependent processes, oxidants and cytokines, resulting in the development of massive proteinuria [3]. Recently, attention to HO-1 has centred on its biological effects for protective and therapeutic use [8,9]. The HO-1 induced in our study caused the effective attenuation of proteinuria via multiple mechanisms, including immunomodulatory, anti-oxidative and anti-apoptotic effects. CoPP-induced HO-1 suppressed the synthesis of pro-inflammatory cytokines such as IL-1 β , IFN- γ and TNF- α . Furthermore, it stimulated the production of the anti-inflammatory cytokine and IL-10. It has been postulated that the degradation products of haeme and its metabolic derivatives (CO in particular) might contribute to the anti-inflammatory functions of HO-1. The CO-mediated anti-inflammatory effect caused by the increasing production of IL-10 and the inhibition of TNF- α and IL-1 β has been reported to be mediated through interactions with the MAPK signalling pathways [8]. In contrast, IL-10 mediates the immunosuppressive effect by an HO-1-dependent pathway [18]. CoPP treatment also significantly reduced the production of serum anti-cBSA antibodies and glomerular immunodeposits in experimental MN mice. It has been demonstrated that the immunomodulatory effect of HO-1 is associated with regulatory T-cell *Foxp3* expression and T-cell proliferation via IL-2 [19,20]. However, the role of HO-1 in the reduction of immunoglobulin production remained unclear. The direct action of induced HO-1 against systemic inflammation, the concomitant decrease in the production of immunoglobulins, together with the subsequent decrease in immunodeposition, and other factors such as complement activation and inflammation may all contribute to the attenuation of proteinuria.

A previous study demonstrated that oxygen radical scavengers can dramatically reduce proteinuria in Heymann

nephritis [21]. In our study, MN mice treated with CoPP showed a dramatic reduction in the generation of highly reactive compounds of lipid peroxidation products, TBARS and in proteinuria. These results indicate that oxidative stress may play a pathogenic role in damaging the glomerular filtration barrier, and could be causally related to proteinuria in experimental MN [3]. The decrease in TUNEL-positive kidney cells after CoPP treatment observed in our study also demonstrates the anti-apoptotic properties of HO-1 [7,8]. The metabolic derivatives of haeme produced by HO-1 (CO and biliverdin) have powerful anti-apoptotic and anti-oxidative properties. Whether the cytoprotective capacity of HO-1 in experimental MN is exerted via these byproducts requires further investigation.

The protective role of HO-1 has been shown in several diseases, such as ischaemic renal injury, cisplatin-induced nephrotoxicity, acute glomerulonephritis and the rejection of renal transplantation in an animal model [7]. Anti-glomerular basement membrane glomerulonephritis and lupus nephritis in mice have also been ameliorated by HO-1 induction therapy [22,23]. However, studies involving the application of induced HO-1 to different disease models have produced diverse outcomes. Most studies demonstrated the protective effects of HO-1 induced by CoPP. However, a study of the progression of murine collagen-induced arthritis revealed that SnPP more effectively reduced the disease severity and extent than did CoPP because it attenuated of TNF- α and IL-1 β to a greater extent [24]. In our study, both CoPP and SnPP decreased the pro-inflammatory cytokines, but only CoPP increased the production of the anti-inflammatory cytokine IL-10. Apart from the regulation of pro-inflammatory and anti-inflammatory cytokines, CoPP also decreased the level of immunoglobulin production, complement activation and oxidative stress, which have been proposed to be major pathogenic factors in MN. This may partially explain why only CoPP, and not SnPP, exerted a therapeutic effect in alleviating experimental MN in our study. In contrast, the deficiency of HO-1 impairs renal haemodynamics and exaggerates systemic inflammatory response in mice [25,26].

In our experiments, HO-1 was highly expressed during the induction of MN, but the cellular origin of this HO-1 is still unclear. Infiltrating macrophages have been identified as the major source of HO-1 in acute renal transplant rejection [11]. However, there was no prominent infiltration

of inflammatory cells in our murine MN model. Therefore, we speculate that the resident kidney cells contribute to the induction of HO-1. Renal vascular and tubular structures express HO-1, particularly in response to injurious conditions [10]. Glomeruli have also been reported to express HO-1 in the human kidney in various renal diseases [27]. It seems that site-specific expression along the nephron or in the interstitium occurs due to the proximity of the stimulus. In our study, it was difficult to clarify whether the protective effect of HO-1 was mediated through systemic or local action because both effects coexisted when the mice received CoPP treatment. Previous studies using either the exogenous administration of HO-1 by gene transfer specifically expressed in the kidney or chemical induction or transgenic mice, all demonstrated the therapeutic effects of HO-1 in different disease models [9,11]. Therefore, we assume that both the systemic immunomodulatory effect, which decreases the production of immunoglobulins and subsequently or directly reduces inflammation, complement activation, oxidative stress and apoptosis, and the local effect in the MN glomeruli contribute to the therapeutic effect of HO-1.

The development of ideal therapeutic agents that can effectively and specifically blunt the pathogenic pathway in MN is an important issue. The efficient administration of CoPP once weekly, which can significantly block the key inflammatory, oxidative and immunomodulatory pathogenesis pathways of MN, make HO-1-inducing therapeutic regimens a plausible new option for future therapeutic interventions in MN. However, there are still no drugs available that have been specifically developed to induce HO-1 and can be applied to human clinical use. Although the therapeutic effects of HO-1 have been demonstrated in several diseases in animal models, including our experimental MN model, whether it can be applied to humans requires further investigation.

Our results suggest that HO-1 induction therapy ameliorates experimental MN via multiple pathways, including anti-oxidative, anti-apoptotic and immunomodulatory effects. HO-1-inducing regimens will probably be considered a new therapeutic intervention for MN in the future.

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