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Heme oxygenase is induced in nephrotoxic nephritis and hemin, a stimulator of heme oxygenase synthesis, ameliorates disease

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Heme oxygenase is induced in nephrotoxic nephritis and hemin, a stimulator of heme oxygenase synthesis, ameliorates disease. Heme oxygenase (HO) catalyses degradation of heme to biliverdin, iron and carbon monoxide (CO). Two isoforms exist, a constitutive form and an inducible form (HO-1). Induction of HO-1 may have protective effects in inflammation. We studied heterologous (HNTN) and accelerated (ANTN) nephrotoxic nephritis in Lewis rats. Hemin, an inducer of HO-1, (30 μmol/kg) was administered 18 hours before induction of nephritis and 72 hours later in ANTN. HO-1 was not detected immunohistochemically in normal glomeruli but was present in HNTN and ANTN in cells with the morphology of macrophages. HO-1 induction was confirmed by RT-PCR. In normal rats hemin induced glomerular HO-1 mRNA at 18 hours. In HNTN hemin markedly reduced proteinuria at 24 hours (10 $\pm$ 4 mg/24 hr; control 54 $\pm$ 16; $P < 0.05$), neutrophil infiltration at two hours (29.8 $\pm$ 1.8 vs. 22.3 $\pm$ 1.5 neutrophils/glomerulus, $P < 0.05$), and glomerular macrophage number at two hours (2.1 $\pm$ 0.1 vs. 3.1 $\pm$ 0.4 cells/glomerulus, $P < 0.05$). In ANTN proteinuria was reduced at day 1 and day 4 (36 $\pm$ 11 vs. 60 $\pm$ 15 and 35 $\pm$ 7 vs. 86 $\pm$ 9 mg protein/24 hr, respectively, $P < 0.001$), glomerular thrombi were reduced by hemin at day 1 and 4 (1.5 $\pm$ 2.7 vs. 2.7 $\pm$ 0.2 and 1.3 $\pm$ 0.01 vs. 2.9 $\pm$ 0.02, respectively, $P < 0.001$) and glomerular macrophage infiltration was reduced on day 4 (11.2 $\pm$ 0.8 cells/glom; control 15.9 $\pm$ 0.8, $P < 0.01$). Possible mechanisms by which HO-1 ameliorates disease include anti-complement or anti-oxidant effects of bilirubin and vasodilator and anti-platelet effects of carbon monoxide.

Heme oxygenase (HO) is the rate limiting enzyme in heme catabolism catalyzing the oxidative cleavage of heme molecules to biliverdin, carbon monoxide and iron (Fig. 1) [1–3]. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. There are two isoforms of HO that are products of separate genes: HO-2 is a constitutive form which is found in tissues such as the brain and testis; HO-1 is an inducible form of the enzyme and its induction is important in the response of tissues to oxidative stress and inflammation. Endotoxin, interleukin 1, heat shock and other stress agents lead to a rapid activation of the HO-1 gene [2], and the promoter for rat HO-1 has been shown to contain a heat shock consensus element [4], and NF-κB [5] and AP-1 [6] binding sites consistent with its induction by inflammatory stimuli. Heme oxygenase-1 is also induced by its substrate hemin (ferriprotoporphyrin IX chloride) [1].

Heme oxygenase-1 has a number of potential protective effects in inflammation [2]. Firstly, bilirubin has both antioxidant [7] and anticomplement [8] effects. Secondly, carbon monoxide, in a fashion similar to nitric oxide, may up-regulate cGMP [9] and lead to vasodilation and inhibition of platelet aggregation. Thirdly, degradation of heme by HO leads to the induction of ferritin synthesis, which may then sequester iron and prevent it from participating in subsequent oxidative injury [10]. *In vitro*, gene transfection leading to increased synthesis of HO-1 has been shown to protect pulmonary epithelial cells against oxidant stress [11] and endothelial cells against hemoglobin toxicity [12]. *In vivo*, increased HO-1 activity was shown in a model of acute inflammation in the rat induced by injection of carageenin into the peritoneal cavity [13], and inflammation was reduced by stimulating HO-1 synthesis with hemin before induction of the disease. Heme oxygenase-1 was predominantly found in macrophages.

Glomerular mesangial cells show rapid induction of HO-1 mRNA when stimulated with phorbol myristate acetate or heat shock [14]. Interleukin-1β (IL-1β) leads to a slower induction of HO-1 and this effect is potentiated by the cyclooxygenase inhibitor indomethacin. Therefore, in view of the potential for HO-1 synthesis by both mesangial cells and macrophages, its antioxidant effects and the importance of oxidative free radical damage in glomerular inflammation [15], we have examined the synthesis of HO-1 in nephrotoxic nephritis and studied the effect of administering hemin, an inducer of HO-1 synthesis.

METHODS

Rats

Inbred male Lewis rats, from St Mary's Hospital Medical School, weighing between 250 and 350 g were used.

Glomerulonephritis

Accelerated nephrotoxic nephritis (ANTN) [16, 17] was induced by intraperitoneal immunization of rats with 1 mg rabbit IgG (Sigma chemicals Ltd, Poole, UK) in Freund's complete adjuvant (Sigma) one week prior to an intravenous, subnephritogenic, dose of rabbit anti-rat nephrotoxic globulin (NTG) 3 mg IgG/100 g body wt. Heterologous nephrotoxic nephritis was induced by a single intravenous nephritogenic dose of rabbit anti-rat NTG (6 mg/100 g body wt IgG).

Key words: heme oxygenase, inflammation, nephrotoxic nephritis, proteinuria, oxidative stress.

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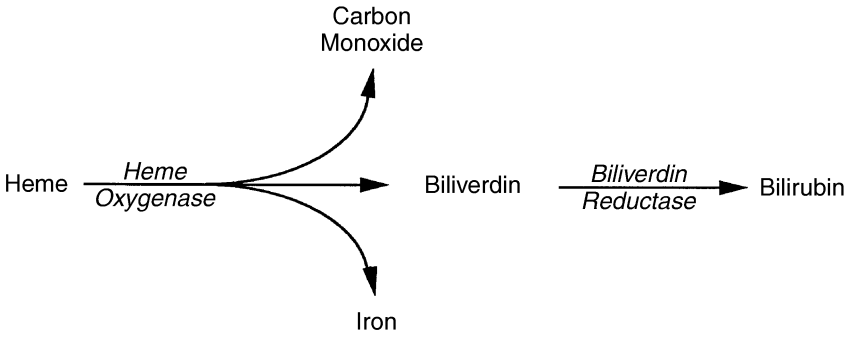

Fig. 1. Pathway of heme degradation by heme oxygenase.

Hemin administration

To investigate the effect of HO-1 induction on these models of nephritis, hemin (30 μmol/kg) (ferriprotoporphyrin IX chloride; Porphyrin products Inc., Logan, UT, USA) a heme oxygenase inducer, or saline vehicle, was administered subcutaneously 24 hours before NTG in both models, and again at day 3 in the accelerated model. In the accelerated model urine was collected from rats placed in metabolic cages at days 0 to 1 and 3 to 4 after NTG, and the proteinuria determined by the sulphosalicylic acid method. A renal biopsy was taken at day 1, and animals killed at day 4. In the heterologous model urine was collected as before for 24 hours; animals were killed at either two hours or 24 hours.

Hemin was also given to normal rats to study induction of HO-1 after 24 hours.

Histology and immunohistochemistry

Kidneys or renal biopsies were fixed in formal saline and paraffin embedded. Sections were stained with hematoxylin and eosin for neutrophil counting and thrombus detection. Thrombi were scored as the mean number of glomerular quadrants affected.

For HO-1 immunohistochemistry, sections were dewaxed, microwaved [18], blocked for one hour in 20% normal swine serum in phosphate buffered saline (NSS/PBS) and incubated overnight with rabbit anti-HO-1 (Stressgen Corp., Victoria, Canada) diluted 1/5000 in NSS/PBS. Endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol for 20 minutes, and the sections then incubated with biotinylated swine anti-rabbit IgG (Dako Ltd., Bucks) at 1/1000 in NSS/PBS for 30 minutes. StreptABComplex-HRP (Dako) was prepared following the manufacturer's instructions and incubated for 30 minutes. 3,3′-Diaminobenzidine-4HCl was used as the chromagen. Controls consisted of either normal rabbit serum in place of the primary antibody, or rabbit anti-rat HO-1 that had been pre-absorbed with HO-1 (10 μg HO-1 protein/ml antibody for 1 hr at 37°C). Macrophages were detected on microwaved sections using ED1 antibody (Serotec Ltd., Oxon, UK) at 1:500.

Reverse transcription-polymerase chain reaction

Glomeruli were isolated by sieving as previously described [17]. In order to preserve the integrity of the glomerular RNA, kidneys were perfused and sieved using diethyl pyrocarbonate (DEPC) treated PBS, sieves were baked at 280°C for four hours, and RNAse free plasticware was used at all stages.

RNA was isolated from glomeruli using RNAzol B (Biogenesis) following the manufacturer's instructions. RNA was quantified and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Paisley, UK), and the resulting cDNA amplified by polymerase chain reaction (PCR). The HO-1 primers were as described by Paschen et al [19] and were purchased from Oswel DNA services. The sense primer was: 5′-TGGAAGAGGAGATAGAGCGA-3′, and the antisense 5′-TGTTGAGCAGGAAGGCGGTC-3′. The amplification product length was 451 bp. The identity of the PCR product was confirmed by digestion with *Nco*I which yielded the expected products of lengths 280 and 171 bp. The conditions for PCR were a hot start of 94°C five minutes, followed by cycling of one minute 60°C, one minute 72°C and one minute 94°C for 30 cycles. The reaction was finished with two minutes at 60°C and 10 minutes at 72°C. Polymerase chain reaction products were analyzed on a 2% agarose gel and visualized with ethidium bromide and ultraviolet transillumination. In order to allow a semiquantitative estimate of mRNA for comparison between samples, each PCR run was carried out with three dilutions of template cDNA.

Statistics

Data are presented as mean ± SEM. Student's unpaired *t*-test was used for comparison between groups.

RESULTS

Heme oxygenase-1 expression in control animals

Control animals (non-immunized and those immunized with rabbit IgG in Freund's adjuvant 7 days previously) were examined by immunohistochemistry for the expression of HO-1. In neither case was staining observed in the glomeruli, tubules or vessels (Fig. 4A). Reverse transcription (RT)-PCR of glomeruli showed no HO-1 mRNA (Fig. 2A).

Induction of heme oxygenase-1 by hemin

Hemin was given subcutaneously to normal rats to examine the induction of HO-1 in the kidney. Reverse transcription (RT)-PCR of the glomeruli demonstrated that HO-1 mRNA was induced 18 hours after hemin administration (Fig. 2B).

Heme oxygenase-1 expression in nephrotoxic nephritis

Heme oxygenase-1 mRNA and protein expression was examined in both accelerated and heterologous NTN. Heme oxygenase-1 mRNA was detected by RT-PCR at days 1, 2, 4, 6 and 7 in ANTN and at two hours in HNTN (Fig. 3). By immunohistochemistry, HO-1 positive cells were observed in the glomeruli of ANTN rats at day 1 and day 4 (2.5 ± 0.4 and 3.8 ± 0.5 cells/glomerulus,

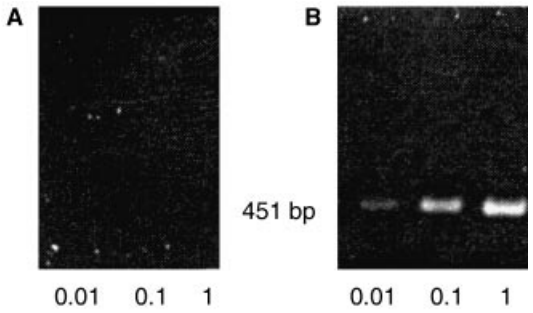

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) for heme oxygenase-1 (HO-1) in rat glomeruli from (*A*) control animals and (*B*) normal animals 24 hours after hemin administration. In each case 1, 0.1 or 0.01 μg of cDNA were used for PCR.

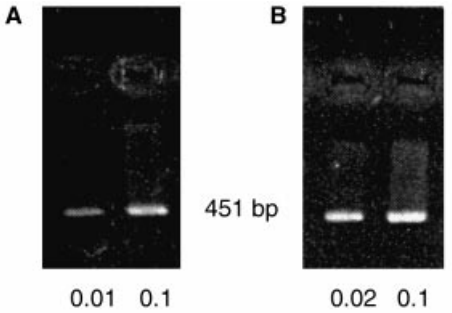

Fig. 3. Reverse transcription-polymerase chain reaction (RT-PCR) for heme oxygenase-1 (HO-1) in rat glomeruli from a representative animal with (*A*) accelerated nephrotoxic nephritis (ANTN) at one day, and (*B*) heterologous nephrotoxic nephritis (HNTN) at two hours. The numbers beneath the lanes show μg of cDNA used for PCR.

respectively), with no significant staining of the tubules at either time point (Fig. 4B). No vascular staining was seen. The staining in glomerular cells appeared to be mainly in mononuclear cells, many of which were intraluminal. These cells appeared identical to those stained by the macrophage marker ED1.

In HNTN there was faint diffuse tubular staining after two hours, but no detectable HO-1 in the glomeruli. After 24 hours strong tubular staining was observed, and 0.6 ± 0.2 HO-1 positive cells were seen per glomerulus (Fig. 4C). No vascular staining was seen. In neither model was staining seen using rabbit IgG in the place of the anti-HO-1 antibody, and staining was completely abolished by pre-absorption of the primary antibody with HO-1 peptide.

Effect of hemin, a heme oxygenase-1 inducer, on nephrotoxic nephritis

Accelerated nephrotoxic nephritis. Preimmunized rats given NTG developed a proliferative glomerulonephritis with proteinuria. At 24 hours the glomerular infiltrate was predominately macrophages, and macrophage infiltration persisted to day 4. Hemin caused a significant reduction in proteinuria of 39.5% at day 0 to 1, and 59% at day 3 to 4 (36 ± 11 vs. 60 ± 15 and 35 ± 7 vs. 86 ± 9 mg protein/24 hr, $P = 0.001$, respectively; Fig. 5A). Protein excretion in normal rats was 7.7 ± 1.8 mg/24 hours ($N = 8$), and in normal rats given hemin was 9.8 ± 1.7 mg/24 hours ($N = 3$). Glomerular thrombosis was also significantly reduced by hemin at days 1 and 4 (1.5 ± 0.1 vs. 2.7 ± 0.2. and 1.3 ± 0.01 vs. 2.9 ± 0.02, respectively, $P < 0.001$; Fig. 5B). Animals receiving hemin showed reduction of glomerular macrophage numbers of 30% at day 4 (11.2 ± 0.8 vs. 15.9 ± 0.8, $P = 0.003$). No change was observed at d1 (13.6 ± 1.4 vs. 13.9 ± 2.7) (Fig. 5C). Reverse transcription-PCR using HO-1 primers demonstrated the expected 451 bp product, with no difference between treated and untreated animals.

Heterologous nephrotoxic nephritis. Hemin administration significantly reduced proteinuria during the first 24 hours by 82% (10 ± 4 vs. 54 ± 16 mg protein/24 hr, $P = 0.03$; Fig. 6A). At two hours, glomerular neutrophil infitration was reduced by hemin administration from 29.8 ± 1.8 to 22.3 ± 1.5 neutrophils/glomerulus ($P < 0.05$; Fig. 6C). Very few neutrophils were observed at 24 hours. Macrophage infiltration (ED-1 positive cells) was reduced by hemin at two hours but not 24 hours (2.1 ± 0.1 vs. 3.1 ± 0.4, $P <$ 0.05, and 8.2 ± 0.6 vs. 8.2 ± 0.9 cells/glomerulus, respectively; Fig. 6B). Heme oxygenase-1 mRNA was detected in glomeruli at two hours but no difference was observed between the two groups. The strong tubular staining seen at 24 hours in HNTN was markedly reduced in animals pretreated with hemin.

DISCUSSION

In this study we show that the inducible form of heme oxygenase, HO-1, is induced in glomeruli during proliferative glomerulonephritis and that administration of hemin, which augments HO-1 synthesis, leads to amelioration of disease. Heme oxygenase-1 was not detectable in normal glomeruli by RT-PCR or immunohistochemistry but was rapidly induced in glomerulonephritis. By RT-PCR, HO-1 mRNA was detected by two hours in HNTN and in ANTN was increased up to seven days. Immunohistochemical staining suggested that macrophages were the major site of glomerular HO-1 synthesis. Although definite staining of intrinsic glomerular cells was not seen, glomerular mesangial cells *in vitro* have been shown to synthesize HO-1 in response to stimulation by phorbol esters, heat shock and IL-1β [14], and we cannot exclude a contribution to glomerular HO-1 from these cells. It may be that our immunohistochemical method is not sensitive enough to detect low levels of expression by intrinsic glomerular cells. This possibility is supported by the fact that hemin administration to normal animals led to glomerular HO-1 mRNA synthesis at 24 hours, although we were unable to detect HO-1 immunohistochemically. In other inflammatory models macrophages have been shown to be a major source of HO-1. In acute pleurisy induced by injection of carageenin into the pleural cavities of rats [13], there was an initial influx of neutrophils followed by increasing numbers of macrophages. Heme oxygenase activity increased as the inflammation progressed and by 48 hours 89% of infiltrating macrophages stained positively for HO-1. The HO-1 activity in macrophages from the pleural cavity was eight times that in peripheral blood monocytes. In a model of renal transplant rejection in the rat, marked induction of HO-1 was found at day 5 and immunohistochemistry showed that the major source was infiltrating macrophages [20]. There are many possible mediators that may induce HO-1 in monocytes that infiltrate the glomeruli in glomerulonephritis including reactive oxygen species [21], IL-1 and tumor necrosis factor [22], and nitric oxide [23, 24].

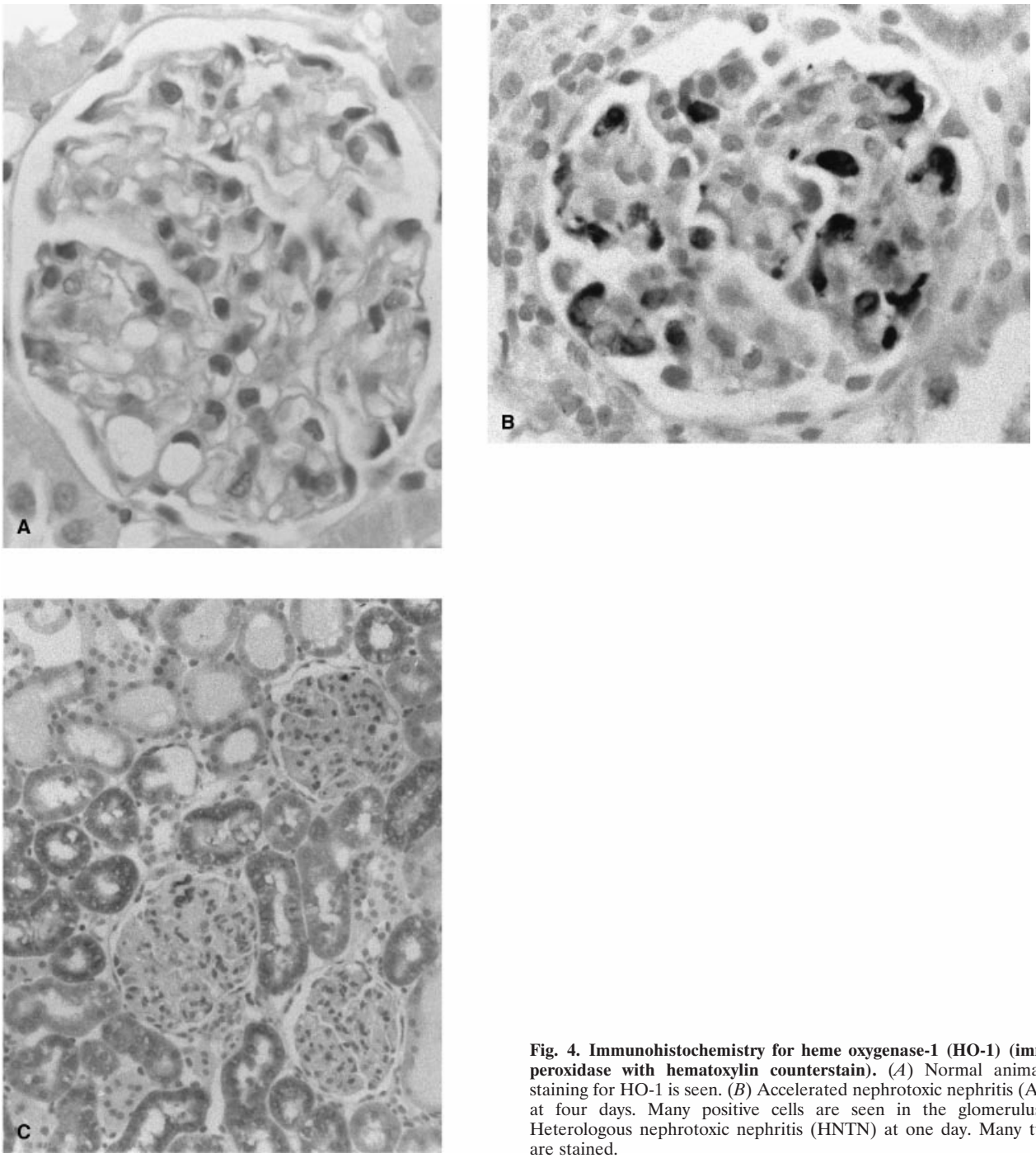

Fig. 4. Immunohistochemistry for heme oxygenase-1 (HO-1) (immunoperoxidase with hematoxylin counterstain). (*A*) Normal animal. No staining for HO-1 is seen. (*B*) Accelerated nephrotoxic nephritis (ANTN) at four days. Many positive cells are seen in the glomerulus. (*C*) Heterologous nephrotoxic nephritis (HNTN) at one day. Many tubules are stained.

In addition, phagocytosis induces HO-1 in mononuclear phagocytes [25]. All of these stimuli are potential inducing agents in the inflamed glomerulus.

This is the first demonstration of the induction of HO-1 in glomeruli, although induction has been shown in the renal tubules of rats with HNTN [26]. Heme oxygenase-1 was demonstrated immunohistochemically in tubular cells at 6 and 24 hours after injection of nephrotoxic globulin, and our results confirm that there is HO-1 staining in tubules at 24 hours in HNTN. However, in this previous study, no HO-1 staining was seen in glomeruli. The reason for this diffence is not clear, but comparison with our model is difficult since there was no description of glomerular histology or of levels of proteinuria. The induction of tubular HO-1 was associated with resistance to glycerol-induced acute renal failure as judged by serum creatinine and histological damage.

There is evidence that HO-1 is protective against oxidative injury, and therefore we hypothesized that its induction in the glomerulus would have an anti-inflammatory effect. To test this we examined the effect of stimulating its synthesis prior to

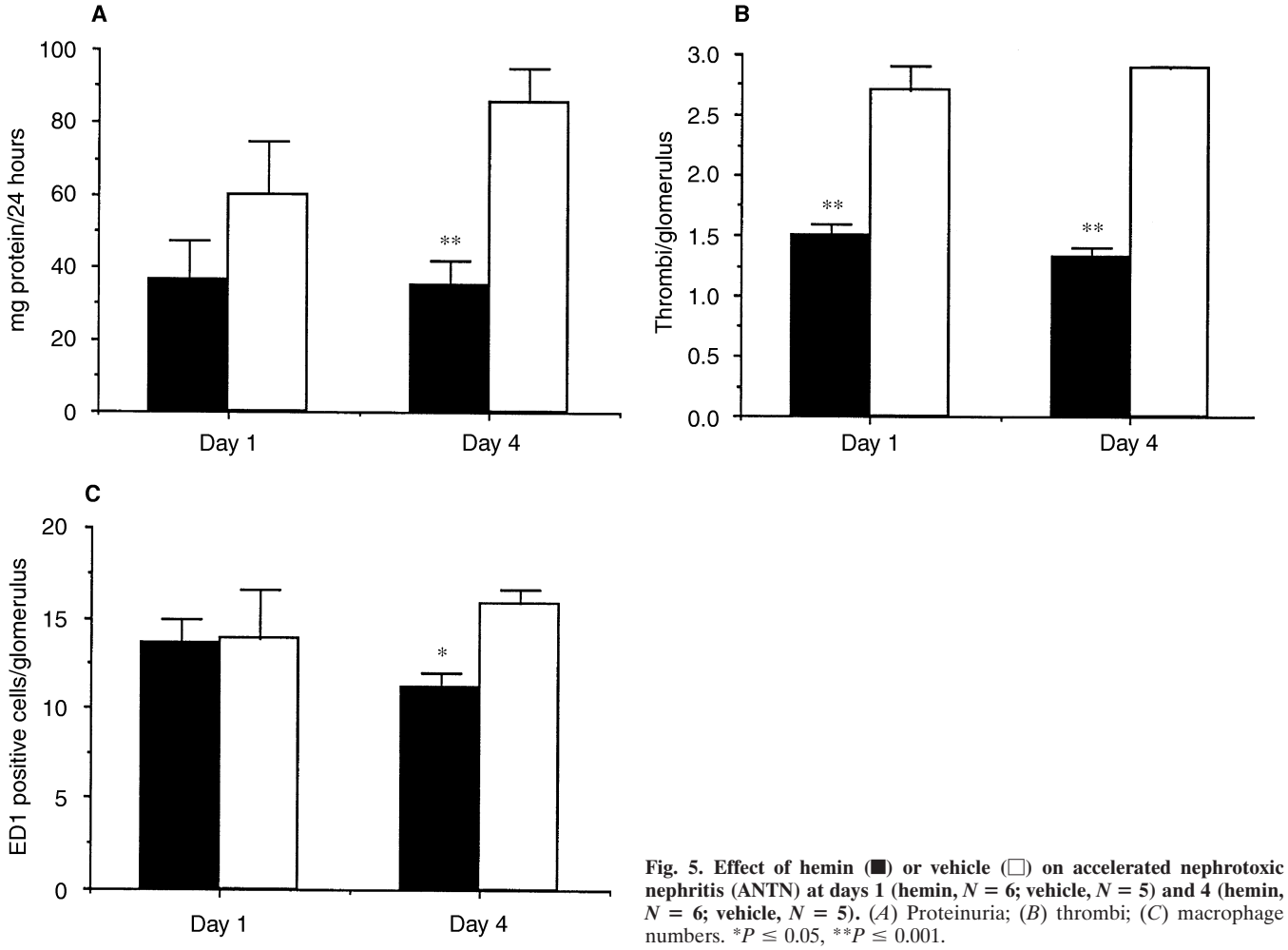

Fig. 5. Effect of hemin (■) or vehicle (□) on accelerated nephrotoxic nephritis (ANTN) at days 1 (hemin, $N = 6$; vehicle, $N = 5$) and 4 (hemin, $N = 6$; vehicle, $N = 5$). (*A*) Proteinuria; (*B*) thrombi; (*C*) macrophage numbers. $^{*}P \leq 0.05$, $^{**}P \leq 0.001$.

induction of glomerulonephritis by administering the natural substrate hemin, which is a well characterized and potent inducer of HO-1. It has been previously shown that hemin administration reduces inflammation in carageenin-induced pleurisy in the rat as measured by inflammatory cell infiltration and exudate volume [13]. In normal rats hemin led to induction of glomerular mRNA for HO-1 24 hours later, without any effect on glomerular histology or urinary protein excretion. In HNTN, prior administration of hemin almost abolished the proteinuria in the first 24 hours, and led to a small but significant reduction in neutrophil and macrophage numbers at two hours. In ANTN hemin also reduced proteinuria, glomerular thrombus formation at 24 hours and macrophage infiltration at four days. However, we recognize that hemin may have other actions and that we are not able to be certain that its benefical effect is through induction of HO-1.

There are several possible reasons why HO-1 induction might have beneficial effects and they may operate together. The proteinuria and neutrophil infiltration in HNTN are dependent on the activation of complement by bound antibody. Biliverdin is an inhibitor of the complement cascade at the level of C1 activation in the classical pathway [27]. The slight reduction we found in infiltrating neutrophils at two hours in HNTN is consistent with a reduction in the generation of chemotactic factors of complement. Reactive oxygen metabolites have been postulated as a major mediators of neutrophil-dependent damage to the glomerular filtration barrier [28, 29]. Induction of HO-1 by hemin *in vitro* has been shown to protect renal epithelial cells against the cytotoxic effect of H_2O_2. There are at least two ways in which HO-1 may protect against damaging effects of reactive oxygen metabolites. Firstly, bilirubin is a potent antioxidant scavenging peroxyl radicals [7] and preventing lipid peroxidation [30] and albumin oxidation [31]. Secondly, synthesis of ferritin, the major iron storage protein is often induced in tandem with heme oxygenase, most probably in response to the free iron [32, 33]. Ferritin is able to sequester large amounts of free iron and may therefore prevent the synthesis of the damaging hydroxyl radical from superoxide and hydrogen peroxide, a reaction which is catalysed by iron [34]. Balla et al [35] showed that endothelial cells respond to heme with induction of HO and ferritin and become insensitive to oxidant damage, an effect that can also be achieved by addition of apoferritin. The relevance of this mechanism to glomerular inflammation is shown by a study in which chelation of iron by administration of desferrioxamine prevented the development of proteinuria in a complement and neutrophil dependent model of heterologous NTN in rabbits [36].

Proteinuria in NTN may depend not only on damage to the capillary wall but also on hemodynamic factors, and in particular the intracapillary pressure that may be affected by afferent and

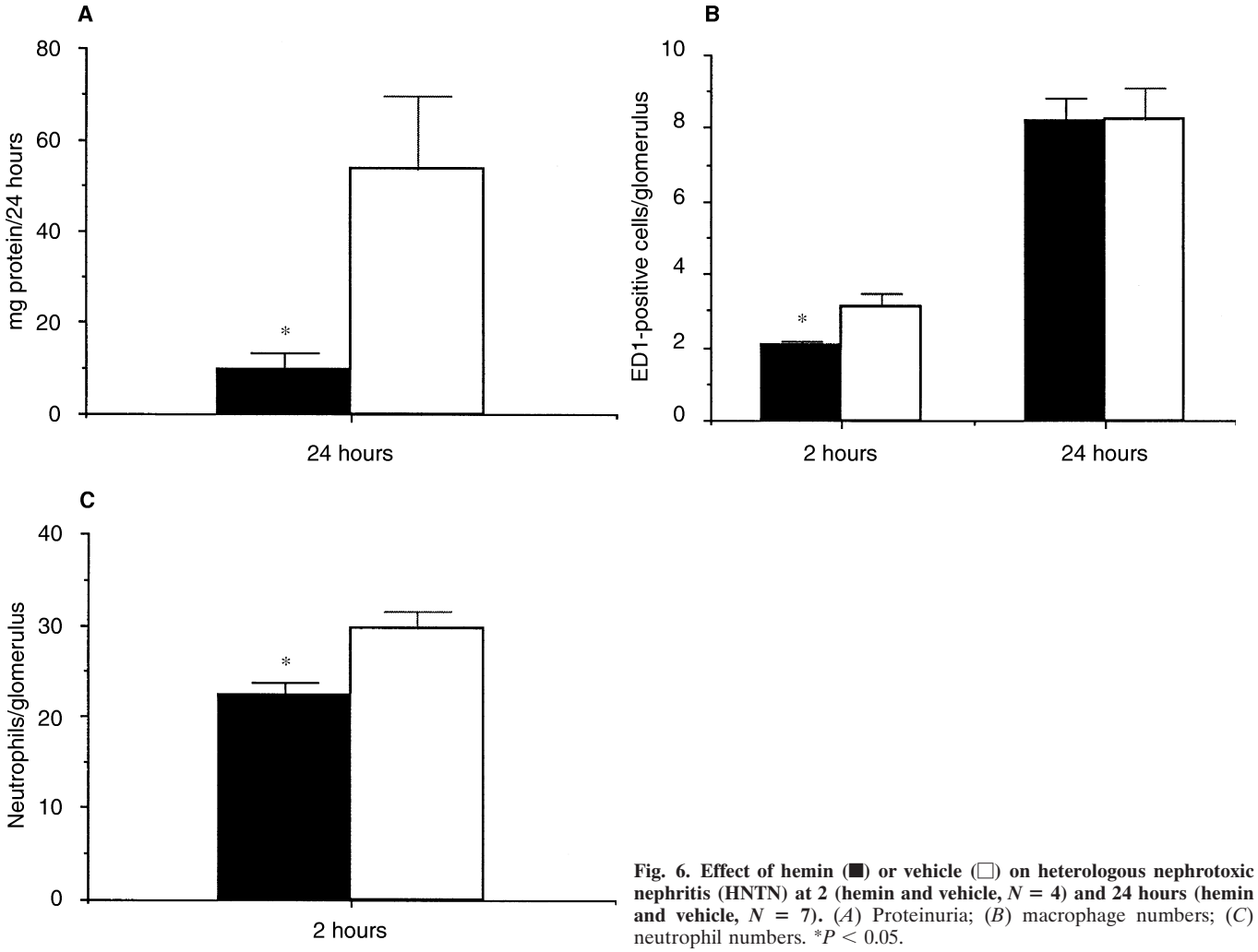

Fig. 6. Effect of hemin (■) or vehicle (□) on heterologous nephrotoxic nephritis (HNTN) at 2 (hemin and vehicle, $N = 4$) and 24 hours (hemin and vehicle, $N = 7$). (*A*) Proteinuria; (*B*) macrophage numbers; (*C*) neutrophil numbers. $^*P < 0.05$.

efferent arteriolar constriction. Elevation of transcapillary hydraulic pressure exacerbates proteinuria in glomerular injury [37, 38]. Carbon monoxide, a product of heme oxygenase, increases smooth muscle cell cGMP and may therefore act to produce vasodilation, reduced intracapillary pressure and reduced proteinuria. The activation of platelet cGMP inhibits platelet aggregation and platelet adhesion to collagen, and therefore carbon monoxide may be expected to have an antithrombotic effect analogous to that of nitric oxide [39]. This may explain the reduction in glomerular thrombosis that we found in the accelerated model of NTN.

We found increased staining for HO-1 at 24 hours in the tubules of rats with HNTN as described previously [26]. In rats treated with hemin this staining was reduced and the most plausible explanation for this is that the induction of HO-1 in the tubules is a secondary response to glomerular inflammation. Possible inducers are pro-inflammatory cytokines such as tumor necrosis factor (TNF) produced in the glomerulus and taken up by the tubules from the urinary space, or the leakage of other plasma proteins such as ferritin into the urine [26]. Whatever the explanation, our study shows that amelioration of the glomerulonephritis and proteinuria by hemin reduces the tubular induction of HO-1.

In conclusion, this is the first demonstration that the inducible form of heme oxygenase, a protein implicated in the protection of tissues against oxidative stress, is up-regulated in glomeruli during glomerular inflammation. A major source of the increased HO-1 appears to be infitrating macrophages. Administration of hemin leads to a reduction in glomerular inflammation as assessed by proteinuria, cellular infiltration and, in the more severe active form of NTN, a reduction in glomerular thrombosis and we hypothesise that this effect of hemin is via HO-1 synthesis. The mechanism of the protective effects of HO-1 induction may include anti-oxidant and anti-complement effects of bilirubin, stimulation of ferritin synthesis by iron and antithrombotic and vasodilatory effects of carbon monoxide.

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