Interactions between inducible nitric oxide synthase and heme oxygenase-1 in glomerulonephritis

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Background. In anti-glomerular basement membrane (GBM) nephritis, inducible nitric oxide synthase (iNOS) and heme oxygenase (HO-1) are co-induced. Moreover, in glomerular mesangial cells iNOS-derived nitric oxide (NO) production stimulates HO-1 while HO-1 activation reduces iNOS expression/activity. These observations prompted us to explore regulatory interactions between iNOS and HO-1 in anti-GBM nephritis.

Methods. Rats with anti-GBM nephritis were pretreated with the iNOS inhibitor L-N6-(1-iminoethyl) lysine (L-NIL) or with the HO-1 inducer hemin. Glomerular HO-1 levels were assessed by Western blot analysis. iNOS activity was assessed by calculating conversion of L-arginine to L-citrulline.

Results. iNOS inhibition reduced glomerular HO-1 levels without altering the inflammatory response to anti-GBM antibody induced injury. Induction of HO-1 reduced glomerular iNOS activity.

Conclusions. In anti-GBM nephritis iNOS up-regulates HO-1 presumably via high output NO production. Suprainduction of HO-1 attenuates iNOS activity. This negative feedback interaction points to HO-1 as a target for pharmacologic manipulation to reduce activity of prooxidant heme containing enzymes such as iNOS.

In experimental glomerulonephritis, as exemplified in the model of anti-glomerular basement membrane (anti-GBM) antibody induced nephritis, there is enhanced expression and enzyme activity of the inducible nitric oxide synthase (iNOS) and of heme oxygenase-1 (HO-1) in glomeruli [1, 2]. iNOS activation results in a sustained high output production of nitric oxide (NO), which can cause oxidative injury either on its own or by interacting with the superoxide anion to form the relatively stable prooxidant peroxynitrite (ONOO⁻) [3]. HO-1 is the inducible isoform of heme oxygenase. It catalyzes the NADPH, O₂, and cytochrome P450 reductase-dependent oxidation of heme to carbon monoxide, iron and biliverdin, which is reduced to bilirubin by biliverdin reductase [4]. Heme is a key constituent of a number of enzymes that are activated in inflammation. Such enzymes include iNOS, cyclooxygenase and cytochrome P450 monoxygenases. Degradation of heme by HO-1 can, therefore, limit heme availability for synthesis or optimal activity of these enzymes. This may reduce synthesis of proinflammatory mediators such as prostaglandins and thromboxane from activation of cyclooxygenase and arachidonate epoxides from activation of cytochrome P450 monoxygenases, and attenuate formation of prooxidant byproducts, that is, NO derived from iNOS activation and reactive oxygen metabolites derived from activation of cytochrome P450 monoxygenases during NADPH-dependent electron transfer. An overall anti-inflammatory/antioxidant effect can thus be expected as a result of HO-1 activation. Supporting this hypothesis, it was shown that HO-1 induction protects from chemically induced inflammation in the pleural cavity [5], and protects renal tubules from myoglobin or hemoglobin induced injury [6]. It was further shown that HO-1 induction prolongs survival of cardiac xenografts in mice and protects against chronic rejection [7, 8]. Finally, two independent studies demonstrated that HO-1 induction attenuates proteinuria in the rat model of anti-GBM nephritis [2, 9]. The mechanism of enhanced HO-1 expression in glomerulonephritis is unknown. In previous studies we reported that NO derived from NO donors or from iNOS activation by cytokines stimulates HO-1 expression and activity in glomerular mesangial cells [10]. This, and the observation that iNOS and HO-1 are coactivated within nephritic glomeruli, prompted us to explore regulatory interactions between these two enzyme systems. We employed the rat model of anti-GBM nephritis to explore whether iNOS activation stimulates HO-1 expression and whether HO-1 induction attenuates iNOS activity.

METHODS

Development of anti-GBM nephritis

Male Lewis rats weighing 175 to 200 g were immunized intraperitoneally with 1 mg of rabbit IgG emulsified in complete Freund’s adjuvant and given as a total volume

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of 0.5 mL. Seven days after this immunization, animals were injected in the tail vein with two subnephritogenic doses (0.3 mL/100 g body wt given 24 hours apart) of rabbit immune serum raised against rat particulate GBM. This protocol results in heavy proteinuria and accelerated onset of glomerular infiltration by macrophages, crescent formation, and scarring [9]. Animals were nephrectomized 24 hours after the second injection of anti-rat GBM serum or of nonimmune rabbit serum (controls) and glomeruli were isolated by differential sieving of minced cortex.

**iNOS inhibition**

The selective iNOS inhibitor l-N6-(1-iminoethyl) lysine (L-NIL) was used (Dr. Mark G. Currie; The Monsanto Co., St. Louis, MO, USA) [11]. This inhibitor has an IC$_{50}$ of 3.3 μmol/L for macrophage iNOS compared to an IC$_{50}$ of 92 μmol/L for rat brain constitutive NOS indicating that L-Nil is 28-fold more selective for inducible NOS. To prevent iNOS activation, 15 mg/kg of L-NIL was injected in the tail vein 30 minutes prior to each injection of the anti-rat GBM immune serum.

**Induction of HO-1**

We employed the HO-1 inducer hemin (Ferriproto-porphyrin IX chloride; Sigma). Animals were injected subcutaneously with hemin, 30 μmol/kg body wt, or with saline vehicle 24 hours before the first and second injection of anti-GBM serum or of rabbit nonimmune serum. A solution of hemin (10 mmol/L) was prepared immediately before use by dissolving in 0.1N NaOH adjusted to pH 7.4 with 0.1N HCl, and further diluted in saline for subcutaneous injections.

**Western blot analyses of HO-1 and ED$_1$ protein levels in glomeruli**

These analyses assessed glomerular levels of HO-1 and of the rat macrophage marker ED$_1$, as an index of the extent of infiltration of nephritic glomeruli by macrophages. Preparation of glomerular protein lysates and electrophoretic transfer of proteins were performed as previously described [9, 10]. Membranes were incubated either with rabbit polyclonal antibody against rat HO-1 (1:1000 dilution) obtained from StressGen Biotechnologies (Vancouver, British Columbia, Canada), or with anti-ED$_1$ antibody (Serotec, Westbury, NY, USA), washed thoroughly and incubated with anti-rabbit horseradish peroxidase-conjugated second antibody. Detection of signal used an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL, USA).

**iNOS enzyme activity**

Activity of iNOS in glomerular lysates was determined by calculating conversion of L-[¹⁴C]arginine to L-[¹⁴C]-citrulline as previously described [9]. Results were expressed as pmol citrulline/min.

**Urine nitrite excretion**

Urinary excretion of nitrite was measured using the Griess reaction method and a commercially available kit (Promega, Madison, WI, USA). Results were expressed as urine nitrite-to-urine creatinine ratio.

**Statistical analyses**

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

**RESULTS**

**iNOS inhibition reduces glomerular HO-1 levels**

Pre-treatment of animals with anti-GBM nephritis with L-NIL reduced urine nitrite excretion to 88.25 ±
Fig. 3. Inducible nitric oxide synthase (iNOS) activity in glomeruli of nephritic rats treated with NIS, anti-GBM serum or hemin + anti-GBM serum.

9.39 μmol per mg creatinine compared to a value of 839.51 ± 315.70 in nephritic animals not pretreated with L-NIL (P < 0.05).

Figure 1 shows a Western blot analysis of ED1 and HO-1 proteins in protein lysates prepared from glomeruli isolated from animals that received anti-GBM serum or from controls that received non-immune serum. In this experiment, two animals received non-immune serum (NIS) (lanes 1 and 2 in the upper and lower panels), three animals received anti-GBM serum (lanes 3, 4, 5 in the upper and lower panels) and three animals received anti-GBM serum and L-NIL treatment (lanes 6, 7, 8 in the upper and lower panels). As expected, ED1 levels were higher in nephritic glomeruli (upper panel; lanes 3, 4, 5, 6, 7, 8) compared to controls (upper panel; lanes 1 and 2). L-NIL treatment reduced HO-1 levels in nephritic glomeruli that received L-NIL (lanes 6, 7, 8 in lower panel) compared to levels in nephritic glomeruli that did not receive L-NIL (lanes 3, 4, 5 in lower panel).

Suprainduction of HO-1 reduces glomerular iNOS activity

Hemin treatment prior to administration of anti-GBM serum increased HO-1 protein level in glomeruli of nephritic animals (Fig. 2, lane 2) compared to the level in glomeruli of nephritic animals that were not treated with hemin (Fig. 2, lane 1). In nephritic animals, iNOS enzyme activity in glomeruli was markedly increased (Fig. 3). Hemin pre-treatment significantly reduced glomerular iNOS activity (Fig. 3).

DISCUSSION

Our previous observations that iNOS and HO-1 are co-activated in the model of anti-GBM antibody induced nephritis [9] and that activation of iNOS stimulates HO-1 in glomerular mesangial cells [10], prompted us to explore whether iNOS activation mediates the increase in glomerular HO-1 levels in nephritic glomeruli. The present studies demonstrate that iNOS activation up-regulates glomerular HO-1 in anti-GBM nephritis. Thus, pretreatment with the selective iNOS inhibitor, L-NIL, in doses sufficient to reduce urine nitrite excretion, attenuated glomerular HO-1 protein levels. There was no effect on extent of glomerular infiltration by macrophages assessed as glomerular levels of the ED1 marker (Fig. 1). This indicates that the reduction in HO-1 levels observed in L-NIL treated animals was due to iNOS inhibition rather than to an alteration of the inflammatory response to anti-GBM antibody-induced injury that could have occurred as a result of the L-NIL treatment.

In its capacity as the rate-limiting enzyme of heme degradation, HO-1 reduces cellular heme levels thereby limiting availability of heme for synthesis or enzyme activity of heme containing enzymes such as iNOS. The present studies explored whether this occurs in the model of anti-GBM nephritis. Pre-treatment with the HO-1 inducer, hemin, augmented glomerular HO-1 levels (Fig. 2) and significantly reduced glomerular iNOS enzyme activity (Fig. 3). This supports the presence of a negative regulatory interaction between HO-1 and iNOS whereby induction of HO-1 reduces iNOS activity.

In summary, our studies demonstrate a negative feedback interaction between iNOS and HO-1 in nephritic glomeruli whereby iNOS activation up-regulates HO-1, presumably via a sustained high output NO production, and HO-1 induction attenuates iNOS activity. These observations point to HO-1 as a potential target for pharmacologic manipulation in order to enhance its expression/activity in glomerulonephritis. This may have a beneficial effect by reducing activity of proinflammatory/prooxidant heme-containing enzymes such as iNOS, cyclooxygenase and the cytochrome P450 monoxygenases.

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