ROLE OF RENAL HEME OXYGENASE-1 IN RESPONSE TO ISCHEMIA/REPERFUSION IN RATS PRECONDITIONED BY HYPOXIA AND/OR REMOTE ISCHEMIA

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Background: Heme oxygenase (HO) is the rate-limiting enzyme for heme degradation, and HO-1 is the inducible isoform. Induction of HO-1 is an adaptive response to injurious stimuli such as renal ischemia reperfusion (I/R). We examined the effect of preconditioning on response to renal I/R injury and the role of HO-1 in this response.

Methods: Female Wistar rats were divided into four groups: sea level (SL, 1 atm = 101.3 kPa); hypoxic preconditioning (HA, 4 wks at 50.7 kPa); remote surgical ischemic preconditioning (SLR); and preconditioning by hypoxia and remote surgical ischemia (HAR). Each group was randomized into control and renal I/R subgroups.

Results: HO-1 protein expression and enzymatic activity was significantly higher in the HA, SLR, and HAR groups than in the SL group. ZnPP IX (HO inhibitor) was administered to assess changes in renal function, as determined by glomerular filtration rate (GFR) and renal blood flow. The renal function of rats undergoing I/R in the SLR, HA, and HAR groups were better than that of rats in the SL group.

Conclusion: Renal preconditioning by hypoxia or a remote surgical procedure increased renal HO-1 expression and enzymatic activity. This appears to have a protective effect on the kidney in response to renal I/R injury. (Acta Nephrologica 2010; 24: 101-108)

Key words: Heme oxygenase, hypoxic preconditioning, ischemic preconditioning, ischemic reperfusion, remote ischemic preconditioning

INTRODUCTION

Preconditioning can be classified as pharmacological, ischemic, or hypoxic. Preconditioning procedures generally protect against subsequent injury (Bonventre JV, 2002, Kidney ischemic preconditioning. Cur Opin Nephrol Hypertens 11: 43-48.), but the mechanisms underlying the protective effects of different types of preconditioning are not yet well established.

In 1986, Murry et al.1 introduced the term “ischemic preconditioning” (IP) to describe a treatment in which the induction of brief ischemia followed by a short period of reperfusion produced a beneficial effect on subsequent long-term ischemia. Initially, IP was reported to affect the myocardium, but subsequent studies have identified beneficial effects in other organs, including the kidney.2,3 Remote ischemic preconditioning, or “preconditioning at a distance”, is a method that protects the myocardium by applying single or repetitive remote ischemia of the small intestine,4 kidney,5 or a hind limb.6 Recent research has shown that brief liver ischemia can protect the kidney from ischemia reperfusion (I/R) injury by reducing cellular oxidative stress.7

Physiological adaptation to chronic hypoxia involves the induction of erythropoietin and an increase in red blood cell mass, resulting in an increased oxygen-
Surgical procedure, but no occlusion of the renal artery).

min and the other subgroup were sham-operated (similar to a period of hypoxia increases the cardiac tolerance to subsequent ischemic insults. In the kidney, the glomerular filtration rate (GFR) is usually well maintained, and elicits renal vasodilatation due to the release of vasodilators such as NO or CO.

Heme oxygenase (HO) is the rate-limiting enzyme for the degradation of heme to biliverdin, and this reaction is accompanied by the release of free iron and carbon monoxide (CO). Biliverdin is further metabolized to bilirubin, a strong antioxidant. By means of soluble quanly cyclase (sGC), CO acts as an intracellular messenger, similar to nitric oxide (NO). There are three HO isoforms: HO-1, HO-2, and HO-3. HO-1, also known as heat shock protein 32, is induced by numerous stimuli, including hypoxia, hyperoxia, angiotensin II, and oxidative stress. The HO-1 promoter contains several regulatory sites, including hypoxia-inducible factor-1 (HIF-1). HO-1 protects against hepatic I/R injury in rats following hypoxic preconditioning. In the kidney, tin-protoporphyrin (SnPP)-induced HO-1 protects the kidney from I/R injury. HO-1 also has a protective effect on renal injury in several animal models of acute renal failure.

However, whether HO-1 is involved in hypoxic preconditioning or remote ischemic preconditioning of the kidney remains unknown. In the present study, we examined the role of HO-1 in the protective effect of remote ischemic and/or hypoxic preconditioning on renal ischemia insult in female Wistar rats.

MATERIALS AND METHODS

Animal Care and Preparation

Female Wistar rats (10-12 weeks old, bodyweight: 200-250 g) were used for all experiments. All animal experiments and care were performed in accordance with the Guide for the Care and Use of Animals (National Academy Press, Washington, DC, 1996) and all protocols were approved by the Laboratory Animal Care Committee of the National Taiwan University College of Medicine.

Rats were randomly divided into four groups: sea level (SL), hypoxia-adapted (HA), SL with remote preconditioning (SLR), and HA with remote preconditioning (HAR). Then, rats of each group were randomly divided into two subgroups. One subgroup, with 6 to 8 animals per subgroup, was given renal ischemia/reperfusion (I/R) injury by left renal artery occlusion for 45 min and the other subgroup were sham-operated (similar surgical procedure, but no occlusion of the renal artery).

Reperfusion for 4 h was initiated by removal of the clamp.

Remote preconditioning was performed by occlusion of the left femoral artery. Experimental groups underwent four cycles of 10-min left femoral artery occlusion and 10-min of reperfusion, followed by 2-h of reperfusion prior to data collection. HA rats were placed in a hypobaric chamber (50.7 kPa, equivalent to an altitude of ~5500 m) for 15 h per day for 4 weeks prior to data collection.

Western Blot Analysis

After functional measurements and sacrifice of the rats via urethane (see below), left kidneys were removed and the renal cortexes and medullas were isolated. For detection of HO-1 immunoreactive proteins, the renal medulla and cortical tissue were homogenized in ice-cold lysis buffer (20 mM Tris HCl, 137.5 mM NaCl, 1% Triton X-100, pH 8.0) with protease inhibitors (10 μg/mL aprotinin, 1 mmol/L phenyl sulfonyl fluoride). The lysate was mixed with an equal volume of double-strength sample buffer (250 mM Tris-HCl, 4% sodium dodecyl sulfate, 10% glycerol, 2% β-mercaptoethanol, 0.0065% bromophenol blue, pH 6.8), boiled for 10 min, sonicated for 30 s, and then centrifuged at 10,000 g for 10 min. The resulting supernatants were collected and subjected to 12% SDS polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes using a Semiphor unit (Hoefer Scientific Instruments, San Francisco, CA), and blocked in Tris-buffered saline (TBS) with 0.05% polyoxyethylene-sorbitan monolaureate (Tween 20; TBS-T buffer) that contained 5% w/v nonfat dry milk powder. Then, the membrane was incubated with anti–HO-1 primary antibodies (Stressgen Biotechnologies, Corp. BC Canada.; 1:500 dilution in TBS-T with 5% dry milk powder) overnight at 4°C. After TBS-T washing for 3 times, the membrane was incubated with goat biotinylated anti-rabbit IgG secondary antibodies (Vector, Burlingame, CA, USA.; 1:200 dilution) for 1 h at room temperature. An enhanced chemiluminescence western blotting system (peroxidase substrate kit, DAB, Vector Laboratories, Inc., Burlingame, CA, USA) was used for detection. Quantification of protein signals was performed by computer-assisted densitometry.

HO Activity Assay

HO activity in the renal medulla and cortical cells were measured by generation of bilirubin. Briefly, microsomes from harvested cells were added to a reaction mixture that contained NADPH, rat liver cytosol (a source of biliverdin reductase), and the substrate hemin. This reaction was performed at 37°C in the dark for 1 h,
and terminated by the addition of 1 mL of chloroform. The extracted bilirubin was calculated as the difference in absorbance between 464 nm and 530 nm (Δε = 40 mM-1 cm-1). HO activity is expressed as picomoles/h/mg protein.

Surgical Procedures
Rats were anesthetized with i.p. urethane (1 g/kg bodyweight). Polyethylene cannulas were placed in the trachea to aid ventilation. The right femoral vein was used for infusion of a saline solution with inulin (Sigma Chemical Co., MO, USA) (0.25 mg/min/kg bodyweight); the left femoral vein was used for administration of anesthesia; the left femoral artery was used for blood sampling; the left carotid artery was used for measuring mean arterial pressure; and the left ureter was used for urine collection. The left kidney was exposed by a frank incision and a flow probe (Probe# 0.5VBB517, Transonic Systems Inc., Ithaca, NY, USA) was placed around the left renal artery for measuring renal blood flow, which was recorded continuously with a T206 recording system (Transonic Systems), and displayed on the PowerLab/16S Data Acquisition System (ADI Instruments, Pty Ltd, Castle Hill, Australia).

Effect of HO inhibition on renal function
Our experiments were designed to determine whether inhibition of HO affects the renal function of SL, SLR, HA, and HAR rats and to determine whether these rats experience renal I/R. Animals were surgically prepared as described above, and renal function was measured at the basal stage, vehicle stage, and in the presence of zinc protoporphyrin IX (ZnPP IX, an HO inhibitor). The inulin-containing saline was infused continuously in the 60-min control period and throughout all three stages. I/R was assessed after the control period, followed by the basal stage, vehicle stage, and ZnPP IX stage. Each stage was performed for 1 h (consisting of two 30-min periods), and renal function was assessed by measurement of GFR and renal blood flow. ZnPP IX was infused at dose of 10 μmol/kg body weight via the left femoral vein during two consecutive 30-min periods (ZnPP IX stage) in the sham and I/R subgroups. The GFR was estimated from the measured renal clearance of inulin. For assessment of GFR, a sustained inulin-containing saline solution was infused at a rate of 0.25 mg/min/kg bodyweight via the right femoral vein. Aterial blood samples were taken from the left femoral arterial catheter at the mid-point of 30-min period in each 1-h stage. The left ureter was cannulated with a PE-10 tube for urine collection from the left kidney.

Data Analysis
The renal function of each stage were averaged. All data are expressed as the means ± SEMs. Statistical analysis was performed using the Newman-Keuls test of analysis of variance for multiple comparisons. Student’s t test was used for paired comparisons between groups. A P value of 0.05 was considered statistically significant.

RESULTS
Effect of remote ischemic and/or hypoxic preconditioning on renal HO-1
Our western blotting results indicate that remote ischemic preconditioning and hypoxic preconditioning significantly increased the expression of HO-1 protein in the renal medulla and cortex of rats (Fig. 1 and 2). In particular, rats in the SL group (lanes 1-3) had significantly lower expression of renal medullar HO and cortical HO than rats in the SLR, HA, and HAR groups.

In agreement with these results, remote ischemic and hypoxic preconditioning also increased the HO enzymatic activity in these two renal regions (Fig. 3). Rats preconditioned by remote ischemia (SLR, HO activity: 100.6 ± 25.0 pmol/mg protein/hr) and/or 4 weeks of a hypoxic environment (HA, HO activity: 96.3 ± 21.4 pmol/mg protein/hr; HAR, HO activity: 100.5 ± 15.9 pmol/mg protein/hr) had significantly higher HO activity than rats in the SL group.

Effect of remote ischemic and/or hypoxic preconditioning on renal function during ischemia/reperfusion
In subgroups that did not undergo renal I/R, the basal levels of GFR in SL, SLR, HA and HAR rats showed no significant difference (Table 1). However, after intravenous infusion of ZnPP IX, the GFR levels of SLR, HA, and HAR rats were significantly lower than the GFR of SL rats.

After 45 min of renal I/R, the GFR levels decreased significantly in all four subgroups (Fig. 4). However, the SLI subgroup had significantly lower GFR than the SLRI, HAI, and HARI subgroups (Fig. 4). Furthermore, rats in the SLRI, HAI and HARI subgroups were more sensitive to administration of ZnPP IX than rats in the SLI subgroup (Fig. 5). In agreement with these results, the SLI group showed greater decrease in renal blood flow than the SLRI, HAI, and HARI groups after renal I/R (Fig. 6 and Fig. 7).

Taken together, our results indicate that preconditioning by hypoxia or occlusion of the left femoral artery reduces the injurious effects of subsequent I/R injury. Inhibition of HO activity by ZnPP IX reduced the protective effects of preconditioning, suggesting that HO plays a role in the protective effect of preconditioning.
Fig. 1. Effects of hypoxic and/or ischemic preconditioning on HO-1 expression in rat renal medulla. (A) Western blots of HO-1 protein expression (3 rats per group). SL: Lanes 1-3; SLR: Lanes 4-6; HA: Lanes 7-9; HAR: Lanes 10-12. (B) Quantitation of HO-1 protein levels. In this and all subsequent figures, SL: sea level (1.0 atm); HA: hypoxia (0.5 atm) adapted; SLR: SL + remote preconditioning; HAR: HA + remote preconditioning. **P < 0.01 vs. SL.

Fig. 2. Effects of hypoxic and/or ischemic preconditioning on HO-1 expression in rat renal cortex. (A) Western blots for HO-1 protein expression (3 rats per group). SL: Lanes 1-3; SLR: Lanes 4-6; HA: Lanes 7-9; HAR: Lanes 10-12. (B) Quantitation of HO-1 protein levels. **P < 0.01 vs. SL.

Fig. 3. HO enzymatic activity in renal medulla and cortex of SL, HA, SLR, and HAR rats. **P < 0.01 vs. SL.
Table 1. Influence of zinc protoporphyrin IX (ZnPP IX, 10 μmol/kg bw) on GFR of rats treated by hypoxic and/or remote ischemic preconditioning. SL: sea level (101.3 kPa); HA: hypoxia (50.7 kPa) adapted, SLR: SL + remote preconditioning; HAR: HA + remote preconditioning. Values are means ± SEs. *Each group after administration of ZnPP IX compared with baseline (**P < 0.01). †HA, SLR, and HAR compared to SL in the same period (†P < 0.05, ††P < 0.01).

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Fig. 4. Effect of renal ischemia/reperfusion on glomerular filtration rate (GFR) of rats preconditioned by hypoxia and/or remote preconditioning. **P < 0.01 vs. SLI.

Fig. 5. Effect of renal ischemia/reperfusion and zinc protoporphyrin IX (ZnPP IX, 10 μmol/kg bw) on GFR of rats preconditioned by hypoxia and/or remote preconditioning. *P < 0.05 vs. SLI.

Fig. 6. Effect of renal ischemia/reperfusion on renal blood flow of rats preconditioned by hypoxia and/or remote preconditioning. *P < 0.05 vs. SLI.

Fig. 7. Effect of renal ischemia/reperfusion and zinc protoporphyrin IX (ZnPP IX, 10 μmol/kg bw) on renal blood flow of rats preconditioned by hypoxia and/or remote preconditioning. *P < 0.05 vs. SLI.
DISCUSSION

The main findings of our study of a rat I/R model are: (i) remote ischemic and/or hypoxic preconditioning increased the level of HO-1 protein and enzyme activity, suggesting a role for HO-1 in the cellular response to preconditioning; (ii) remote ischemic and/or hypoxic preconditioning protects the kidney from subsequent I/R injury; and (iii) the protective effects induced by remote ischemic and/or hypoxic preconditioning are reduced by inhibition of HO-1 enzyme activity, further supporting a role for HO-1 in the response to preconditioning.

The local protective effects of IP are well known (3). In particular, Riera et al. reported that 5, 10, 15 or 20 min of renal ischemia preconditioning, followed by 10 min of reperfusion, results in protection against 40 min of renal ischemia, as reflected by reduced levels of serum creatinine. Another study found that the renal protective effect of IP may result from a decrease in renal cell apoptosis that is mediated by the production of NO or HO. It has recently been suggested that IP also affects more distant organs. Ates et al. demonstrated functional protection of the kidney when rats were given remote ischemic preconditioning by brief hepatic ischemia. They also found that preconditioning reduced the renal cellular oxidative stress subsequent to renal ischemia injury, and that preconditioning reduced the increases in both BUN and creatinine that normally follow renal I/R.

Other studies of IP have indicated that receptor-mediated triggers may include adenosine, opioid, bradykinin, and NO, the postreceptor protein kinase C (PKC) signaling pathway, or an end-effector, such as ATP-sensitive potassium channels. Our study demonstrated a direct protective role of HO-1 in renal ischemic preconditioning.

It was first shown in 1958 that ischemic tolerance of the heart increased following long-term exposure of animals to intermittent hypoxia. The cardiac protective effects of HA may persist longer than other hypoxia-induced adaptive responses, such as polycythemia and pulmonary hypertension. Walker et al. reported that vascular HO-1 and renal HO-1. Our study demonstrated that induction of renal HO-1 by hemin and SnCl2 attenuated afferent arteriolar autoregulation by increasing CO production from tubular epithelial cells. Renal autoregulation maintains GFR and the afferent arterioles are vessels that provide the most resistance. Myogenic tone and tubuloglomerular feedback, which contribute to renal autoregulation, were also modulated by HO-1. Another study found that induction of HO-1 by Sn-protoporphyrin also had a protective effect on subsequent renal I/R.

Thus, in contrast to remote ischemic renal preconditioning, the mechanism of cardiac protection induced by HA does not involve the activation of adenosine receptors or the PKC pathway, suggesting a different signaling pathway. Recent studies have examined the protective mechanisms of HA. Asemu et al. found that the mitochondrial K-ATPase plays a role in the protection afforded by HA. Ladilov et al. reported that HP exerts PKC-independent protection and that protein phosphatase 1 is a possible mediator. Sasaki et al. proposed that HA triggers angiogenesis and thereby enhances functional reserve of the ischemic heart. In agreement with our results, Lai et al. found that HO-1 is an important mediator in the protection effects of HA on the liver.

Hypoxic stress is known to induce hypoxia-inducible factor-1 (HIF-1), a nuclear factor that is required for hypoxic activation of transcription of erythropoietin, inducible nitric oxide synthase, and vascular endothelial growth factor. In long-term hypoxia, HIF-1 regulates HO-1 gene expression by binding to hypoxia response elements on the enhancer sequences. Our study, which found that HO-1 is an important mediator of the protective effects induced by remote ischemic preconditioning, suggests that HIF-1 may also be involved in remote ischemic preconditioning, although there is currently no direct evidence for this.

Botros et al. demonstrated that induction of renal HO-1 by hemin and SnCl2 attenuated afferent arteriolar autoregulation by increasing CO production from tubular epithelial cells. Renal autoregulation maintains GFR and the afferent arterioles are vessels that provide the most resistance. Myogenic tone and tubuloglomerular feedback, which contribute to renal autoregulation, were also modulated by HO-1. Another study found that induction of HO-1 by Sn-protoporphyrin also had a protective effect on subsequent renal I/R.

The renal medulla is most susceptible to hypoxia because the countercurrent mechanism of the kidney preserves high medullary solute concentrations, resulting in low concentrations of oxygen. Thus, anaerobic metabolism is predominant in medullary tissues, and the lowest PO2 is in the inner medulla. In rats, Zou reported higher HO-1 expression in the renal medulla and that CO (its metabolic product) controls renal medullary circulation. Our results indicate that both GFR and RBF decreased after renal I/R, and that remote ischemic and/or hypoxic preconditioning protected the kidney from I/R injury. The differences in reduction between GFR and RBF may be due to the differences in expression of vascular HO-1 and renal HO-1.

HO-1 induction has been shown to protect against renal failure in numerous experimental animal models, including the rat renal I/R model. Induction of renal
HO-1 increases the levels of CO and bilirubin, decreases NADPH oxidase-mediated oxidative stress, and inhibits 20-HETE synthase and thromboxane synthase. Shimizu et al. demonstrated significant induction of HO-1 in an animal model of unilateral renal I/R injury and that inhibition of HO activity by Sn-mesoporphrin increased the level of microsomal heme and aggravated renal injury.

The mechanism by which HO-1 induces cytoprotection against I/R injury of the kidney are not yet well established, but it has been shown that cells that over-express HO-1 have low levels of free iron due to an increase in ferritin and excretion of iron into the extracellular space. Cellular iron contributes to the formation of free radicals, resulting in damage of DNA, proteins, and lipids. Thus, the elimination of iron from the cell may be the mechanism by which HO-1 protects against oxidative stress. In addition, CO (a product of HO-1) also confers protection against cellular injury.

In summary, our study of a rat model indicates that remote ischemic or/hypoxic preconditioning leads to increased expression of HO, and protects against subsequent I/R injury of the kidney. Our results suggest that HO-1 plays a role in the protective mechanism of remote ischemic or/hypoxic preconditioning. We suggest that future studies should examine the mechanism by which HO-1 confers protection against I/R injury.

REFERENCES


