Heme oxygenase-1 induction improves ischemic renal failure: role of nitric oxide and peroxynitrite

Miguel G. Salom,1 Susana Nieto Ceron,2 Francisca Rodriguez,1 Bernardo Lopez,1 Isabel Hernandez,1 Jose Gil Martinez,3 Adoracion Martinez Losa,4 and Francisco J. Fenoy1

1Departamento de Fisiologia, Facultad de Medicina, Universidad de Murcia and 2Servicio de Analisis Clinicos, Unidad de Investigacion, 3Servicio de Cirugia, and 4Servicio de Nefrologia, Hospital Universitario “Virgen de la Arrixaca,” Murcia, Spain

Submitted 23 August 2007; accepted in final form 19 September 2007

Am J Physiol Heart Circ Physiol 293: H3542–H3549, 2007. First published September 21, 2007; doi:10.1152/ajpheart.00977.2007.—The present study evaluated the effects of heme oxygenase-1 (HO-1) induction on the changes in renal outer medullary nitric oxide (NO) and peroxynitrite levels during 45-min renal ischemia and 30-min reperfusion in anesthetized rats. Glomerular filtration rate (GFR), outer medullary blood flow (OMBF), HO and nitric oxide synthase (NOS) isoform expression, and renal low-molecular-weight thiols (→SH) were also determined. During ischemia significant increases in NO levels and peroxynitrite signal were observed (from 832.1 ± 129.3 to 2,928.6 ± 502.0 nM and from 3.8 ± 0.7 to 9.0 ± 1.6 nA before and during ischemia, respectively) that dropped to preischemic levels during reperfusion. OMBF and →SH significantly decreased after 30 min of reperfusion. Twenty-four hours later, an acute renal failure was observed (GFR 923.0 ± 66.0 and 253.6 ± 55.3 μL·min⁻¹·g kidney wt⁻¹ in sham-operated and ischemic kidneys, respectively; P < 0.05). The induction of HO-1 (CoCl2 60 mg/kg sc, 24 h before ischemia) decreased basal NO concentration (99.7 ± 41.0 nM), although endothelial and neuronal NOS expression were slightly increased. CoCl2 administration also blunted the ischemic increase in NO and peroxynitrite (maximum values of 1,315.6 ± 445.6 nM and 6.3 ± 0.5 nA, respectively; P < 0.05), preserving postischemic OMBF and GFR (686.4 ± 45.2 μL·min⁻¹·g kidney wt⁻¹). These beneficial effects of CoCl2 on ischemic acute renal failure seem to be due to HO-1 induction, because they were abolished by stannous chloride, a HO inhibitor. In conclusion, HO-1 induction has a protective effect on ischemic renal failure that seems to be partially mediated by decreasing the excessive production of NO with the subsequent reduction in peroxynitrite formation observed during ischemia.

cobalt chloride; nitric oxide stores; Western blot; nitric oxide synthase; peroxynitrite amperometry; nitric oxide voltammetry

ISCHEMIC INJURY TO THE KIDNEY is a leading cause of acute renal failure, associated with high mortality rates of ~50% and characterized by low glomerular filtration rate (GFR), tubular necrosis (mainly affecting the outer medullary S3 portion of proximal tubules), and increased renal vascular resistance. These changes have been attributed to the generation of reactive oxygen species during reperfusion (6, 18–21), causing endothelial dysfunction (25) and persistent postischemic hypoperfusion, mainly affecting the outer medulla, a renal zone with high oxygen requirements but physiologically on the verge of hypoxia (3, 4, 29).

Reperfusion of an ischemic tissue produces an abrupt rise in oxygen concentration that is traditionally thought to be followed by an oxidative burst producing superoxide anion and other reactive oxidant species responsible for tissue damage (20, 21). However, superoxide anion and 3-nitrotyrosine (the footprint of peroxynitrite) formation have also been observed during hypoxic and ischemic conditions (8, 16, 21). The renal content of 3-nitrotyrosine increases during ischemia (30), and because peroxynitrite is formed by the reaction of nitric oxide (NO) with superoxide anion, NO must also be increased, as previously demonstrated (24, 26). Because 3-nitrotyrosine increases in the first minutes of ischemia preceding the development of renal injury and failure (30), peroxynitrite must be generated early in the course of ischemia, when it may cause at least part of the injury associated with renal ischemia-reperfusion (I/R). This implies that the increase in NO concentration observed during ischemia can contribute to I/R injury by promoting the formation of peroxynitrite. However, the time course of the renal changes in NO and peroxynitrite during I/R and how these changes correlate with postischemic renal vasocostriction and severity of renal failure are presently unknown.

Oxidative stress destabilizes heme proteins, leading to free heme release, which has prooxidant effects through free radical formation, and lipid peroxidation with damaging renal tubular effects (1). Oxidative stress also promotes the upregulation of the inducible form of heme oxygenase (HO-1) that degrades heme to produce equimolar quantities of CO, iron, and biliverdin, the latter reduced to bilirubin by biliverdin reductase. HO-1 is considered to be protective against oxidative damage (1, 16) and ischemic acute renal failure (16, 27) by degrading heme and producing CO and bilirubin. The vasodilation produced by CO may preserve tubular blood flow during reperfusion. However, because HO-1-derived CO appears to inhibit NO synthesis (28), we hypothesized that the protective effect of HO-1 induction on ischemic acute renal failure could be partially mediated by decreasing the excessive production of NO observed during renal ischemia (26), with the subsequent reduction in peroxynitrite formation. Therefore, the present study evaluated the role of HO-1 induction on the renal outer medullary changes in NO and peroxynitrite observed during 45-min renal ischemia and 30-min reperfusion in
anesthetized rats. Renal low-molecular-weight thiol (—SH) content was also evaluated, because we previously showed (26) that the increase in renal NO concentration observed during ischemia originates from thiol-dependent tissue stores. Finally, to assess the functional relevance of HO-1 induction, the decrease in renal outer medullary blood flow (OMBF) and GFR normally observed during early and late reperfusion, respectively, were also determined.

METHODS

The experiments were performed on 99 anesthetized male Sprague-Dawley rats (200–250 g body wt) bred in the Animal Care Facility at the University of Murcia after being approved by the Bioethical Committee of the University of Murcia. All procedures were in accordance with the recommendations of Helsinki and the “Guiding Principles in the Care and Use of Animals” and approved by the Council of the American Physiological Society.

Outer medullary blood flow measurements. Two hours before ischemia, the animals were anesthetized with an intramuscular injection of ketamine (30 mg/kg; Rhône Merieux, Athens, GA) plus an intraperitoneal injection of Pentothal Sodium (50 mg/kg; Abbott, Madrid, Spain) and surgically prepared, and the left kidney was immobilized in a plastic holder as previously reported (26). An intravenous infusion of saline (0.9%) at a rate of 2 ml·100 g body wt·h⁻¹ was started until the end of the experiment. OMBF (arbitrary units) was measured by laser-Doppler flowmetry (laser blood flow monitor model MFB3D, Moor Instruments) as previously described (14). Briefly, basal OMBF was measured for two consecutive 10-min periods. Renal ischemia was then induced by occluding the left renal pedicle (26) in saline (n = 5), CoCl₂ (n = 6), or CoCl₂ + stannous mesoporphyrin (SnMP, 30 mg/kg ip 90 and 30 min before ischemia; n = 5)-pretreated animals. After 45 min of ischemia, the clamp was removed and OMBF was measured for an additional 30-min period.

Glomerular filtration rate determinations (n = 36). The animals were anesthetized with isoflurane, the left kidney was accessed through a flank incision, and the renal vascular pedicle was occluded for 45 min with a clamp. When the clamp was removed the flank incision was sutured, and the animals were placed under a heat source until they recovered from anesthesia. Twenty-four hours later animals were anesthetized and GFR was measured in ischemized and nonischemized kidneys from rats infused with saline (n = 6 and n = 8, respectively), CoCl₂ alone (60 mg/kg sc 24 h before ischemia; n = 7 and n = 9, respectively), or CoCl₂ + SnMP (30 mg/kg ip 90 and 30 min before ischemia; n = 6 and n = 9, respectively) with [3H]inulin as previously described (14). The GFR measurement in nonischemic kidneys of CoCl₂ and CoCl₂ + SnMP groups was performed in the same animals (n = 9).

Western blot analysis of HO (n = 4) and nitric oxide synthases (n = 4). The expressions of HO-1, HO-2, and endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) nitric oxide synthase isoenzymes were determined by Western blot (23) in outer medullary tissue from normal rats pretreated with either 0.9% NaCl (control) or CoCl₂ (60 mg/kg sc in NaCl) to induce HO-1 protein expression (13). Twenty-four hours after the subcutaneous injection of saline or CoCl₂ the animals were anesthetized and laparotomized, and the kidneys were perfused with cold phosphate-buffered saline (PBS) for several minutes at 120 mmHg to eliminate all blood and then excised. The outer medulla was dissected and quickly stored at −80°C until being used for Western blot analysis. Outer medullary tissue was later homogenized, and 50 µg of protein from outer medullary extracts from control and CoCl₂-treated rats was resolved by reductive SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, blocked, and incubated with antibodies against HO-1, HO-2 (Stressgen Biotechnologies), or eNOS, nNOS, and iNOS (BD Transduction Laboratories). Labeled proteins were revealed with the corresponding horseradish peroxidase-conjugated antibodies. Finally, blots were developed by the enhanced chemiluminescence method (GE Healthcare), and immunocomplexed bands were visualized and quantified by densitometric analysis (GelPro Analyzer Software version 3.1; Media Cybernetics). Data are expressed as the protein isoform-to-β-actin ratio.

Nitric oxide and peroxynitrite measurements (n = 39). NO generated in vivo in the outer medulla of the left kidney was measured by differential normal pulse voltammetry as previously described (26).

Peroxynitrite generated in vivo in the outer medulla of the left kidney was measured by differential pulse amperometry (31) using a three-electrode potentiostatic system similar to that used for NO measurements (26). The peroxynitrite sensor (30 µm × 500 µm; Textron) was prepared by electropolymerizing an inorganic macro-molecular film of manganese(II) phthalocyanine onto a carbon fiber microelectrode (30 µm) and then coating with poly(4-vinylpyridine). The response of all electrodes to peroxynitrite was tested with a peroxynitrite solution (15.1 µM in NaOH, pH 12.5) in PBS pH 7.4 (Fig. 1). Because of the very short half-life of this compound at neutral pH (t½ = 1 s at pH 7.4, 37°C) (22), the signal obtained disappears so fast it cannot be recorded easily. To be able to record a peroxynitrite signal, we reduced the total volume of PBS solution to 1 ml and then quickly injected 10, 20, or 40 µl of the peroxynitrite solution with a spring-loaded Hamilton syringe. This is the only way we could obtain a near-instantaneous mixture of the peroxynitrite standard into the PBS, thus obtaining a reliable and reproducible

Fig. 1. Typical response of a peroxynitrite microelectrode to a peroxynitrite solution (15.1 µM, pH 12.5). Top: amperometric recordings obtained after 3 consecutive injections using the same volume and concentration (20 µl). Bottom: amperometric recordings obtained after injections of 10, 20, and 40 µl of the same peroxynitrite solution. Determinations were performed in PBS pH 7.4 at 37°C in aerobic conditions.
signal (see Fig. 1). However, the signal disappears too fast to allow for any meaningful calibration. The resulting peroxynitrite current is negative. However, because presentation of negative current may be misleading we present data as relative current measured. To compensate for the differences in the response among microelectrodes, the signal of each microsensor was obtained in PBS (pH 7.4) before insertion in the outer medulla, and the mean value obtained was then subtracted from all the values determined during the experiment. The insertion of NO and peroxynitrite microelectrodes and the location of the probe tip in the outer medulla were performed as previously described (26).

NO concentration and peroxynitrite current were measured in saline (n = 5 and n = 7, respectively), CoCl₂ (n = 6 in both groups), or CoCl₂ + SnMP (30 mg/kg ip 90 and 30 min before ischemia; n = 6 and n = 5, respectively)-pretreated animals. To determine whether the effects of HO-1 induction on NO levels can be reproduced by NO synthesis inhibition, four rats were given nitro-l-arginine methyl ester (l-NAME, 70 mg·kg⁻¹·day⁻¹ in tap water for 2 days) and changes in NO concentration were then determined before and during the I/R experiment. NO concentration was calculated as previously described (26).

Reduced low-molecular-weight thiol measurements. We previously demonstrated (26) that the increase in renal NO concentration observed during ischemia originates from thiol-dependent tissue stores. Thus, in several rats of each group, at the end of the experiments the kidneys were perfused with cold PBS for several minutes at 120 mmHg to eliminate all blood and then excised and quickly removed and frozen at −80°C until the determination of renal reduced low-molecular-weight thiol (—SH) concentration by the reaction of tissue-dereproteinized extracts with 5,5'-dithiobis(2-nitrobenzoic acid) as previously described (5, 26).

Statistical methods. Data are presented as means ± SE. The significance of differences in the measured values between groups of NO and peroxynitrite was analyzed by two-way ANOVA for repeated measures followed by Duncan’s multiple-range test. The significance of differences in the measured values of GFR and sulfhydryl groups was analyzed by one-way ANOVA followed by Duncan’s multiple-range test. The differences in density of HO and NOS isoforms (Western blot) were analyzed by unpaired t-test. A value of P < 0.05 (2-tailed test) was considered statistically significant. The Pearson correlation coefficient between NO concentration and peroxynitrite signal was calculated by using the mean values of NO and peroxynitrite in each experimental period of the saline, CoCl₂, and CoCl₂ + SnMP groups (14 periods in each of the 3 groups for a total n = 42 pairs of values).

RESULTS

Outer medullary blood flow during renal ischemia-reperfusion. The basal values of OMBF in saline, CoCl₂, and CoCl₂ + SnMP groups were not significantly different (64.8 ± 8.3, 79.0 ± 8.6, and 82.6 ± 10.8 arbitrary units, respectively). The changes in OMBF observed during a renal I/R in the same three groups are depicted in Fig. 2. OMBF fell during ischemia in all rats and remained below basal values during reperfusion in the saline group (71.6 ± 4.9% of the basal value at end of experiment; P < 0.05). In contrast, CoCl₂-pretreated rats fully recovered on reperfusion (114.7 ± 9.9% of basal value; P < 0.001 vs. control rats), an effect that was abolished when SnMP, a HO inhibitor, was infused before ischemia (65.4 ± 6.2% at end of experiment).

Gomelar filtration rate. The GFR values in all groups are presented in Fig. 3. Pretreatment with CoCl₂ or CoCl₂ + SnMP had no significant effect on basal GFR (sham operated 923.0 ± 66.0, CoCl₂ 760.3 ± 88.0, and CoCl₂ + SnMP 617.5 ± 111.3 μL·min⁻¹·g·kidney wt⁻¹). Twenty-four hours after ischemia GFR was significantly lower in ischemized kidneys from rats given saline than in sham-operated rats (253.6 ± 55.3 vs. 923.0 ± 66.0 μL·min⁻¹·g·kidney wt⁻¹, respectively; P < 0.05). Pretreatment with CoCl₂ prevented the decrease in GFR observed in saline-infused rats after ischemia (686.4 ± 45.2 vs. 253.6 ± 55.3 μL·min⁻¹·g·kidney wt⁻¹, respectively; P < 0.05); this beneficial effect was abolished when SnMP, a HO inhibitor, was infused before ischemia in rats given CoCl₂.

Effect of CoCl₂ on outer medullary expression of heme oxygenase (n = 4) and NOS (n = 4) isoforms. Under basal conditions HO-1, HO-2, eNOS, and nNOS proteins were expressed in control, nonischemized kidneys, but iNOS was...
undetectable (Figs. 4 and 5). CoCl₂ strongly induced the renal outer medullary expression of HO-1 (0.2 ± 0.03 and 9.9 ± 1.2 HO-1-to-β-actin ratio in saline- and CoCl₂-treated rats, respectively). The neuronal and endothelial isoforms of NOS were slightly increased after CoCl₂ (0.36 ± 0.04 and 0.70 ± 0.06 eNOS/β-actin and 0.20 ± 0.05 and 0.56 ± 0.11 nNOS/β-actin, respectively). HO-2 expression was not modified and iNOS remained undetectable in rats given CoCl₂.

Changes in renal NO levels during renal ischemia-reperfusion.
Renal ischemia produced a striking increase in NO levels of control rats that was maintained during ischemia (2,360.8 ± 527.4 and 2,560.0 ± 623.4 nM after 15 and 45 min of ischemia), rapidly dropping below basal values on reperfusion (Fig. 6). In CoCl₂-pretreated animals, basal NO concentration was lower than in control rats (99.7 ± 41.0 and 937.9 ± 152.6 nM, respectively), and the increase in NO concentration observed during ischemia was slower and significantly blunted (943.4 ± 225.5 and 1,315.7 ± 445.6 nM after 15 min and 45 min of ischemia). NO synthesis inhibition for 2 days (L-NAME, 70 mg·kg⁻¹·day⁻¹ in drinking water) mimicked the
A significant correlation \((r = 0.914, P < 0.0001)\) was observed between outer medullary NO concentration and peroxynitrite signal, with a strong dependence between both parameters \((r^2 = 0.835; \text{Fig. 8})\).

Effects of ischemia, CoCl\(_2\) and CoCl\(_2 + \text{SnMP}\) on renal content of reduced low-molecular-weight thiols. The renal contents of reduced low-molecular-weight ---SH are presented in Fig. 9. CoCl\(_2\) had no effect on renal ---SH levels in nonischemic kidneys \((0.718 \pm 0.106 \text{ and } 0.694 \pm 0.069 \mu \text{mol/g kidney wt in sham-operated and CoCl}_2\)-infused rats). Renal ischemia produced a similar ---SH depletion in ischemic kidneys of control, CoCl\(_2\), or CoCl\(_2 + \text{SnMP}\) rats.

DISCUSSION

In the present study, renal ischemia caused outer medullary hypoperfusion during reperfusion, indicating impaired oxygen supply. Because the outer medulla is physiologically on the verge of hypoxia, this postischemic vasoconstriction (thought to be caused by endothelial dysfunction) may negatively affect the recovery of renal function, contributing to the hypoperfusion and hypoxia of the outer medulla and the profoundly
Depressed renal function observed 24 h after ischemia (25) characterizing the postischemic acute renal failure. This reduced renal function was preceded by a significant and correlating increase in outer medullary levels of NO and peroxynitrite during ischemia, indicative of oxidative and nitrosative stress in the outer medulla. Preinduction of HO-1 with CoCl2 ameliorated oxidative and nitrosative stress and improved OMBF and GFR during reperfusion, beneficial effects that were prevented by HO inhibition.

Inducible HO-1 is known to participate in cellular defense mechanisms. HO-1 induction is an indicator of oxidative stress and HO-1 protein overexpression protects the kidney from free radical-mediated injury (16). The present study aimed to determine the effects of HO-1 induction on the changes in outer medullary levels of NO and peroxynitrite observed during I/R and whether these changes protect renal function. The results show that CoCl2 produced a strong increase in outer medullary HO-1 expression and a significant decrease in basal outer medullary NO concentration, in contrast with the fact that the expression of eNOS and nNOS proteins were slightly increased. However, the NOS enzyme is active only as a dimeric complex, and SDS-PAGE electrophoresis only detects the monomeric, inactive NOS protein. In this regard, Cordelier et al. (7) recently reported that the decrease in NO production induced by NOS inhibition was accompanied by an increase in the inactive monomeric nNOS protein in isolated CHO/SST5 cells, indicating that the monomerization of NOS may be an important regulatory mechanism reducing NOS activity. Therefore, the increase in monomeric eNOS and nNOS expression observed in the present study is compatible with the low outer medullary NO concentration present in rats given CoCl2. This reduced in vivo NOS activity may also be due to degradation of the heme located in the active site of NOS by the induced HO-1 (9, 15). Alternative explanations for these changes include the fact that high levels of CO may inhibit NOS by binding to the heme group of the NOS isoforms (28). Therefore, HO induction can reduce NO synthesis through a variety of mechanisms, and the reduced in vivo NOS activity may take place in the presence of increased monomeric, inactive NOS protein.

In control rats renal ischemia was followed by an immediate 2.5-fold increase in NO concentration, reaching a maximum after 30 min of ischemia that was maintained until reperfusion, when a fast drop in NO levels near preischemic values was observed. We recently reported (26) that this increase in NO levels originates in thiol-dependent tissue stores (likely nitrosothiols), which slowly decompose, releasing NO and increasing its tissular levels in anoxic conditions; this is associated with cellular oxidative stress that depletes sulfydryl groups (mainly intracellular reduced glutathione) during ischemia. These changes in NO levels were significantly blunted in HO-1-preinduced animals despite the fact that they had basal and postischemic levels of renal —SH similar to control rats. This apparent contradiction can be explained by taking into account that in vivo NOS activity is very low in HO-1-induced animals. Because the cellular NO stores (nitrosothiols) depend on the reaction of NO with —SH groups, low levels of NO will probably generate small amounts of nitrosothiols, even if the renal thiol content is normal. The fact that the chronic inhibition of NO synthesis with L-NAME produces effects similar to CoCl2 (low basal NO concentrations and blunted ischemic NO increase) supports this interpretation. Moreover, the fact that SnMP, an inhibitor of HO-1, prevented the changes in NO produced by CoCl2 indicates that this effect may be due to HO-1 induction.

In the present study, differential pulse amperometry has been used to measure peroxynitrite. This technique was developed to discard capacitive currents that produce noise when the electrodes move within the tissue. When the electrode is introduced into the kidney of control rats, a basal current is present in the outer medulla, suggesting that there is a physiological level of peroxynitrite formation in renal tissue. This is in agreement with previous reports of basal generation of superoxide in renal extracts (12) and also with the presence of 3-nitrotyrosine in normal kidneys (30). However, because of the very short half-life of peroxynitrite at neutral pH, the calibration of these electrodes was not possible. In addition, part of the basal signal measured can be originated by the electrical conductivity of renal tissue without analytical significance, making any estimation of basal peroxynitrite concentration impossible; therefore, only the changes of peroxynitrite signal have been considered as meaningful.

Renal ischemia was followed by a rapid increase in peroxynitrite generation that peaked at 30 min of ischemia, reach-
CoCl₂ pretreatment also promoted a full recovery of OMBF participation of other pathways induced by CoCl₂ (such as avoiding postischemic renal vasoconstriction (2). However, the kidney may also be due to increased CO that may contribute to addition, the protective effect of HO-1 induction on ischemic nitrosative stress due to HO-1 overexpression may also be decreased peroxynitrite and hence of oxidative and nitrosative stress during renal ischemia and preventing outer medullary hypoperfusion during reperfusion. The decrease in peroxynitrite and hence of oxidative and nitrosative stress due to HO-1 overexpression may also be originated by superoxide inactivation because of the well-known antioxidant effects of biliverdin and bilirubin (11). In addition, the protective effect of HO-1 induction on ischemic kidney may also be due to increased CO that may contribute to avoid posts ischemic renal vasoconstriction (2). However, the participation of other pathways induced by CoCl₂ (such as those dependent on hypoxia-inducible factor-1α) cannot be excluded (17). These results also indicate that the oxidative and nitrosative stress produced during ischemia (and not only on reperfusion, as traditionally hypothesized) may be an important factor contributing to the posts ischemic deterioration of renal function. In summary, the results of the present study demonstrate that the administration of CoCl₂ decreases basal NO concentration and blunts the increase in NO and peroxynitrite levels during renal ischemia in the renal outer medulla, prevents posts ischemic outer medullary vasoconstriction, and improves the ensuing renal failure. These beneficial effects seem to be dependent on HO-1 induction, which blunts the increase in NO and the subsequent peroxynitrite generation during ischemia. Therefore, HO-1 induction may be potentially applicable to kidney donors or to patients under treatment for pathological conditions that may lead to oxidative insults including I/R. Further research involving HO and its downstream products is necessary to expand our understanding of the cellular and molecular mechanisms involved in I/R injury and acute renal failure, thus allowing us to improve the preservation of organ function and, ultimately, the well-being and survival of these patients.

ACKNOWLEDGMENTS

We thank Dr. Juan Cabezas for his kind assistance in the Western blot analysis.

GRANTS

This work was supported by grants from the Ministerio de Ciencia y Tecnología (BFIO2001-0497), from the Fundación Séneca (PI-31/00895/01), and from the Fondo de Investigaciones Sanitarias (FIS PI052737).

REFERENCES


