Inducible Nitric-oxide Synthase Is an Important Contributor to Prolonged Protective Effects of Ischemic Preconditioning in the Mouse Kidney

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Ischemic preconditioning renders the mouse kidney resistant to subsequent ischemia. Understanding the mechanisms responsible for ischemic preconditioning is important for formulating therapeutic strategies aimed at mimicking protective mechanisms. We report that the resistance afforded by 30 min of bilateral kidney ischemia persists for 12 weeks after preconditioning. The protection is reflected by improved postischemic renal function, reduced leukocyte infiltration, reduced posts ischemic disruption of the actin cytoskeleton, and reduced posts ischemic expression of kidney injury molecule-1 (Kim-1). The protection is observed in both BALB/c and C57Bl/6J strains of mice. Thirty minutes of prior ischemia increases the expression of inducible nitric-oxide synthase (iNOS) and endothelial NOS (eNOS) and the expression of heat shock protein (HSP)-25 and is associated with increased interstitial expression of α-smooth muscle actin (α-SMA), an indication of long term posts ischemic sequelae. Treatment with N-nitro-L-arginine (L-NNa), an inhibitor of NO synthesis, increases kidney susceptibility to ischemia. Gene deletion of iNOS increases kidney susceptibility to ischemia, whereas gene deletion of eNOS has no effect. Pharmacological inhibition of NO by L-NNa or L-N6-(1-iminoethyl) lysine (L-NIL, a specific inhibitor of iNOS) mitigates the kidney protection afforded by 30 min of ischemic preconditioning. Fifteen minutes of prior ischemic preconditioning, which does not result in the disruption of the actin cytoskeleton, impairment of renal function, increased interstitial α-SMA, or increased iNOS or eNOS expression, but does increase HSP-25 expression, partially protects the kidney from ischemia on day 8 via a mechanism that is not abolished by L-NIL treatment. Thus, iNOS is responsible for a significant component of the long term protection afforded the kidney by ischemic preconditioning, which results in persistent renal interstitial disease, but does not explain the preconditioning seen with shorter periods of ischemia.

Ischemic preconditioning is the phenomenon whereby a prior ischemic stress renders the organ resistant to a subsequent ischemic insult (1–4). In the heart preconditioning, induced by short episodes of ischemia and reperfusion treatment, has two distinct phases: an early phase that lasts from a few minutes to 2 h and a late phase that is sustained for 2–4 days (5). Recently, we demonstrated that prior 30 min of bilateral ischemia, which results in severe functional and histological injury, protects the mouse kidney from subsequent ischemia 8 or 15 days later. The degree of protection is dependent on the duration of ischemia, and the protection is associated with increased heat shock protein-25 (HSP-25) expression, reduced post ischemic phosphorylation of stress-activated protein kinase (SAPK) 1/2 and p38 mitogen-activated protein kinases (MAPKs), and reduced post ischemic leukocyte accumulation and outer medullary congestion (2, 6). We also observed that protection against ischemia was also afforded by prior ureteral obstruction in the mouse (6). The identification of the mechanisms responsible for ischemic preconditioning is important not only for an understanding of the pathophysiology of ischemic injury but also for formulating therapeutic strategies aimed at mimicking the protective mechanisms with pharmacological or genetic manipulation.

Nitric oxide down-regulates inflammatory reactions, which are important contributors to ischemia/reperfusion injury (6–11). NO regulates neutrophil recruitment by inhibiting the expression of adhesion molecules in the vascular endothelium resulting in increased blood flow to ischemic regions (12). NO production is dependent on the activities of NOS enzymes, whose expression is, in turn, modulated by signaling pathways implicated in inflammation, such as NF-κB and MAPKs (9–11, 13, 14). NO donors protect the kidney in diverse models of renal failure, including ischemic renal failure (15, 16), obstructive nephropathy (17), or renal allografts (18). Inhibition of NO synthesis increases susceptibility to kidney ischemia (16, 19, 20). By contrast, some studies have demonstrated that inhibition of iNOS protects organs against ischemia (20, 21). iNOS/NO has been implicated in protection induced by preconditioning. Takano et al. (22) reported in the heart that iNOS enzymes are implicated in protection 24 h after ischemic pre-

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1 The abbreviations used are: HSP, heat shock protein; MPO, myeloperoxidase; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; Kim-1, kidney injury molecule-1; L-NIL, L-N6-(1-iminoethyl) lysine; NOS, nitric-oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; i.p., intraperitoneal; L-NNa, N-nitro-L-arginine; SMA, smooth muscle actin.
conditioning induced by short episodes of ischemia and reperfusion (22). An iNOS inhibitor eliminated the infarct-sparing effect of preconditioning afforded by isoflurane or halothane anesthesia 24 h previously (23). Prior brain ischemia protects isolated aortic ring reactivity in an iNOS-dependent manner (24). Thus, while these and other data suggest that expression of iNOS plays a role in the protection induced by mild sublethal ischemic preconditioning against a second exposure to ischemia/reperfusion 24 or 48 h later in heart, brain, and kidney (5, 14, 25–27), little is known in any organ about the role of NOS or other molecules in preconditioning when the initial insult results in prolonged long term protection, as defined by protection afforded for longer than 48 h.

In our studies we characterized the time characteristics of the protective effect of ischemic preconditioning, whether the protective mechanisms differ according to the strength of ischemic preconditioning, and whether ischemic preconditioning in the kidney is dependent upon NOS expression. Our results indicate that prior ischemic preconditioning protects the kidney from ischemia/reperfusion insults up to 12 weeks later with the degree of protection decreased as length of time between ischemic periods increases. Thirty minutes of ischemic preconditioning results in a sustained increase in iNOS expression and sustained damage to the kidney as reflected by α-smooth muscle actin (SMA) accumulation. Pharmacological inhibition of NO synthesis or genetic deletion of the iNOS gene, but not the eNOS gene, increases mouse kidney susceptibility to ischemia and mitigates the protection afforded by 30 min but not 15 min of ischemic preconditioning. Fifteen minutes of ischemia does not lead to increases in iNOS or eNOS expression. Thus increases of iNOS expression account for an important component of long term ischemic preconditioning in the kidney when the initial ischemia results in persistent tissue injury. While others have studied the role of iNOS in preconditioning in other organs as indicated above, our data go beyond these studies in a number of ways. No one previously, in any organ, has reported nor explored the mechanisms of preconditioning that persist up to 12 weeks after the initial event. Furthermore we report persistent long term renal interstitial changes after ischemia in the mouse kidney, and for the first time implicates these changes in long term protection against subsequent ischemia. We have thus identified iNOS as an important factor in prolonged protection that follows ischemic preconditioning. This role of iNOS depends upon the initial ischemic time in the mouse kidney.

EXPERIMENTAL PROCEDURES

Animal Preparation—Experiments were performed in male BALB/c mice obtained from Charles River Laboratory, C57BL/6J (eNOS +/+), eNOS gene deletion (eNOS −/−) (28), C57BL/6J (iNOS +/+), and iNOS gene deletion (iNOS −/−) male mice (29) obtained from the Jackson Laboratory. Mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and administered 1 ml of 0.9% NaCl (37 °C) on the day of surgery (day 0). Body temperature was maintained at 36–38 °C. Kidneys were

![Fig. 1. Length of time that preconditioning leads to protection of renal function after a subsequent ischemia/reperfusion. BALB/c male mice were subjected to either sham-operation (S) or 30 min of bilateral ischemia (I) on day 0. A and B, after the surgery on day 0, plasma creatinine levels and body weight were determined at indicated times. C, the animals were subjected to either sham-operation (S) or 30 min of bilateral ischemia (I) 1, 3, 4, 6, 10, or 12 weeks after the initial surgery on day 0. Plasma creatinine concentration was measured 24 h after the second operation. Values are expressed as mean ± S.E. for 4–8 animals. *, p < 0.01 compared with before ischemia; #, p < 0.01 compared with S/I.](http://www.jbc.org/content/27257/1/27257/F1.expansion.png)
exposed through flank incisions. Mice were subjected to 15, 25, or 30 min of bilateral renal ischemia (preconditioning) or sham-surgery (non-preconditioning) on day 0. Ischemia was induced by clamping both renal pedicles with nontraumatic microaneurysm clamps (Roboz). The incisions were temporarily closed during ischemia or sham surgery. After the clamps were removed reperfusion of the kidneys was visually confirmed. Preconditioned or non-preconditioned animals were subjected to 30 min of either bilateral ischemia or sham-surgery at the times indicated in the figures. Some animals were administrated either 0.9% NaCl (vehicle, i.p.), Nω-nitro-L-arginine (L-NNA, 12 mg/kg, i.p.), L-N6-(1-iminoethyl) lysine (L-NIL, 10 mg/kg, i.p.) or l-arginine (30 mg/kg, i.p.) 30 min before and 30 min after 30 min of either bilateral ischemia or sham-operation on day 8.

Kidneys were harvested at the times indicated in the figure legends. Kidneys were snap frozen in liquid nitrogen for myeloperoxidase (MPO) activity and Western blot analysis or were rinsed in PBS and fixed in 4% paraformaldehyde for histological analysis.

**Renal Functional Parameters**—Seventy microliters of blood were taken from the retroorbital vein plexus at the times indicated in the figures. Plasma creatinine concentration was measured using a Beckman Creatinine Analyzer.

**Immunocytochemistry**—After perfusion via the left ventricle with 30 ml of PBS for 2 min at 37 °C and then PLP (4% paraformaldehyde, 75 mM l-lysine, 10 mM sodium phosphate) fixative for 5 min, kidneys were excised and placed in PLP overnight at 4 °C. Kidneys were then washed and stored in PBS containing 0.02% sodium azide at 4 °C. Fixed tissue was washed with PBS three times for 5 min each, placed overnight in PBS containing 30% sucrose, embedded in oxtetraycine compound (Sakura FineTek, Torrance, CA), frozen in liquid nitrogen, and then cut into 5-μm sections using a cryomtome. Sections were mounted on Fisher Superfrost Plus (Fisher) microscope slides, dried in air, and stored at −20 °C. For staining with phalloidin, which stains the actin cytoskeleton, sections were dried, washed in PBS for 10 min, incubated in blocking buffer (PBS containing 3% nonfat dry milk) for 40 min at room temperature, and washed three times in PBS for 5 min each. Sections were incubated in blocking buffer containing TRITC-labeled phalloidin (Sigma, 1:500) for 20 min at room temperature, washed twice in PBS containing 1.9% NaCl, once in PBS and mounted with a 1:1 mixture of Vectashield (Vector Laboratories) and NaCl pH 8.9.

To detect kidney injury molecule-1 (Kim-1), whose expression is markedly up-regulated in dedifferentiated proximal tubular cells in the outer stripe after ischemia or ureteral obstruction (30, 31), sections were dried, washed in PBS, incubated in blocking buffer (PBS containing 2% bovine serum albumin) for 20 min at room temperature, incubated with rabbit polyclonal anti-Kim-1 antibody (1:1000) overnight at 4 °C, and then washed with PBS. The sections were incubated with FITC-labeled anti-rabbit IgG for 40 min at room temperature, washed with PBS three times for 10 min each, and mounted with Vectashield. To detect α-smooth muscle actin, sections were boiled with 10 mM sodium citrate buffer, at pH 6.0, for 15 min and then washed with PBS. Monoclonal anti-α-smooth muscle actin antibody,1,250, Sigma, was used as primary antibody. Subsequent procedures were carried out as described above with the exception that the secondary antibody was goat anti-mouse IgG antibody conjugated with FITC. Images were viewed on a Nikon FXA epifluorescence microscope.

**MPO Activity**—MPO activity, an index of tissue leukocyte infiltration, was measured in 1.5 and 24 h postischemic kidneys as previously described (6, 32). Activity was normalized to protein concentration.

**Western Blot Analysis**—Proteins were extracted from kidneys as previously described (33). Protein samples were separated on 7.5 or 10% SDS-PAGE gels and then transferred to an Immobilon membrane (Millipore, Bedford, MA). Membranes were incubated with rabbit polyclonal anti-HSF-27 or 25 (Upstate Biotechnology), monoclonal anti-α-smooth muscle actin (Sigma) or mouse monoclonal anti-iNOS or eNOS (BD Transduction Laboratories) antibodies. As a positive control for iNOS expression, mouse macrophage RAW 264.7 cells were stimulated with interferon-γ (10 ng/ml) and lipopolysaccaride (1 μg/ml) for 12 h. Macrophage cell lysates were provided by BD Transduction Laboratories. Secondary antibodies, conjugated with horseradish peroxidase (Santa Cruz Biotechnology), were detected with the ECL system (Amersham Biosciences).

**Statistics**—All results are expressed as mean ± S.E. A p value of <0.05 was taken as statistically significant. Each group consisted of more than four animals as indicated in the figure legends.

**RESULTS**

**Thirty Minutes of Ischemic Preconditioning Reduces Postischemic Kidney Functional Impairment and Disruption of the Actin Cytoskeleton**—We have reported that prior ischemia confers resistance of the BALB/c male mouse to subsequent ischemia 8 or 15 days later (2). To examine how long the acquired resistance persists, male BALB/c mice were subjected to either sham-operation or 30 min of bilateral ischemia on day 0, and the animals were exposed to a second 30-min period of bilateral ischemia on day 8 or week 3, 4, 6, 10, or 12 after the initial
surgery. The initial 30 min of bilateral ischemia results in dramatic increases in plasma creatinine levels that return to basal levels by 1 week after the surgery (Fig. 1A). Sham-operation does not result in a change in plasma creatinine concentration (Fig. 1A). After the initial 30 min of bilateral ischemia, the body weight of all animals was slightly reduced followed by an increase over time. While sham-operated animals have higher growth curves than animals exposed to ischemia, weights differ by only ~5% 12 weeks later (Fig. 1B).

Thirty minutes of bilateral ischemia on day 8 in the non-preconditioned mice results in a significant increase in plasma creatinine concentration (Fig. 1C). In contrast, 30 min of bilateral ischemia imposed on day 8 in the preconditioned mice has no effect on plasma creatinine levels (Fig. 1C). Even when the preconditioned mice were exposed to 30 min of bilateral ischemia at 3 weeks after the initial ischemia, a postischemic increase of plasma creatinine is not observed (Fig. 1C). When the preconditioned mice were subjected to 30 min of bilateral ischemia 4, 6, 10, or 12 weeks after the preconditioning, there is a postischemic increase of plasma creatinine, but this increase is significantly less than the increase in plasma creatinine seen in the non-preconditioned mice (Fig. 1C).

Renal ischemia/reperfusion results in disruption of the actin cytoskeleton (6, 34). We evaluated the effect of ischemic preconditioning on postischemic actin cytoskeleton changes using immunocytochemistry techniques. Kidney sections were stained for phalloidin to identify the actin cytoskeleton (35). There is normal apical phalloidin staining in the proximal tubules of the sham-operated kidneys (Fig. 2A). Twenty-four hours after 30 min of ischemia, severe disruption of the actin cytoskeleton is observed in the non-preconditioned kidney (Fig. 2B). The disruption is particularly severe in the S3 segment proximal tubular cells in the outer stripe of the outer medulla (Fig. 2). At 8 days or 6 or 12 weeks after ischemia, the disruption of the actin cytoskeleton has largely reversed (Fig. 2, C, E, and G). At 24 h after a second period of ischemia imposed on day 8, the changes in the actin cytoskeleton were much less pronounced in the preconditioned kidneys than in the non-preconditioned kidneys (compare Fig. 2, D and B). With increasing time between ischemic insults the postischemic disruption of the actin cytoskeleton increases (Fig. 2).

We evaluated the effect of 30 min of ischemic preconditioning on Kim-1 expression after subsequent ischemia as an additional measure of injury to the proximal nephron. We wanted to evaluate whether there are effects of ischemia that persisted well beyond the time that serum creatinine returned to normal. Kim-1 is markedly up-regulated in the S3 segment of the proximal tubule in the outer stripe of the outer medulla after ischemia and ureteral obstruction (30, 31). In the normal kidney there is no staining with an anti-Kim-1 antibody (Fig. 3). Ischemia results in the marked expression of Kim-1 in the S3 segment of the proximal tubules (Fig. 3). Kim-1 expression peaked 24 h after ischemia and reperfusion and then decreased over time. Increased expression of Kim-1 was seen for at least 3 weeks after 30 min of ischemia suggesting there was persistent tubular injury (Fig. 3) despite the absence of any increase in plasma creatinine (Fig. 1). When a second ischemic insult was imposed either 3 or 6 weeks after the first the postischemic expression of Kim-1 in the preconditioned kidney was much lower when compared with the expression normally seen 24 h after ischemia in the non-preconditioned kidney (Fig. 3).

Thirty Minutes of Ischemic Preconditioning Reduces Postischemic Tissue MPO Activity—To evaluate whether protection against ischemic injury could be related to postischemic leukocyte infiltration, the extent of tissue leukocyte infiltration was determined by tissue MPO activity. When 30 min of ischemia was induced on day 8, MPO levels markedly increased in the non-preconditioned kidneys 1.5 and 24 h after (Fig. 4). By comparison there was no increase of MPO activity in the preconditioned kidneys (Fig. 4).

Thirty Minutes of Ischemia Increases Endothelial and Inducible NOS Protein Expression—Endothelial or iNOS protein ex-
pression was evaluated by Western blot analysis. Thirty minutes of ischemia results in a significant increase in the expression of eNOS and iNOS in whole kidney lysate (Fig. 5). Sham-surgery does not result in a change in the expression of eNOS and iNOS (Fig. 5). The increased expression of eNOS and iNOS after ischemia/reperfusion persists for 12 weeks (Fig. 5). The expression of iNOS peaks at 1 week after ischemia and gradually diminishes over time, but remains above baseline levels for 12 weeks (Fig. 5, A and C).

Since the sustained long term iNOS expression suggests the presence of a persistent stimulus, we evaluated whether the initial ischemic period resulted in long term changes in the kidney, which were not severe enough to cause measurable functional changes in plasma creatinine. α-Smooth muscle actin expression is a characteristic feature of renal fibrosis (36). Thirty minutes of ischemia results in an increase of α-smooth muscle actin expression in the interstitium 1, 6, and 12 weeks later (Fig. 6A). This increase in renal α-smooth muscle actin expression is also manifest on Western blots taken from kidneys at 1, 3, 6, and 12 weeks after 30 min of bilateral ischemia (Fig. 6B).

Effect of L-Arginine or L-NNA on IschemiaReperfusion Injury—To evaluate whether NO/NOS enzymes contribute to the protection afforded by ischemic preconditioning, we evaluated ischemic preconditioning in eNOS −/−, iNOS −/−, and inos+/− mice. We used both 25- and 30-min periods of ischemia so that we could be sure that an effect was not masked by too much injury after 30 min of ischemia. In iNOS −/− mice, 25 min of bilateral ischemia significantly increased plasma creatinine levels 24 h after ischemia (Fig. 8A). In contrast, there is a small increase in plasma creatinine levels in inos+/− mice (Fig. 8A). Thirty minutes of bilateral ischemia markedly increases plasma creatinine levels in both iNOS −/− and inos+/− mice 24 h after ischemia. (Fig. 8B). Twenty-four hours after either 25 or 30 min of ischemia the plasma creatinine levels are significantly higher in iNOS −/− mice than in iNOS +/+ mice (Fig. 8, A and B). Sham-operation in both inos−/− and inos+/− does not result in a change in plasma creatinine levels (Fig. 8, A and B). Eight days after 30 min of ischemic preconditioning survival
rate is 84.6% in iNOS +/+ (n = 23) and 44.4% in iNOS −/− (n = 27) mice, respectively (Fig. 8C). All mice survived for 8 days after 25 min of initial ischemia (data not shown). Eight days after either the 25 or 30 min of bilateral ischemia or sham-operation, the animals were subjected to 30 min of bilateral ischemia (Fig. 8, A and B). Thirty minutes of bilateral ischemia on day 8 results in an increase in plasma creatinine levels in both iNOS −/− and iNOS +/+ mice which are preconditioned by 25 min of bilateral ischemia on day 0, but the postischemic increase in plasma creatinine is less in iNOS +/+
than iNOS $-/-$ mice (Fig. 8A). Thirty minutes of bilateral ischemic preconditioning of iNOS $+/-$ mice completely prevents the increase of plasma creatinine induced by 30 min of bilateral ischemia on day 8 (Fig. 8B), whereas in iNOS $-/-$ mice there is only partial protection of the kidney against this second insult (Fig. 8B). On day 9 postischemic tissue MPO activity is greater in the iNOS $-/-$ than in the iNOS $+/-$ mice 24 h after a second ischemic period (I/I) (Fig. 8D). Treatment with L-NIL, a specific inhibitor of iNOS, 30 min prior to and after the second procedure mitigates the kidney protection afforded by 30 min of prior ischemia (Fig. 8E).

When 30 min of bilateral ischemia is induced in the eNOS $+/-$ or eNOS $-/-$ mice, the levels of plasma creatinine markedly increased to equivalent levels at 24 and 48 h after ischemia in mice of both genotypes (Fig. 8F). There are no differences in the levels of plasma creatinine between eNOS $+/-$ mice.

**Fig. 8.** Effect of prior ischemia on the levels of plasma creatinine (A, B, E, and F), survival (C) and MPO activity (D) after ischemia in iNOS $-/-$, iNOS $+/-$, eNOS $-/-$, eNOS $+/-$, or BALB/c male mice. Mice were subjected to either sham-operation (S), 25 min (A) or 30 min (B–F) of bilateral ischemia (I) on day 0. Animals were then subjected to either sham-operation or 30 min of bilateral ischemia 8 days after the initial surgery. D, MPO activity was measured 24 h after the second procedure ($n = 4$–5). E, 8 days after the 30 min of bilateral ischemia on day 0, the BALB/c mice were subjected to ischemia. L-NIL (10 mg/kg BW, i.p.) was administrated to the mice 30 min before and 30 min after ischemia. Values are expressed as mean ± S.E. *$p < 0.05$ versus baseline; #, $p < 0.05$ versus each wild type mice; †, $p < 0.05$ versus S/S.
after the first surgery in iNOS experiments. The expression of HSP-25 was evaluated with anti-HSP-25 antibody using Western blot analysis. The mice were harvested 1, 6, or 12 weeks after the initial surgery. 

Sham-operation (S) on day 0. 

S/H11002 and eNOS expression were evaluated with 30 min of bilateral ischemia in iNOS−/− and eNOS+/+ mice. The kidneys were harvested 24 h after the second surgery. The expression of HSP-25 was evaluated with anti-HSP-25 antibody using Western blot analysis. Each blot is representative of 3–4 independent experiments.

and eNOS−/− at any time point (Fig. 8F). Sham-operation in both eNOS−/− and eNOS+/+ does not result in a change in plasma creatinine levels (Fig. 8F). When the animals are subjected to 30 min of bilateral ischemia on day 8, significant postischemic increases of plasma creatinine levels were not seen in either eNOS−/− and eNOS+/+ animals (Fig. 8F).

Thirty Minutes of Ischemia Increases HSP-25 Expression—We previously reported that increased HSP-25 expression is associated with the late phase of ischemic preconditioning (2, 6). When the levels of HSP-25 expression were evaluated by Western blot analysis, 30 min of bilateral ischemia increased HSP-25 expression, and the increase in HSP-25 protein expression was sustained for at least 12 weeks after the preconditioning in BALB/c male mice (Fig. 9A). After peaking 24 h after ischemia, the increased expression of HSP-25 decreased over time (Fig. 9A). The level of expression at 8 days after 30 min of bilateral ischemia is elevated to nearly equivalent levels in iNOS−− and eNOS+/+ mice, at a time when there are major differences in functional changes in response to subsequent ischemia between iNOS+/+ and iNOS−/− mice (Fig. 9B).

Effect of Fifteen Minutes of Ischemic Preconditioning on Postischemic Renal Function, Postischemic Disruption of Actin Cytoskeleton, Expression of iNOS, eNOS, α-Smooth Muscle Actin, or HSP-25 or Response to L-NIL Treatment—Since 15 min of prior ischemia does not result in an increase in plasma creatinine and partially protects the kidney from ischemia (2), we evaluated whether the protection afforded by 15 min of ischemia involves NO production or expression of iNOS protein. Fifteen minutes of prior bilateral ischemia results in partial protection of the kidney from 30 min of bilateral ischemia 8 days later (Fig. 10A). Fifteen minutes of bilateral ischemia does not increase plasma creatinine (Fig. 10A), disrupt the actin cytoskeleton (Fig. 10B), increase the expression of iNOS or eNOS protein (Fig. 10, C and D), or result in fibrosis as reflected by α-smooth muscle actin expression (Fig. 10, E and G). Fifteen minutes of ischemia results in an increase of HSP-25 expression, and the increased HSP-25 expression is sustained for 8 days after the ischemia (Fig. 10F). In contrast to the effect of L-NIL observed with 30 min of prior ischemia (Fig. 8E), the protection afforded by 15 min of prior ischemia was not mitigated by L-NIL (Fig. 10H).

DISCUSSION

We have previously reported in the BALB/c mouse kidney that the resistance afforded by prior ischemia or ureteral obstruction was seen up to 8 or 15 days after the initial procedure (2, 6). We now report that ischemic preconditioning is likely a general phenomenon in mice since it is observed in different strains of mice. In addition our current studies reveal that the protection afforded by prior ischemia is observed 1, 3, 4, 6, 10, or 12 weeks later, as reflected by decreased in plasma creatinine, tissue leukocyte accumulation, expression of Kim-1, and disruption of the actin cytoskeleton of the proximal tubular cells after a second ischemic exposure. Protection afforded by preconditioning is reduced as the length of time between ischemic periods is increased, but remains quite significant at least for 12 weeks. Previous studies have not addressed the length of time that a “preconditioning” ischemic insult can effectively protect against subsequent ischemia. There are data, however, that show a toxic insult can induce protection against a second exposure to ischemia over extended periods of time. MacNider (37) reported protection against a second exposure to uranium at twice the dose of the first exposure when the second uranium injection occurred two months after the first. Hayes et al. (38) reported functional protection when animals were injected with glycerol 35 days after the initial exposure to glycerol. The mechanisms responsible for preconditioning with toxins or ischemia in the kidney have not been hitherto defined.

Ischemia/reperfusion results in enhanced leukocyte-endothelial adhesion interactions in the small vessels of the outer medulla (7). These interactions can alter blood flow characteristics in the outer stripe of the outer medulla, and further impair oxygen supply to the proximal straight tubule, the major site of injury in this model of ischemic renal failure (39, 40). Our laboratory has reported that treatment with anti-neutrophil serum or anti-intercellular adhesion molecule-1 (ICAM-1) antibody or deletion of ICAM-1 from mice protects animals from ischemic renal failure (32, 41). Recently, we also observed preconditioning due to prior ureteral obstruction, which is associated with a reduced subsequent postischemic leukocyte infiltration and congestion in the outer medulla (6). While our observations do not distinguish between leukocyte trapping as a cause or effect of the protection, they suggest that the reduced postischemic inflammatory response may be responsible for the protection.

Ischemia/reperfusion in the kidney activates NOS enzymes (42) and increases the expression of NOS proteins (20, 40, 43), Kim-1 (30, 31), and HSPs (2, 6, 20, 21, 40, 44). In the present study we observed that 30 min of ischemic preconditioning increases the expression of eNOS, iNOS, and HSP-25 and the increased expression of eNOS, iNOS, and HSP-25 is sustained for 12 weeks. The increases in iNOS, Kim-1, and HSP-25 expression are reduced over time postischemia. The sustained expression of these proteins might be related to the presence of irreversible injury and inflammatory responses causing ongoing generation of reactive oxygen species and a persistent dedifferentiation and proliferation of tubular epithelial cells. Since Kim-1 is expressed in dedifferentiated cells (30), the sustained Kim-1 expression might reflect ongoing responses of kidney epithelial cells to persistent inflammatory stimuli and/or repair processes of the kidney epithelial cells after ischemic preconditioning. Recently Basile et al. (45, 46) reported in rats that severe ischemia results in irreversible injury and progresses to chronic renal disease. In the present studies in mice 30 min of ischemia results in renal fibrosis indicating persistence of renal injury. This persistent interstitial response with fibrosis may
Fig. 10. Effect of 15 min of ischemic preconditioning on plasma creatinine (A), the disruption of the actin cytoskeleton (B), expression of iNOS (C), expression of eNOS (D), expression of α-smooth muscle actin (α-SMA) (E and F), response to L-NIL treatment (G), and expression of HSP-25 (H). BALB/c male mice were subjected to either 15 or 30 min of sham-operation (S) or 15 or 30 min of ischemia (I) on day 0. Eight days after the first surgery the mouse kidneys were exposed to either 30 min of sham-operation or 30 min of bilateral ischemia. A, plasma creatinine concentrations at baseline, 1 day, 8 days, and 9 days (1 day after the second procedure). B, phalloidin staining of the outer medulla 24 h after 15 min of ischemia. C and D, 24 h after the first or second procedure iNOS (C) or eNOS (D) expression was determined by Western blot analysis. E and F, α-SMA (E) or HSP-25 (F) expression was evaluated by Western blot analysis 1 or 8 days after sham (S) or ischemia (I). Bands were quantitated by the NIH Image Program. G, α-SMA expression 8 days after either 15 min of sham-operation or 15 min of ischemia showing no differences. Green color indicates α-SMA. H, plasma creatinine levels in animals sham-treated or preconditioned with 15 min of ischemia and subsequently treated with L-NIL or vehicle, i.p., 30 min before and 30 min after the second sham or ischemic period, which also lasted 30 min. Plasma creatinine was determined 24 h after the second procedure. *, p < 0.05 compared with baseline; #, p < 0.05 compared with S (15 min); †, p < 0.05 compared with S (15 min)/S (30 min).
lead to the persistent increase in iNOS, Kim-1, and HSP-25 expression.

Our data indicate that pharmacological inhibition of NOS proteins by l-NNA enhances the susceptibility to ischemia. Genetic deletion of iNOS increases the kidney susceptibility to ischemia. By contrast gene deletion of eNOS in mice has no effect. l-NIL mitigates the protection afforded by 30 min of prior ischemia. Thus, genetic deletion of iNOS or pharmacological inhibition of NOS proteins by l-NNA or iNOS by l-NIL mitigates the protection afforded by 30 min of ischemic preconditioning, whereas genetic deletion of the eNOS gene does not affect the protection. l-NNA or l-NIL treatment, or iNOS gene deletion, however, does not completely abolish the protection indicating that NO/iNOS is important for ischemic preconditioning but does not account for the entire phenomenon. Furthermore l-NIL has no effect on the protection afforded by 15 min of prior ischemia indicating that the mechanism involved in the protection 8 days after a prior short ischemic period does not involve iNOS.

Many studies have demonstrated that the increased activity of NOS is associated with reduced ischemia/reperfusion-induced injury and an increase of blood flow in the ischemic region (47). By contrast, Ling et al. (21) reported that genetic deletion of the iNOS gene in mice, in part, attenuates postischemic kidney dysfunction through higher postischemic expression of HSP-72 (21). The discrepancy between our results and their report might be due to the different levels of NO production associated with the degree or method of ischemia/reperfusion injury. Noiri et al. (20) reported that treatment with N\textsuperscript{\textbeta}-nitro l-arginine methyl ester (l-NAM), an inhibitor of NOS worsens the postischemic renal function, whereas treatment with antisense oligodeoxynucleotides targeting iNOS protects the kidney. NO may have a protective effect due to its anti-apoptotic action and effects to decrease leukocyte-endothelial interactions. Nitric oxide can result in vasodilatation and inhibition of platelet plug formation, as well as reduction of the inflammatory response. In contrast NO can induce injury via lipid peroxidation, DNA damage, and pro-apoptotic effects, which are implicated in ischemia/reperfusion injury (19). Goligorsky et al. (19) demonstrate that cellular effects of NO depend on its concentration, site of release and duration of action. Low levels of NO may be protective but higher levels may be detrimental (19).

Bolli and co-workers (48, 49) suggest that prior short episodes of ischemia/reperfusion in the heart without severe injury increases iNOS expression leading to production of NO. These investigators proposed that the early production of NO stimulates iNOS expression through intracellular signal pathways, and the induction of iNOS protein mediates the late phase protection afforded by ischemic preconditioning in heart. Recently, Bolli et al. (5, 14, 25) have reported that iNOS inhibition by pharmacological or genetic modulation of mice abolished the protection afforded by short episodes ischemia/reperfusion preconditioning at 24–48 h in heart. In kidney, Jefayri and colleagues reported that there is increased NO release secondary to increased NOS expression 6 h after 4 cycles of 4 min of ischemia followed by 11 min of reperfusion (43). In comparison to our studies these prior studies are examining relatively short term effects not studied beyond 48 h.

Since the genetic deletion of the eNOS gene in our studies does not abolish the protection afforded by preconditioning, our results indicate that the increase of eNOS expression is not required for the late phase of protection in the kidney. The NO generated from iNOS after the initial ischemia/reperfusion may react with reactive oxygen species (ROS), such as superoxide \( O_2^- \) to generate other oxidant species such as ONOOand/or OH. ROS have been found to be essential for preconditioning in the heart (50). In the present studies, the protection afforded by 30 min of ischemic preconditioning, which increases iNOS expression, is partially inhibited by inhibition of iNOS, whereas the protection afforded by 15 min of ischemia, which did not increase iNOS expression, is not inhibited by treatment with l-NIL. With the longer period of ischemia there is persistent renal injury, which is not present with the shorter ischemic periods. Thus the prolonged protection induced by 30 min of ischemic preconditioning is partially mediated by the increase in generation of NO by iNOS. This persistent increase in iNOS expression may be caused by recurrent low grade tissue injury.

As an extension of our previous reports (2, 6) we observed that enhanced HSP-25 expression persists for 12 weeks after the initial ischemia. HSPs confer cytoprotection against ischemia. ATP depletion or reactive oxygen species in many organs and cultured cells through stabilization of the actin cytoskeleton and/or reduction of the inflammatory reaction (51–54). We also reported that the overexpression of HSP-25 protein in renal epithelial LLC-PK1 cells using adenoviral vectors protects cells from injury due to oxidants and chemical anoxia (6). HSPs suppress cytokine-induced IL-8 and TNF-\( \alpha \) expression and the translocation of the p65 subunit of NF-\( \kappa \)B, which regulates iNOS expression (13, 55). In iNOS \(-/-\) mice, however, where the preconditioning effect is mitigated, the expression of HSP-25 is elevated in both iNOS \(-/-\) and iNOS \(+/+\) animals. Fifteen minutes of ischemic preconditioning also results in an increase of HSP-25 expression and the increased HSP-25 persists for 8 days later. It is possible that the residual protection seen in iNOS \(-/-\) mice and the protection afforded by short periods of ischemia are related to up-regulation of HSP-25.

In summary, we have demonstrated that the mouse kidney is profoundly protected against ischemia/reperfusion injury imposed up to 12 weeks after an initial 30-min ischemic exposure. Preconditioning occurs in more than one mouse strain indicating it is not strain-specific. Preconditioning induces iNOS expression. iNOS inhibition by pharmacological inhibitors or genetic deletion partially abolishes the protective effects of preconditioning, but eNOS gene deletion does not. Inducible NO inhibition does not mitigate protection 8 days after a shorter 15 min of ischemia under conditions where the ischemia results in much less chronic interstitial accumulation of \( \alpha \)-smooth muscle actin. These results indicate that iNOS plays an important role in kidney protection afforded by prolonged ischemic preconditioning which may be explained by chronic interstitial inflammation, which stimulates iNOS to generate NO, which in turn attenuates postischemic interactions between leukocytes and endothelium.

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