Protection against ischemic kidney injury is afforded by 24 h of ureteral obstruction (UO) applied 6 or 8 days prior to the ischemia. Uremia or humoral factors are not responsible for the protection, since unilateral UO confers protection on that kidney but not the contralateral kidney. Prior UO results in reduced postsischemic outer medullary congestion and leukocyte infiltration. Prior UO results in reduced postsischemic phosphorylation of c-Jun N-terminal stress-activated protein kinase 1/2 (JNK1/2), p38, mitogen-activated protein kinase (MAPK) kinase 4 (MKK4), and MKK3/6. Very few cells stain positively for proliferating cell nuclear antigen after obstruction, indicating that subsequent protection against ischemia is not related to proliferation with increased numbers of newly formed daughter cells more resistant to injury. UO increases the expression of heat shock protein (HSP)-25 and HSP-72. The increased HSP-25 expression persists for 6 or 8 days, whereas HSP-72 does not. HSP-25 expression is increased in the proximal tubule cells in the outer stripe of the outer medulla postobstruction, prior to, and 24 h after ischemia. In LLC-PK1 renal epithelial cells, adenovirus-expressed human HSP-27 confers resistance to chemical anoxia and oxidative stress. Increased HSP-27 expression in LLC-PK1 cells results in reduced H$_2$O$_2$-induced phosphorylation of JNK1/2 and p38. In conclusion, prior transient UO renders the kidney resistant to ischemia. This resistance to functional consequences of ischemia is associated with reduced postsischemic activation of JNK, p38 MAP kinases, and their upstream MAPK kinases. The persistent increase in HSP-25 that occurs as a result of UO may contribute to the reduction in phosphorylation of MAPKs that have been implicated in adhesion molecule up-regulation and cell death.

Prior ischemia leads to resistance against subsequent ischemia in a number of organs (1–4). Recently, we observed in kidney that the acquired resistance persists for at least 15 days and is associated with a reduced activation of the JNK and p38 MAPKs and their upstream MAPK kinases (5). Renal ischemia causes tubular necrosis, and the damaged tubular cells are replaced by regenerating cells. Since dividing cells are less susceptible to oxidative stress than quiescent cells (6) and the aged heart is more susceptible to ischemic-induced DNA damage than the young heart (7), it is possible that epithelial cell regeneration may explain resistance to ischemia. To gain insight into mechanisms responsible for the protection observed with preconditioning, we evaluated whether other interventions that did not result in mitogenesis could protect the kidney against ischemia. Transient ureteral obstruction can result in renal failure in the absence of tubular necrosis (8). Zager (9) has reported that proximal tubular segments, isolated 24 h after ureteral ligation, are resistant to hypoxia/reoxygenation injury. This protection did not correlate with tubular proliferation (as determined by proliferating cell nuclear antigen (PCNA) staining) and was without any observed changes in HSP-70 or antioxidant enzyme expression (9). The mechanisms responsible for preconditioning remain poorly understood. Since ischemic acute renal failure continues to be associated with a very high mortality rate in humans, it is important to understand how the kidney uses endogenous processes to protect itself. With this understanding, it might be possible to mimic these processes using exogenous influences and hence prevent and/or alter the course of the disease. We characterized the protection afforded by ureteral obstruction in the intact kidney and explored potential mechanisms responsible for the protection.

Prior heat shock confers cytoprotection against ischemia or ATP depletion in many organs and cultured cells (10–13), although this is not a universal finding (14). In the kidney, the effect of the heat shock response and HSPs to protect against ischemic injury remain controversial (9, 14, 15). Recently, it has been suggested that prior heat shock reduces the inflammatory reaction (16), which is one of the major mediators of ischemia/reperfusion injury (17, 18), and attenuates a posts ischemic microcirculatory disturbance (19, 20). Prior heat shock suppresses cytokine-induced interleukin-8 and tumor necrosis factor-$
\alpha$ expression and the translocation of the p65 subunit of NF-$\kappa$B (18). Heat shock protects monocytes against oxidant-induced toxicity (21). This reduction in activation may contribute to a decrease in outer medullary congestion (22).

The MAPKs have been implicated in posts ischemia/reperfu-

mitogen-activated protein kinase; PCNA, proliferating cell nuclear an-
tigen; ERK, extracellular signal-regulated kinase; PBS, phosphate-
buffered saline; HSP, heat shock protein; LDH, lactate dehydrogenase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MOI, multiplicity of infection.
sion cell survival, necrosis, and apoptosis (23–27). Prior heat shock has been shown to both activate these kinases in organs and isolated mammalian cells and suppress their activation in the presence of other stresses (28, 29). HSP-27 is a terminal substrate of the p38 MAPK cascade and is phosphorylated by activation of MAPK-activated protein kinases 2/3 (30).

We examined whether the protective effect of prior ureteral obstruction is associated with expression of HSP-25 and/or activation of the ERK, JNK, and p38 MAPK pathways. We report that prior transient ureteral obstruction protects the kidney against remote ischemia, and the protection correlates with persistent up-regulation of HSP-25 in the S3 proximal tubular cells of the outer stripe of the outer medulla, reduced postischemic leukocyte infiltration, and reduced postischemic activation of JNK1/2 and p38 signal pathway cascades. Furthermore, exogenous expression of HSP-27, the human ortholog of HSP-25, results in reduced phosphorylation of JNK1/2 and p38 and confers protection against oxidative stress and chemical anoxia in LLC-PK1 epithelial cells.

**MATERIALS AND METHODS**

**Animal Preparation**—All in vivo experiments were performed in male BALB/c mice (Charles River Laboratory) weighing 20–25 g. Mice were allowed free access to water and standard mouse chow. Plasma creatinine was determined prior to the experiment. Animals were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally) and administered 1 ml of 0.9% NaCl (37 °C) intraperitoneally on the day of surgery (day 0). Body temperature was maintained at 36.0–37.5 °C throughout the procedure. Animals were divided into seven (I–VII) groups (Table I). Kidneys were exposed through flank incisions. Animals in groups I, III, and VII underwent sham surgery. Other animals were subjected to bilateral (group II) or unilateral (groups IV–VII) ureteral obstruction by clamping the ureter with nontraumatic microanastomosis clamps (Roboz, Rockville, MD). After 24 h, the clamps were removed. At day 8, group I and II mice were exposed to 30 min of bilateral ischemia. In groups III and V, left kidney in group IV) was removed, and the other kidney was subjected to 25 min of bilateral ischemia at day 6.

Kidneys of experimental groups were harvested at the times indicated in the figures. Some kidneys were snap frozen in liquid nitrogen and subsequently used for Western analysis or determination of myeloperoxidase activity.

**Renal Functional Parameters**—Seven microliters of blood were taken from the retrobulbar vein plexus at the times indicated in the figures. Plasma creatinine concentrations were measured using a Beckman Creatinine Analyzer II. Myeloperoxidase (MPO) Activity—MPO activity, an index of tissue leukocyte infiltration, was measured in 24-h postischemic kidney as previously described (17). Activity was normalized to protein concentration.

**Immunocytochemistry**—Kidneys were perfused via the left ventricle with 30 ml of phosphate-buffered saline (PBS) for 2 min at 37 °C and then PLP (2% paraformaldehyde, 75 mM L-lysine, 10 mM sodium periodate) fixative. Kidneys were excised and placed in PLP overnight at 4 °C. The 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 ootrycylane compound (Sakura FineTek, Torrance, CA), frozen in liquid nitrogen, and then cut into 5-μm sections using a cryotome. Sections were mounted on Fisher Superfrost Plus (Fisher) microscope slides, dried in air, and stored at -20 °C.

To detect gp330 (a proximal tubule marker), sections were dried, incubated in PBS containing 0.1% SDS for 5 min, washed in PBS for 10 min, and incubated in blocking buffer (PBS containing 2% bovine serum albumin) for 20 min at room temperature. Sections were then incubated with antibody to gp330, diluted in blocking buffer in a humidified chamber for 1 h at room temperature. Sections were washed with PBS twice for 5 min each, with PBS containing 1.9% NaCl (high salt PBS) for 5 min and with PBS for 5 min. For negative controls, primary antibodies were replaced with blocking solution.

Secondary antibodies were diluted in blocking buffer containing 4’6-diamino-2-phenylindole (1:4,000), a nuclear marker, placed on sections for 1 h at room temperature. Sections were then washed twice in high salt PBS and once in PBS. For double staining with phallolidin, which stains the actin cytoskeleton, the sections were incubated in blocking buffer containing fluorescein isothiocyanate-labeled phallolidin for 20 min at room temperature, washed three times in PBS for 5 min each, and mounted with a 1:1 mixture of Vectashield (Vector Laboratories) and 0.3 μM Tris–HCl, pH 9.9. Images were viewed on a Nikon FXA epifluorescence microscope and collected using a digital camera (Hamamatsu). In some cases, images were merged by using IP Lab spectrum software.

To prepare sections for immunostaining of PCNA or HSP-25, double staining for HSP-25 and Na⁺-ATPase, or double staining for HSP-25 and aquaporin-1, dried sections were washed in PBS three times for 5 min each at room temperature, fixed in 100% methanol for 10 min at 4 °C, washed again in PBS three times for 5 min at room temperature, boiled in 10 mM sodium citrate buffer, at pH 6.0, for 10 min using a microwave oven, and gradually cooled to room temperature for 30 min. Sections were again washed in PBS three times for 5 min each at room temperature and incubated in blocking buffer for 1 h at room temperature. Subsequent procedures were carried out as described above.

**Antibodies for Immunocytochemistry**—PCNA antibody (diluted 1:100) was obtained from DAKO AS (Denmark). Aquaporin-1 antibody (1:300 (31)) was obtained from Dr. A. S. Van Hoek (Massachusetts General Hospital). gp330-megalin antibody (1:1,000 (32)) was obtained from Dr. R. T. McCluskey (Massachusetts General Hospital). The α1 subunit of Na⁺-K⁺-ATPase antibody (6F; 1:5 (33)) was obtained from Dr. C. J. Woolf (Massachusetts General Hospital) (34). LLC-PK1 cells infected with Ad-LacZ. The secondary antibodies used were Cy3-conjugated donkey anti-rabbit IgG (1:400) obtained from Jackson Immunoresearch Laboratories, Inc., and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100) obtained from Sigma.

**Cell Culture and Adenovirus-mediated HSP-27 Gene Transfer**—LLC-PK1 cells were maintained in culture at 95% air, 5% CO₂ at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. An adenoviral construct expressing HSP-27 (Ad-HSP-27) was obtained from Dr. C. J. Woolf (Massachusetts General Hospital) (34). LLC-PK1 cells were infected at an MOI of 100, and cells were studied 48 h after infection. For controls, cells were infected under the same conditions with adenovirus containing the lacZ gene (Ad-LacZ).

**Cell Injury in Vitro**—LLC-PK1 cells infected with Ad-HSP-27 or Ad-LacZ, were treated with 10 mM deoxyglucose and 10 mM sodium cyanide in metabolic substrate-free Kreb’s-Henseleit buffer for 4 h or with 1 mM H₂O₂ in Hanks’ balanced salt solution for 2 h. Cell injury was evaluated by lactate dehydrogenase (LDH) release. LDH activity was measured with the LDH assay kit (Sigma) according to the manufacturer’s protocol.

**Western Blot Analysis**—Proteins were extracted from kidneys or

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**Table I**

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Initial procedure (day 0)</th>
<th>Second procedure (day 6 or day 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7</td>
<td>Sham ureteral obstruction (L, R)</td>
<td>30-Min bilateral ischemia (day 8)</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>Ureteral obstruction (L, R)</td>
<td>30-Min bilateral ischemia (day 8)</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>Sham ureteral obstruction (L)</td>
<td>Nephrectomy (R) and 25-min ischemia (L)</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>Ureteral obstruction (L)</td>
<td>Nephrectomy (L) and 25-min ischemia (R)</td>
</tr>
<tr>
<td>V</td>
<td>7</td>
<td>Ureteral obstruction (L)</td>
<td>25-Min bilateral ischemia</td>
</tr>
<tr>
<td>VI</td>
<td>6</td>
<td>Ureteral obstruction (L) and sham ureteral obstruction (R)</td>
<td>25-Min bilateral ischemia</td>
</tr>
<tr>
<td>VII</td>
<td>5</td>
<td>Sham ureteral obstruction (L, R)</td>
<td>25-Min bilateral ischemia</td>
</tr>
</tbody>
</table>

Prior Kidney Obstruction Protects against Ischemic Injury
LLC-PK₁ cells as previously described (26). Protein samples were separated on either 10% or 12% SDS-PAGE gels and then transferred to an Immobilon membrane (Millipore Corp., Bedford, MA). Membranes were incubated with antibodies against phospho-JNK1/2 (Thr183/Tyr185), phospho-p38 (Thr 180/Thr182), phospho-ERK1/2 (Thr 202/Tyr204), phospho-SEK1/MKK4, phospho-MKK3/6, phospho-MEK1/2, total MEK1/2, and total MKK3 (Cell Signaling); ERK1/2, JNK1, p38, and MKK4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); HSP-27 and HSP-72 (Upstate Biotechnology, Inc.). Secondary antibodies, conjugated with horseradish peroxidase (Santa Cruz Biotechnology), were detected by the ECL system (Amersham Pharmacia Biotech).

Statistics—All results were expressed as mean ± S.E. *p < 0.05 was taken as statistically significant. The number of animals in each group was 5–8, as indicated in Table I.

RESULTS

Effects of Prior Ureteral Obstruction on Renal Function after Subsequent Ischemia/Reperfusion—Twenty-four hours of bilateral ureteral obstruction results in a marked increase in plasma creatinine levels. Plasma creatinine returns to baseline within 3 days after relief of the obstruction (Fig. 1A). Sham-treated animals subsequently exposed to 30 min of bilateral renal ischemia have a marked increase in plasma creatinine 24 h after the ischemia. In contrast, in animals previously exposed to 24 h of bilateral ureteral obstruction, 30 min of bilateral renal ischemia 7 days after the release of the obstruction had no effect on the levels of plasma creatinine (Fig. 1A). To evaluate whether the systemic effects of uremia might play a role in the protection, animals (groups IV and V) were subjected to 24 h of unilateral ureteral obstruction on day 0, a maneuver that does not result in an increase in plasma creatinine. On day 6, one kidney was removed, and the remaining kidney was rendered ischemic for 25 min. Prior ureteral obstruction protected the previously obstructed kidney but not the contralateral kidney against ischemia, even in the absence of an increase in plasma creatinine (Fig. 1B). Thus, uremic factors are not responsible for the protective effects. To evaluate whether uninephrectomy itself contributed to the protection, animals were subjected to 24 h of unilateral ureteral obstruction at day 0 followed by 25 min of bilateral ischemia 6 days later without nephrectomy (group VI). Twenty-four hours after ischemia, plasma creatinine was not increased due to protection afforded by unilateral obstruction in the ipsilateral kidney when the contralateral kidney is in place. In group VII animals, which were subjected to unilateral sham surgery on day 0, 25 min of bilateral ischemia had a marked effect on plasma creatinine levels 24 h later (Fig. 1C). In no group did unilateral ureteral obstruction (groups IV–VI) nor sham operation (group III and VII) have any effect on plasma creatinine concentration (Fig. 1, B and C).

Reduction of Postischemic Outer Medullary Congestion and Postischemic Leukocyte Infiltration by Prior Ureteral Obstruction—Twenty-four hours after ischemia, kidneys were perfusion-fixed with PLP fixative and hemisected. Severe postischemic congestion was observed in the outer medulla of nonpreconditioned kidneys (group VI), whereas there was no significant congestion in the preconditioned kidneys in which ischemia was induced 5 days after release of obstruction (group VI; Fig. 2A).

When the extent of tissue leukocyte infiltration was determined by tissue MPO activity at 24 h after ischemia, there was 0. Eight days after the first surgery, animals were subjected to bilateral ischemia for 30 min. B and C, 6 days after the first surgery one kidney was nephrectomed, and the contralateral kidney was exposed to 25 min of ischemia (groups III–VI) (B), or both kidneys were subjected to 25 min of ischemia (groups VI and VII) (C). The number of animals in each group was 5–7 as indicated in Table I. Values are expressed as mean ± S.E. *p < 0.05 versus before ischemia. I, ischemia; L, left kidney; Neph, nephrectomy; R, right kidney; S, sham.

**Fig. 1.** Effect of prior ureteral obstruction on plasma creatinine after ischemia. A, animals were subjected to either sham surgery (group I) or 24 h of bilateral ureteral obstruction (group II) on day 0–7.
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Effect of Prior Ureteral Obstruction on the Postischemic Phosphorylation of MAPKs (JNK1/2, p38, and ERK1/2) and MAPK Kinases (MEK1/2, MKK3/6, and MKK4)—MAPK signal pathways have been implicated in ischemia/reperfusion injury (5, 26, 27). We examined whether prior ureteral obstruction affects the postischemic activation of these kinase cascades. Ischemia/reperfusion resulted in activation of JNK1/2, p38, and ERK1/2 as previously observed (5, 26). In kidneys previously exposed to ureteral obstruction, ischemia on day 6 results in much less activation of JNK1/2 and p38 than is seen in postischemic kidneys not previously obstructed (Fig. 5A). In contrast, prior obstruction did not alter the initial response of ERK1/2 to ischemia/reperfusion (Fig. 5A). In kidneys not previously obstructed, however, the postischemic activation of ERK1/2 persists longer than in kidneys previously obstructed (Fig. 5A). The upstream regulators of these kinases, MKK4 (an upstream activator of JNK1/2 and possibly p38), MKK3/6 (an upstream activator of p38), or MEK1/2 (an upstream activator of ERK1/2), were markedly activated by ischemia/reperfusion (Fig. 5B). MKK4 and MKK3/6 phosphorylation are markedly reduced in postischemic kidneys previously obstructed. Like its downstream kinases ERK1/2, activation of MEK1/2 in the kidneys previously sham-obliterated persists longer than in kidneys previously obstructed. The activation patterns of the MAPK kinases parallel the phosphorylation of MAPKs, suggesting that the activation of JNK1/2, p38, and ERK1/2 are potentially explained by changes in activation patterns of their respective MAPK kinases.

Postobstructive and Postischemic PCNA Staining—To examine whether the resistance to ischemic injury induced by prior ureteral obstruction may be related to mitogenesis, with possibly decreased sensitivity of daughter cells to ischemic injury, we evaluated the expression of PCNA, a marker of cell proliferation, in postobstructed kidneys. There was no significant increase in nuclear PCNA staining in the proximal tubular cells of the outer medulla of postobstructed kidneys 24 h or 5 days after relief of the obstruction (Fig. 6). Obstruction alone did not result in significant tubular cell damage. Ischemia on day 6 significantly increased the expression of PCNA detected 24 h later in kidneys previously obstructed or sham-treated, but the number of cells expressing PCNA is significantly less in the obstruction-preconditioned kidneys than in sham-pre-treated kidneys (Fig. 6, A and B).

Postobstructive and Postischemic Expression of HSP-25 and HSP-72—Since the expression of HSP-27, the human ortholog of HSP-25, is up-regulated in the postischemic proximal tubule (5, 42) and this small heat shock protein stabilizes the cytoskeleton (43), we considered that prior ureteral obstruction might increase HSP-25 expression and that the increased HSP-25 expression might protect against postischemic cellular injury. Five days after release of the ureteral clamp, expression of...
immunoreactive HSP-25 is greater in the proximal tubular cells in the outer stripe of the outer medulla when compared with these structures in kidneys sham-operated on day 0 (Figs. 3A and 4A). At 24 h after ischemia, imposed on day 6, there is increased HSP-25 expression in the proximal tubular cells in the outer medulla of both kidneys previously sham-operated or obstructed (Figs. 3B and 4B). There was no HSP-25 expression in the dead cells without nuclear integrity. When the levels of HSP-25 and HSP-72 expression were evaluated by Western blot analysis, ureteral obstruction increased the HSP-25 and HSP-72 expression. The significant increase of HSP-25 expression by ureteral obstruction persists for 6 or 8 days (Fig. 7), whereas the increase of HSP-72 expression does not persist (Fig. 7), indicating that HSP-25 expression might be important for the remote protection. Twenty-four hour postischemic HSP-25 expression is greater in the preconditioned kidneys than in the nonpreconditioned kidneys, whereas there are no significant differences in the postischemic elevation of HSP-72 between kidneys previously sham-operated and kidneys previously obstructed (Fig. 7, B and C).

Effects of HSP-27 Expression Mediated by Adenoviral HSP-27 Gene Transfer on Chemical Anoxia and Oxidative Stress in LLC-PK1 Renal Epithelial Cells—To examine whether the postobstructive resistance to ischemic injury might be contributed to by enhanced HSP-25 expression, we infected LLC-PK1 renal epithelial cells with an adenovirus expressing the human ortholog of HSP-25, HSP-27. LLC-PK1 cells were incubated with Ad-HSP-27 at an MOI of 100 or 500 or Ad-LacZ at an MOI of 100 for 48 h. When HSP-27 expression was measured by Western blot analysis at 48 h after infection, there was a dose-dependent expression of HSP-27 in cells infected with Ad-HSP-27, whereas HSP-27 expression is not observed in the cells infected with Ad-LacZ or noninfected cells (Fig. 8A). Increased HSP-27 expression confers cell resistance to chemical anoxia produced by 10 mM deoxyglucose and 10 mM sodium cyanide or oxidative stress induced by 1 mM H2O2. LDH release induced by chemical anoxia or oxidative stress is significantly reduced when compared with cells previously infected with Ad-HSP-27 (Fig. 8B). The increase of HSP-27 expression results in a decrease in LDH release from cells.

FIG. 3. Immunocytochemical assessment of Na⁺-K⁺-ATPase, aquaporin-1, and HSP-25 expression 6 days after ureteral obstruction (A) and 24 h after subsequent ischemia (B). Animals were subjected to either sham operation (S) or 24 h of ureteral obstruction (UO) on day 0, and kidneys were harvested at day 6 (A). Other animals, sham-treated (S) or obstructed (UO) on day 0, were exposed to 25 min of bilateral ischemia (I) at day 6, and these kidneys were harvested on day 7 (B). Na⁺-K⁺-ATPase and HSP-25 antibodies were applied to the same sections, and serial sections were used for aquaporin-1 staining. Sections were taken from outer medulla. Bar, 50 μm. The arrows identify an S3 proximal tubule, and asterisks identify a thick ascending limb.
exposed to 1 mM H$_2$O$_2$ (Fig. 8C). In either cells treated with deoxyglucose/cyanide or H$_2$O$_2$ the expression of HSP-27 does not, however, prevent completely a significant increase in LDH release.

Effects of HSP-27 Expression Mediated by Adenoviral HSP-27 Gene Transfer on MAPK Activation Induced by H$_2$O$_2$ Treatment in LLC-PK1 Renal Epithelial Cells—To determine whether increased HSP-27 expression might account for the reduction in postischemic activation of MAPKs, we exposed LLC-PK1 cells, infected with either Ad-HSP-27 or Ad-LacZ at 100 MOI, to 1 mM H$_2$O$_2$. Cells expressing HSP-27 had a reduction in levels of phospho-JNK1/2 and phospho-p38, when compared with levels in the cells infected with Ad-LacZ (Fig. 9).

**DISCUSSION**

Our studies demonstrate that prior transient ureteral obstruction results in protection against ischemic injury imposed 5–7 days after release of the obstruction. Ureteral obstruction results in a persistent increase of HSP-25 expression and reduced postischemic activation of JNK1/2 and p38. An imposed increased expression of the human ortholog of HSP-25, HSP-27, in LLC-PK1 renal epithelial cells, using an adenoviral construct, leads to partial protection against injury mediated by chemical anoxia or oxidative stress and reduced phosphorylation of JNK1/2 and p38 induced by H$_2$O$_2$.

Zager (9) has studied the effects of prior obstruction on susceptibility to hypoxic/reoxygenation injury of tubules isolated directly from obstructed kidneys. In those studies, Zager reports that the proximal tubules isolated from the kidneys obstructed for 24 h are resistant to hypoxic injury and that the resistance does not require prior uremia, increased HSP-70 expression, or tubular proliferative response (9). We previously developed an ischemic preconditioning model and concluded that a local effect within the kidney is probably responsible for protection against subsequent ischemia (5). In the present studies, we confirm our conclusion derived in the ischemic preconditioning model that prior uremia is not necessary for the protection and that a systemic circulating factor is not
responsible for the effect, since protection is observed in the ipsilateral kidney, which sustained unilateral obstruction, but not in the contralateral kidney.

Although both bilateral ureteral obstruction and ischemia results in acute renal failure, the cellular basis of development of renal failure is different (4). Transient ureteral obstruction does not result in necrotic cell death, whereas ischemia does (8). After ischemia, the damaged tubule cells are replaced with regenerating cells (22, 44). Cells resulting from this regenerative process may be resistant to injury (6). In the studies reported here, we observed that after ureteral obstruction very few proximal tubule cells express PCNA. Thus, transient ureteral obstruction does not enhance cell mitogenesis, indicating that the resistance acquired by ureteral obstruction is not due to epithelial cell regeneration with more resistant "younger" cells.

Ischemia results in the disruption of the actin membrane cytoskeleton of renal proximal tubule cells (36, 39). The actin cytoskeleton has been implicated in structural and functional alterations including loss of surface membrane polarity, apical membrane bleb formation, detachment of cells, and redistribution of the cortical actin network throughout the cytoplasm (35, 36, 38, 41). In the present studies, we observed that postischemic changes in the actin cytoskeleton and histological damage are much less apparent in the preconditioned kidneys than in nonpreconditioned kidneys. In the proximal tubular cells of kidneys previously obstructed, immunocytochemical staining for Na^+-K^+-ATPase, aquaporin-1 and gp330 protein reflect a pattern that is more normal than in the kidneys previously sham-obstructed. The actin cytoskeleton is important for localization of these membrane proteins (35, 36, 38, 41). Zager et al. (45) have argued that a change in the cholesterol content of the cell membrane may contribute to the protection they see in proximal tubules that have acquired cytoresistance. It is thus possible that prior ureteral obstruction increases membrane resistance against ischemic insults, unrelated to the differentiation status of the cell.

The MAPKs have been implicated in postischemia/reperfusion cell survival, necrosis, and apoptosis (5, 24, 25). It has been proposed that activation of JNK and p38 kinases contribute to cell death. Reduction of JNK and p38 activation reduces ATP depletion and ischemic injury (28). Ischemia/reperfusion acti-
vates p38 MAPK, which results in activation of MAPK-activated protein kinase-2/3 and phosphorylation of its substrate HSP-25/27. It has been shown in studies in hearts that inhibition of p38 MAPK blocks preconditioning (46, 47). We have reported that the protection afforded the kidney as a result of remote ischemic preconditioning correlates with a reduced postischemic activation of JNK1/2 and p38 (5).

Heat shock proteins are induced by various stresses including heat shock, oxidant radicals, chemical toxins, and ischemia/reperfusion (5, 48). It is known that prior heat shock confers resistance against damage induced by ischemia or ATP depletion in many organs including kidney and cultured cells (28, 49), although this is not a universal finding and there is a good deal of inconsistency with respect to protection against ischemic injury to the kidney. Among HSPs, HSP-25/27 stabilizes the actin cytoskeleton (43). Zager (9) reported that the protection seen in proximal tubules from obstructed kidneys occurred without the expression of HSP-70. Kelly et al. (13) found that renal resistance against ischemia conferred by heat stress is dependent on the timing of ischemia relative to heat stress and was not observed when HSPs were not induced. In our ischemic preconditioning model, we reported that protection induced by prior ischemia against subsequent ischemic insult was correlated with the levels of HSP-25 expression, which depended on the length of prior ischemia (5). In the present studies, we observed that HSP-72 and -25 expression both increased after obstruction. The increased HSP-25 expression was sustained for 6 or 8 days, but HSP-72 was not. The increased HSP-25 was localized to proximal tubular cells in the outer stripe of outer medulla, which are particularly sensitive to ischemia. HSP-25 is expressed in the surviving proximal tubular cells, which maintain an intact nucleus and some degree of cytoskeletal integrity. It is possible that the increased HSP-25 expression might confer stabilization of the actin cytoskeleton.

In the present studies, we observed that prior ureteral obstruction reduced the posts ischemic activation of JNK1/2, p38, MKK4, and MKK3/6. While Western blot analysis of kidney, with its many cell types, does not permit definitive conclusions.

**FIG. 7.** Western blot analysis of HSP-25 and HSP-72 in kidneys. A and B, animals were exposed to either sham operation (S) or 24 h of ureteral obstruction (UO) on day 0. A, kidneys were harvested at 1, 2, 4, or 6 days after sham operation or ureteral obstruction. B, 8 days after sham operation or ureteral obstruction, kidneys were exposed to either sham operation or 30 min of bilateral ischemia (I), and then the kidneys were harvested 24 h after ischemia, on day 9. C, the right kidney (R) was sham operated, and the ureter of left kidney (L) was obstructed for 24 h. Six days later, kidneys were exposed to either sham operation or 25 min of bilateral ischemia. Kidneys were then removed at 0.5, 1.5, or 24 h after the procedure. The density of Western blot bands was quantified by the NIH image program. Data are presented as fold increases relative to sham control. Values are expressed as the mean from 3–5 separate experiments.

**FIG. 8.** Effect of the increased HSP-27 expression on cell injury by chemical anoxia or oxidative stress in LLC-PK1 renal epithelial cells. A, LLC-PK1 cells were infected with an adenoviral vector expressing HSP-27 (Ad-HSP-27) at an MOI of 100 or 500, or control adenoviral vector expressing β-galactosidase (Ad-LacZ) at an MOI of 100 for 48 h. HSP-25 expression was detected by Western blot analysis. B and C, cells were infected with either Ad-HSP-27 or Ad-LacZ at an MOI of 100 for 48 h. Cells were incubated with Krebs-Henseleit buffer containing either 20 mM dextrose as a control or 10 mM sodium cyanide plus 10 mM 2-deoxyglucose for 4 h (B) or Hanks’ balanced salt solution (HBSS) containing 1 mM H2O2 for 2 h (C). After the incubation, LDH activities were measured in the cell supernatant and total lysate. The percentage release of total LDH is presented as mean ± S.E. * and #, p < 0.05 versus control without H2O2 treatment and Ad-LacZ-infected with H2O2 treatment, respectively.
to be drawn regarding a change in kinase activation in a particular cell, it is possible that HSP-25/27 is involved in reduced activation of JNK1/2, p38, and their upstream MAPK kinases in vulnerable outer medullary proximal tubule cells. Rogalla and colleagues reported that large oligomers of HSP-27 are necessary for chaperone action and resistance against oxidative stress. Phosphorylation down-regulates these activities by dissociation of HSP-27 complexes to tetramers (50). Gabai et al. (28) reported that heat shock suppresses the subsequent heat stress-induced activation of JNK and results in thermotolerance. In the present studies, we found that increased expression of HSP-27 by adenovirus-mediated HSP-27 gene transfer reduced, but did not prevent completely, the renal epithelial cell injury induced by ATP depletion or H$_2$O$_2$. Thus, increased expression of HSP-27 alone is not sufficient to protect cells from oxidative or chemical hypoxic stress. Increased HSP-27 expression suppresses the JNK1/2 and p38 activation induced by 1 mM H$_2$O$_2$ treatment. Thus, evidence obtained from in vitro and in vivo studies are consistent with a role for HSP-25/27 up-regulation in the reduction in stress kinase activation, thus reducing their adverse effects. Since it is known that stress kinases are expressed in the proximal tubule cells in which we find HSP-25 expression, the protection afforded by HSP-25 may occur at the level of the proximal cell itself.

HSP-25 may also reduce the inflammatory response. It is well known that an inflammatory reaction is important in ischemic injury (17, 22). Adhesion molecules, such as intercellular adhesion molecule 1, contribute to leukocyte-endothelial interactions important for this inflammatory reaction (17). Congestion in the outer medulla is a consequence of enhanced leukocyte-endothelial interactions and associated inflammation (19, 20, 22). Our results reveal that prior ureteral obstruction reduces postischemic outer medullary congestion and tissue MPO activity. HSPs may decrease production of cytokines (18), reducing leukocyte-endothelial interactions (20) and mitigating congestion in the outer medulla, resulting in less hypoxic injury to the outer medullary tubules (22). Prior induction of HSPs suppresses cytokine-induced interleukin-8 and tumor necrosis factor-α expression and the translocation of the p65 subunit of NF-κB (18, 51). Kelly et al. (13) reported that prior heat stress reduces the postischemic leukocyte infiltration in rat. It is unlikely that HSP-25/27 alone is responsible for the protection seen with prior ureteral obstruction, but our data indicate that it may contribute to this protection.

In summary, we have demonstrated that the mouse kidney is profoundly protected against ischemia/reperfusion injury imposed 5–7 days after a ureteral obstruction. This protection is associated with up-regulation of HSP-25, prevention of outer medullary vascular congestion, reduction of infiltration of leukocytes, and mitigation of postischemic activation of JNK1/2 and p38 and their upstream MKK4, MEK3/6 MAPK kinases. The increased HSP-25 expression might contribute to the protection through reduced postischemic activation of JNK1/2 and/or p38 and attenuation of the postischemic inflammatory reaction.

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**References**