ATP releases HSP-72 from protein aggregates after renal ischemia

CHRISTOPH AUFRICHT, ELLEN LU, GUNILLA THULIN, MICHAEL KASHGARIAN, NORMAN J. SIEGEL, AND SCOTT K. VAN WHY
Departments of Pediatrics and Pathology, Yale University
School of Medicine, New Haven, Connecticut 06520-8064

Aufricht, Christoph, Ellen Lu, Gunilla Thulin, Michael Kashgarian, Norman J. Siegel, and Scott K. Van Why. ATP releases HSP-72 from protein aggregates after renal ischemia. Am. J. Physiol. 274 (Renal Physiol. 43): F268–F274, 1998.—The pattern of 72-kDa heat-shock protein (HSP-72) induction after renal ischemia suggests a role in restoring cell structure. HSP-72 activity in the repair and release from denatured and aggregated proteins requires ATP. Protein aggregates were purified from normal and ischemic rat renal cortex. The addition of ATP to cortical homogenates reduced HSP-72, Na⁺-K⁺-ATPase, and actin in aggregates subsequently isolated, suggesting that their interaction is ATP dependent. Altering ATP hydrolysis in the purified aggregates, however, had different effects. ATP released HSP-72 during reflow and preserved Na⁺-K⁺-ATPase association with aggregates at 2 h but had no effect in controls or at 6 h reflow and caused no change in actin. These results indicate that HSP-72 complexes with aggregated cellular proteins in an ATP-dependent manner and suggests that enhancing HSP-72 function after ischemic renal injury assists refolding and stabilization of Na⁺-K⁺-ATPase or aggregated elements of the cytoskeleton, allowing reassembly into a more organized state.

MATERIALS AND METHODS

Animal Preparation

All experiments were performed on anesthetized male Sprague-Dawley rats weighing 225–300 g as previously described (28, 29). In brief, bilateral renal ischemia was accomplished by selective occlusion of the right renal artery and aorta just proximal to the left renal artery. After 45 min, the clamps were removed, and reperfusion was visually confirmed. After reflow intervals of 15 min, 2 h, or 6 h, the kidneys were rapidly removed. Nons ischemic control kidneys were obtained from animals immediately after induction of anesthesia (28, 29).

Cell Protein Fractionation

Triton X-100 extraction. Renal cortex was homogenized in chilled extraction buffer containing 0.1% Triton X-100, 60 mM piperazine-N,N'-bis(2-ethanesulfonic acid), 2 mM trans-1,2-diaminocyclohexane-N,N',N'-tetraacetic acid (CDTA), 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N',N'-tetraacetic acid, 100 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.75 mg/l leupeptin, and 0.1 mM dithiothreitol, using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 680 g for 10 min at 4°C to pellet nuclei and large cellular fragments. The supernatant was centrifuged at 35,000 g for 14 min at 4°C to separate the Triton-soluble from the insoluble protein fraction.

Isolation of aggregated proteins. Aggregates were isolated by different centrifugation by modifying a protocol reported by Oberg et al. (22) for purification of mutant and overexpressed recombinant proteins with similar physico-
chemical properties. The Triton-insoluble subfraction was twice resuspended in extraction buffer, sonicated, and pelleted at 17,000 g for 30 min at 4°C. The resultant pellet was again twice resuspended in extraction buffer, sonicated, and centrifuged at 5,000 g for 30 min at 4°C, and the final purified pellet (aggregates) was stored in extraction buffer at -70°C.

Enhancement and inhibition of ATP hydrolysis. The effect of enhancing versus inhibiting HSP-72 activity was examined by adding excess Mg-ATP to promote ATP hydrolysis or by adding CDTA to inhibit ATP hydrolysis in parallel aliquots from the same extract. In one series of experiments ATP hydrolysis was altered in homogenates of renal cortex before isolation of aggregates and in a separate series after isolation of aggregates.

Alteration of ATP hydrolysis before isolation of aggregates. CDTA was omitted from the initial extraction buffer. Mg-ATP (5 mM) was then added to half of the homogenate while endogenous ATP hydrolysis was inhibited in the other half of the homogenate by the addition of 2 mM CDTA. Both homogenates were incubated for 60 min at room temperature before the isolation of aggregates by the fractionation procedure as described above.

ATP levels were determined by luciferase assay (9) in homogenates from 2-h reflow cortex. There was a 30% decline in homogenate ATP over the 1-h incubation in the samples that contained CDTA. Parallel addition of Mg-ATP to a separate aliquot of the same homogenates resulted in a greater than 300-fold increase in homogenate ATP levels compared with the CDTA group. Subsequent to the Mg-ATP addition, levels fell by 98% over the 1-h incubation interval, indicating enhanced ATP hydrolysis in the Mg-ATP augmented homogenates compared with the CDTA homogenates in which ATP hydrolysis was inhibited.

Alteration of ATP hydrolysis after isolation of aggregates. After completion of the fractionation procedure, CDTA was omitted from the final extraction buffer used to resuspend the aggregates. Mg-ATP (5 mM) was then added to one-half of the suspension while endogenous ATP hydrolysis was blocked in the other half via the addition of 2 mM CDTA. Both resuspended aggregates were incubated for 60 min at room temperature before the aggregates were recovered by a repeat of the final centrifugation at 17,000 g and the final purified aggregate subfraction was isolated. Minimal native BSA, but 90% of denatured rcm-BSA, was recovered in aggregate subfraction.

Protein determinations were performed in duplicate in each subfraction, according to Lowry et al. (13), using BSA as a standard. Protein samples were electrophoresed through 0.1% SDS-7.5% polyacrylamide gel with 4% stacking gel and electrophoretically transferred to nitrocellulose as previously described (28). Nonspecific binding sites were blocked, and the membranes were incubated for 1 h with antibodies against α-subunit of Na⁺-K⁺-ATPase (29), Na⁺-K⁺-ATPase (Stress-Gen, Victoria, BC, Canada) or actin (Biomedical Technologies, Stoughton, MA). Detection was with secondary antibodies, reagents, and protocols for enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). Computerized densitometry of the specific bands on all blots was performed using image analysis software IM-4000 from Georgia Instruments (Roswell, GA) as previously described (29). All reagents were obtained from Sigma Chemical (St. Louis, MO), except where indicated.

Statistics

Analysis of variance, with the least significant difference approach and the Dunnett multiple comparison test, was used where appropriate. Values for each reflow interval were compared with the respective control and considered to be significantly different if P < 0.05.

RESULTS

Na⁺-K⁺-ATPase Extractability and HSP-72 Induction

Postischemic dissociation of Na⁺-K⁺-ATPase from the membrane-cytoskeletal complex is demonstrated by increased Na⁺-K⁺-ATPase in the Triton-soluble fraction at 15 min reflow and to a lesser extent at 2 h reflow (Fig. 2). Restoration of the normal Na⁺-K⁺-ATPase distribution coincides with the progressive increase in expression of the heat-shock protein HSP-72 in both soluble and insoluble protein fractions.

Na⁺-K⁺-ATPase and HSP-72 in Protein Aggregates During Reflow

After a decrease in Na⁺-K⁺-ATPase content in aggregates at 15 min reflow, Na⁺-K⁺-ATPase returned to control levels over the next 2–6 h. HSP-72 expression was very low in aggregates from control animals and showed a marked induction with discernible protein
abundance as early as 15 min and a progressive increase at 2 and 6 h (Fig. 3).

Effects of Enhancement or Inhibition of ATP Hydrolysis

The Coomassie stain (Fig. 4) demonstrates that ATP incubation (增加了ATP) did not result in nonspecific degradation of proteins in any of the isolated subfractions. There was no difference in protein content and pattern of aggregates isolated under conditions of inhibited ATP hydrolysis (抑制了ATP)，regardless of whether the incubation occurred before (Fig. 4A) or after (Fig. 4B) the isolation of the aggregates. The redistribution of the high-molecular-mass band (at 160–180 kDa) was recently described as being typical for cellular protein aggregates (11).

Alteration of ATP Hydrolysis in Homogenates Before Isolation of Aggregates

Enhancing ATP hydrolysis (增加了ATP) in cortex homogenates reduced the deposition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, actin and HSP-72 in the protein aggregates subsequently isolated (Fig. 5). This occurred not only in the postischemic kidney cortex when HSP-72 was increased but also in control kidneys with very low HSP-72 expression. Altering ATP hydrolysis in the homogenates caused no change in the quantity of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, HSP-72, or actin in the Triton-soluble fraction, again indicating that nonspecific degradation of these three proteins under conditions of enhanced ATP hydrolysis did not occur.

Alteration of ATP Hydrolysis After Isolation of Aggregates

Alterating ATP hydrolysis after purification of the aggregates caused quite different changes in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and HSP-72 composition of the aggregated proteins. Whether ATP hydrolysis was enhanced or inhibited, no change in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase or actin could be found in aggregates isolated from control kidneys. At 2 h reflow, inhibiting ATP hydrolysis (抑制了ATP) prevented release of HSP-72, whereas incubation with ATP (增加了ATP) increased release of HSP-72 from the aggregate into the supernatant. Moreover, at 2 h reflow, inhibiting ATP hydrolysis (抑制了ATP) resulted in increased dissociation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase from the aggregate into the supernatant compared with nonischemic controls. En-
establishment of Na⁺-K⁺-ATPase anchorage to the cytoskeleton may be facilitated by the action of HSP-72.

Increasing evidence suggests that denatured proteins are not only the substrate but also the trigger for the heat-shock response (2, 10, 14, 15, 20, 26). The common denominator appears to be some feature of the denatured protein recognized by HSP-70 proteins, such as exposure of normally hidden hydrophobic domains. In support of this hypothesis microinjection of denatured BSA, but not of the respective native protein, into Xenopus laevis oocytes caused the activation of a co-injected HSP-70 hybrid gene (1). More recent studies have shown that the best predictor of stress response intensity was the extent of denatured protein aggregation (14, 15). Therefore, alterations of physicochemical properties of proteins, such as aggregation and solubility, seem to parallel the ability to elicit a stress response.

To determine whether a similar dynamic interaction occurs in the injured kidney, a protocol was adapted to purify aggregated proteins from renal cortex to examine potential interactions between HSP-72 and cell proteins known to be disrupted by renal ischemia (22). We also sought to evaluate whether the cell proteins isolated in this aggregate fraction might have relevance to the previously established link between denatured proteins and the HSP-70 chaperones. Two groups have used reductive carboxymethylation to denature and aggregate BSA in vitro (rcm-BSA) to examine potential interactions between HSP-72 and the heat-shock response (2, 10, 14, 15, 20, 26). The common denominator appears to be some feature of the denatured protein recognized by HSP-70 proteins, such as exposure of normally hidden hydrophobic domains.

Our results confirm that nonlethal renal ischemia causes transient dissociation of Na⁺-K⁺-ATPase from the membrane-cytoskeleton complex associated with increased expression of the inducible cytosolic heat-shock protein, HSP-72. Moreover, these results directly demonstrate the temporal relationship between the Triton X-100 extractability of Na⁺-K⁺-ATPase and HSP-72 elaboration (12, 28, 29). During recovery from ischemia, HSP-72 expression increases while detergent-soluble Na⁺-K⁺-ATPase decreases, suggesting that re-

**DISCUSSION**

Our results confirm that nonlethal renal ischemia causes transient dissociation of Na⁺-K⁺-ATPase from the membrane-cytoskeleton complex associated with increased expression of the inducible cytosolic heat-shock protein, HSP-72. Moreover, these results directly demonstrate the temporal relationship between the Triton X-100 extractability of Na⁺-K⁺-ATPase and HSP-72 elaboration (12, 28, 29). During recovery from ischemia, HSP-72 expression increases while detergent-soluble Na⁺-K⁺-ATPase decreases, suggesting that re-

**Fig. 5.** Alteration of ATP hydrolysis in homogenates before isolation of aggregates. Na⁺-K⁺-ATPase, HSP-72, and actin in aggregates and Triton-soluble fraction obtained from rat renal cortex that had been incubated with Mg-ATP (+ATP) or CDTA (−ATP): immunoblots demonstrating results from n = 3 determinations of each condition represented. Na⁺-K⁺-ATPase, actin, and HSP-72 all decrease in the aggregate subfraction with ATP incubation (+ATP). This effect was observed not only in postischemic kidney cortex when HSP-72 was increased but also in control kidneys with minimal HSP-72 expression. Altering ATP hydrolysis had no effect on Na⁺-K⁺-ATPase, HSP-72, or actin in Triton-soluble fraction.

**Fig. 6.** Alteration of ATP hydrolysis after isolation of aggregates: purified aggregates were incubated with ATP (+ATP) or CDTA (−ATP), and proportion of Na⁺-K⁺-ATPase, HSP-72, and actin remaining in aggregates or release into supernatant was assayed by Western analysis. CDTA incubation (−ATP) resulted in decreased Na⁺-K⁺-ATPase in aggregates obtained from 2 h reflow samples compared with control with a concomitant increased release of Na⁺-K⁺-ATPase into supernatant from the same aggregates. ATP incubation (+ATP) had no effect on controls but resulted in stabilization of Na⁺-K⁺-ATPase in aggregates obtained at 2 h reflow with less dissociation into the supernatant. At 2 h reflow, ATP released HSP-72 from the aggregate into the supernatant. There were no significant changes in actin from control or reflow samples.
proteins. Thus we would expect to find Na\textsuperscript+-K\textsuperscript+-ATPase units at this reflow interval (29). At 2 h reflow, the aggregates likely contain significantly more disrupted Na\textsuperscript+-K\textsuperscript+-ATPase, since at this interval aggregated Na\textsuperscript+-K\textsuperscript+-ATPase had risen above levels at 15 min even though transcription for new enzyme remains severely depressed (29). In addition, the aggregated Na\textsuperscript+-K\textsuperscript+-ATPase in the injured samples behaves differently from both the control and later recovery samples upon addition of ATP. The present studies, then, indicate that the aggregates contain Na\textsuperscript+-K\textsuperscript+-ATPase in different structural forms, nascent proteins under control conditions and disrupted forms after ischemia.

These results may be further interpreted by considering the essence of Na\textsuperscript+-K\textsuperscript+-ATPase interaction with the cytoskeleton. Classically, increased solubilization of Na\textsuperscript+-K\textsuperscript+-ATPase by Triton X-100 extraction is regarded as the marker for dissociation of the membrane-cytoskeleton complex (8, 16–19). However, the cytoskeletal anchorage of Na\textsuperscript+-K\textsuperscript+-ATPase may represent a continuum ranging from complete assembly to a partly assembled but unstable complex (both Triton insoluble) to complete dissociation (Triton soluble). Renal ischemia may result in a shift of this continuum toward higher degrees of instability and dissociation. In this study, the decreased content of Na\textsuperscript+-K\textsuperscript+-ATPase in the aggregates early after the injury (at 15 min reflow, Fig. 3), when Triton-soluble enzyme is maximally increased (Fig. 2), suggests the aggregates contain partially denatured, unstable, or incompletely assembled membrane protein-cytoskeleton complexes.

Support for functional interaction of HSP-72 with specific proteins can be provided by taking advantage of a cardinal feature of stress protein activity. Molecular chaperones such as HSP-72 readily bind to other proteins in the absence of ATP hydrolysis but do not act and release the attached protein without hydrolysis of ATP (4–6, 21, 25, 30). These HSPs use the energy of ATP hydrolysis to undergo a conformational change, which may result in 1) refolding or partial stabilization of denatured proteins and 2) release of reformed proteins (24). Repeated cycles of this kind would result in the repair of more complex structures, with the released substrates then reassimilating into native form. In a similar manner, cellular proteins disrupted by renal ischemia may be restored by ATP-dependent action of stress proteins. Such HSP-mediated repair should be evident in a cellular subfraction, which contains denatured proteins and HSP-72, such as the aggregates that were isolated in this study.

Enhancement of ATP hydrolysis in homogenates of renal cortex before the isolation of aggregates reduced HSP-72 content in aggregates obtained during reflow, concomitant with decreases of Na\textsuperscript+-K\textsuperscript+-ATPase and actin. This effect is consistent with ATP-dependent functional interactions between these proteins in this aggregated cell protein subfraction. Although the postischemic studies suggest that HSP-72 was released from its substrate (4, 6), similar effects of ATP hydrolysis on actin and Na\textsuperscript+-K\textsuperscript+-ATPase were seen in aggre-
gates obtained under control conditions with low expression of inducible HSP-72. Thus, in this series of experiments, HSP-72-mediated effects during reflow were not separable from constitutive effects of ATP hydrolysis, raising the possibility that other protein chaperones are involved as well.

However, the effects of altering ATP hydrolysis after isolation of the aggregates were unique to those purified after ischemia. Inhibition and enhancement of ATP hydrolysis effected no change in the aggregated Na\(^+\)-K\(^+\)-ATPase or actin in controls, indicating that effects observed in the reflow samples were specific for postischemic recovery and that nonspecific degradation or redistribution of the proteins of interest by the separate incubations did not occur. Inhibition of ATP hydrolysis prevented and enhancement of ATP hydrolysis increased release of HSP-72 from the aggregate into the supernatant at each reflow interval, as would be expected if there were specific, functional interaction between HSP-72 and denatured substrates in the aggregates (4, 6). At 2 h reflow, inhibiting ATP hydrolysis allowed increased dissociation of Na\(^+\)-K\(^+\)-ATPase from the aggregates into the supernatant compared with nonischemic controls, but enhancing ATP hydrolysis preserved the association of Na\(^+\)-K\(^+\)-ATPase with the aggregates. These effects of altering ATP hydrolysis on Na\(^+\)-K\(^+\)-ATPase in the aggregates were less prominent at 6 h reflow.

Together these results suggest that, following renal ischemia, HSP-72 interacts either directly with Na\(^+\)-K\(^+\)-ATPase or with other elements of the membrane cytoskeleton-complex in a manner dependent upon ATP hydrolysis. Enhanced HSP-72 function may have assisted refolding and stabilization of Na\(^+\)-K\(^+\)-ATPase or other nonnative elements of the membrane-cytoskeleton complex, allowing increased binding and preserved association of Na\(^+\)-K\(^+\)-ATPase with proteins in the isolated aggregates and thereby resulting in reassembly into a more organized state. This interpretation is further supported by the maximal effect of altering ATP hydrolysis in the aggregates found at 2 h reflow compared with the diminished effect at 6 h reflow. On the basis of the known pattern of recovery of cell polarity where at 2 h the cell is highly disorganized but by 6 h polarity is largely restored (12, 29), one would expect that at later reflow (6 h) the membrane-cytoskeleton complex in aggregates would be more intact and organized, thus diminishing the reparative effects of ATP hydrolysis. The continued release of HSP-72 at 6 h reflow, when the ATP effect on Na\(^+\)-K\(^+\)-ATPase is diminished, suggests that this stress protein may be complexed with other denatured proteins in the aggregates at this time. However, it cannot be excluded that ATP incubation resulted in release of HSP-72 from its substrate before it could complete its chaperone functions. In this case, the removal of other adjunctive cytosolic factors by the aggregate isolation procedures and the subsequent release of HSP-72 by ATP might have resulted in the formation of more aggregable complexes of nonnative Na\(^+\)-K\(^+\)-ATPase from the injured renal epithelia. Further studies are needed to characterize the pattern and sequence of posts ischemic disruption of the injured membrane-cytoskeletal complex and to identify other specific aggregated proteins that are substrates for HSP-72-mediated repair processes.

In conclusion, this study confirms the temporal associations between induction of HSP-72 and changes in Na\(^+\)-K\(^+\)-ATPase in renal cortex after ischemic injury, demonstrating that restoration of Na\(^+\)-K\(^+\)-ATPase anchorage to the cytoskeleton coincides with increased expression of HSP-72. It further demonstrates that a cellular subfraction of aggregated proteins can be isolated after renal ischemia. Moreover, functional interaction between HSP-72 and Na\(^+\)-K\(^+\)-ATPase during the recovery process is suggested by the effects of manipulating ATP hydrolysis on the release of HSP-72 from and the association of Na\(^+\)-K\(^+\)-ATPase with this aggregate subfraction. HSP-mediated and ATP-dependent repair processes may thus play an integral role in the restoration of cellular integrity during recovery from renal ischemia.

We are grateful for help and advice of Andrea S. Mann and for the excellent assistance of Marie Campbell and Melanie-Dawn Belanger with preparation of the manuscript and figures.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-44336 and DK-17433. C. Aufricht was a recipient of a grant from the Max Kade Foundation. This work was performed during the tenure of a Clinician-Scientist Award (to S. K. Van Why) from the American Heart Association.

Present address of C. Aufricht: Universitäts-Kinderklinik Wien, Allgemeines Krankenhaus der Stadt Wien, University of Vienna, Wahringer Gurtel 18–20, A-1090 Vienna, Austria.

Address for reprint requests: S. K. Van Why, Yale Univ. School of Medicine, Dept. of Pediatrics, 333 Cedar St., P.O. Box 208064, New Haven, CT 06520-8064.

Received 27 January 1997; accepted in final form 7 October 1997.

REFERENCES


