Heat shock prevents simulated ischemia-induced apoptosis in renal tubular cells via a PKC-dependent mechanism

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Meldrum, K. K., D. R. Meldrum, S. F. Sezen, J. K. Crone, and A. L. Burnett. Heat shock prevents simulated ischemia-induced apoptosis in renal tubular cells via a PKC-dependent mechanism. Am J Physiol Regulatory Integrative Comp Physiol 281: R359–R364, 2001.—Heat shock produces cellular tolerance to a variety of adverse conditions; however, the protective effect of heat shock on renal cell ischemic injury remains unclear. Protein kinase C (PKC) has been implicated in the signaling mechanisms of acute preconditioning, yet it remains unknown whether PKC mediates heat shock-induced delayed preconditioning in renal cells. To study this, renal tubular cells (LLC-PK1) were exposed to thermal stress (43°C) for 1 h and heat shock protein (HSP) 72 induction was confirmed by Western blot analysis. Cells were subjected to simulated ischemia 24 h after thermal stress, and the effect of heat shock (delayed preconditioning) on ischemia-induced apoptosis (terminal deoxynucleotidyl transferase dUTP nick-end labeling) and B cell lymphoma 2 (Bcl2) expression (Western) was determined. Subsequently, the effect of PKC inhibition on HSP72 induction and heat stress-induced ischemic tolerance was evaluated. Thermal stress induced HSP72 production, increased Bcl2 expression, and prevented simulated ischemia-induced renal tubular cell apoptosis. PKC inhibition abolished thermal induction of HSP72 and prevented heat stress-induced ischemic tolerance. These data demonstrate that thermal stress protects renal tubular cells from simulated ischemia-induced apoptosis through a PKC-dependent mechanism.

HEAT SHOCK PRODUCES cellular tolerance against a variety of adverse conditions. This phenomenon is thought to be due, in part, to the production of heat shock proteins (HSPs). HSPs are rapidly synthesized in response to cellular stress and function as molecular chaperones, regulating the synthesis, translocation, and degradation of proteins (2, 7, 13, 43). The HSP70 family includes the constitutively expressed HSP73, present in normal cells, and the highly stress-inducible HSP72 (43). In the kidney, HSP72 production localizes primarily to renal tubular cells (40). Induction of HSP72 occurs 24 h after heat stress, ischemia, and exposure to cellular toxins (3, 8, 29, 39, 41, 45), and its expression confers cellular resistance to further heat stress and toxin exposure (37, 45). Although HSP72 expression has also been shown to protect cells from ischemic injury (25, 44), its cytoprotective role in renal ischemia remains controversial. The ability of heat shock and HSP72 to protect renal cells from ischemia has been inconsistent in both animal models of renal ischemia-reperfusion injury (I/R) injury (15, 31) and in vitro models of renal tubular cell hypoxia-ischemia (17, 37, 41, 42).

Renal tubular epithelial cells have been demonstrated to be the cell type most susceptible to renal ischemic injury (6, 11, 22, 34). Ischemic intervals <1 h primarily result in renal tubular cell death through apoptosis (22, 34), a process by which cells use their own energy sources and proteins to undergo nonnecrotic “suicide” (16, 35, 36). As apoptosis is a gene-driven process, it is uniquely suited to molecular modulation and possible inhibition. Recently, investigators demonstrated that HSP72 inhibits apoptosis through a variety of mechanisms (4, 9, 10, 14, 21, 30), and Wang and colleagues (41) further demonstrated that heat stress inhibits apoptosis in ATP-depleted renal tubular cells (41).

The mechanisms of HSP72 induction remain unclear. Lee et al. (20) showed that protein kinase C (PKC) inhibition prevents heat stress-induced thermotolerance in human colon carcinoma cells, and Yamashita and colleagues (44) established that PKC inhibition suppresses the cardioprotective effect of heat stress in animals. PKC is a known mediator of acute ischemic preconditioning in myocardium (1, 5, 26–28, 32) and, indeed, may prove to be an important mediator of HSP72-induced delayed preconditioning and cytoprotection. With the use of an in vitro model of renal...
tubular cell ischemia in LLC-PK1 cells, the purposes of this study were, therefore, to determine whether 1) heat shock induces renal tubular HSP72, 2) HSP72 induction prevents ischemia-induced apoptosis, and 3) PKC mediates heat stress-induced ischemic tolerance.

MATERIALS AND METHODS

Cell culture. The renal tubular epithelial cell line LLC-PK1 established from pig renal cortex was cultured in MEDIUM 199 EBSS (LTI, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). The cells were passaged weekly by trypsinization (0.25% trypsin, 0.02% EDTA) after formation of a confluent monolayer and were placed in serum-free media (0.25% FBS) 24 h before stimulation.

Simulated ischemia. After formation of a confluent epithelial cell sheet, the cells were washed twice with PBS and the monolayer was immersed in mineral oil, simulating ischemic conditions by restricting cell nutrients and oxygen and limiting metabolite washout (12, 38). The cells were exposed to 1 h of simulated ischemia followed by 24 h of substrate replacement (reimmersion in MEDIUM 199 + 0.25% FBS).

Heat shock. After formation of a confluent epithelial sheet, the cells were immersed in a 43°C water bath for 1 h, with or without 2 μM chelerythrine chloride or 2 μM bisindolylmaleimide [PKC inhibitors, Sigma, St. Louis, MO; doses previously shown to be effective in renal tubular cells (24, 33)] pretreatment. Twenty-four hours after thermal stress, the cells were either collected for Western blot analysis or exposed to 1 h of simulated ischemia and 24 h of substrate replacement with subsequent Western blot analysis or apoptosis quantification.

Western blotting. Western blot analysis was performed on control samples, samples exposed to thermal stress in the presence or absence of PKC inhibition, samples exposed to simulated ischemia in the presence or absence of thermal preconditioning, and samples exposed to simulated ischemia in the presence of both thermal preconditioning and PKC inhibition. Each 100-mm confluent plate of cells was washed twice with PBS, scraped, and centrifuged. The supernatant was removed, and the cell pellet was lysed using a 2% SDS solution in protease inhibitor buffer (6.05 g Tris, 0.38 g EDTA, and 0.38 g EGTA in 1.0 l, pH 7.4) supplemented with aprotinin (5 μg/ml), leupeptin (1 μg/ml), and pefabloc (1 mM). After filtration through a QIAshredder (Qiagen, Valencia, CA), the protein extracts (200 μg/lane) were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed by incubating each membrane in 5% dry milk for 1 h, followed by incubation with either an anti-HSP72 rabbit polyclonal antibody (1:1,000, StressGen, British Columbia, Canada) or an anti-B cell lymphoma (Bcl-2) mouse monoclonal antibody (1:1,000, LabVision, Fremont, CA) for 2 h. After washing twice in Tris-PBS, each membrane was incubated for 2 h with a peroxidase-conjugated secondary antibody (1:10,000, StressGen). The membranes were then developed using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Quantitation of apoptosis. Cells were plated in four-well chamber slides at 1.5 × 10^4 cells per well and grown in 1 ml of MEDIUM 199 supplemented with 10% FBS until a confluent monolayer was obtained. The cells were then washed twice in PBS, and the monolayer was immersed in 1 ml of mineral oil. The cells were exposed to either 1 ml of media alone (control), 1 ml of media supplemented with 100 μl of mineral oil (control), 1 ml of media supplemented with either 2 μM chelerythrine chloride or 2 μM bisindolylmaleimide (controls), 1 h of simulated ischemia followed by 24 h of substrate replacement, or 1 h of heat stress (with or without 2 μM chelerythrine chloride or 2 μM bisindolylmaleimide pretreatment) followed by 1 h of simulated ischemia and 24 h of substrate replacement (reimmersion in MEDIUM 199 + 0.25% FBS). Four treatment chambers in each experimental group were quantified for apoptosis using a kit from Boehringer Mannheim (Indianapolis, IN). The kit is based on terminal deoxynucleotidyl transferase incorporation of fluorescein-dUTP to detect DNA strand breaks in the nuclei of cells undergoing apoptosis. The cell nuclei in each well were then counterstained with bis-benzimide (10 μg/ml in PBS) for 30 s. The number of apoptotic nuclei was counted in three nonoverlapping ×320 microscope fields/well and averaged. The entire protocol was then repeated in triplicate.

Cell viability. Cells were seeded at 5.0 × 10^5 and grown in 3 ml of media in 60-mm petri dishes until a confluent monolayer was obtained. With the use of the treatment groups described above, a cell suspension in PBS was prepared from each culture dish. Trypan blue (0.4%) was added to each cell suspension, and the mixture was allowed to stand at room temperature for 5 min. The number of viable cells was counted in each sample and expressed as a percentage of the total cell count.

Statistical analysis. Data are presented as mean values ± SE. Differences at the 95% confidence level were considered significant. The experimental groups were compared using ANOVA with post hoc Bonferroni-Dunn (StatView 4.5, Berkeley, CA).

RESULTS

Induction of HSP72 after heat stress. HSP72 induction by thermal stress was verified by Western blot analysis. Control samples expressed low levels of HSP72, whereas 1 h of thermal stress induced a marked increase in HSP72 expression (Fig. 1).

Evaluation and quantitation of apoptosis after simulated ischemia. Cells exposed to media alone, media supplemented with 100 μl of mineral oil, or media supplemented with either 2 μM chelerythrine chloride or 2 μM bisindolylmaleimide did not undergo apoptosis (0.667 ± 0.333, 1.33 ± 0.882, 0.777 ± 0.619, and 2.7 ± 1.2 apoptotic nuclei/high power field (hpf), respectively). In contrast, 1 h of simulated ischemia and 24 h of substrate replacement induced a significant degree of apoptosis (85 ± 14 apoptotic nuclei/hpf, P < 0.05 vs. control, Figs. 2B and 3). Cells exposed to media alone, media supplemented with 100 μl of mineral oil, and media supplemented with either 2 μM chelerythrine chloride or 2 μM bisindolylmaleimide demonstrated...
Effect of thermal stress on simulated ischemia-induced apoptosis. Heat preconditioning of renal tubular cells prevented simulated ischemia-induced apoptosis as shown in Figs. 2C and 3 (14 ± 9 apoptotic nuclei per high powered field). This effect was blocked by administering a PKC inhibitor before thermal stress (2 μM Chel; 2 μM Bis).

90 ± 2.1, 84 ± 2.8, 83 ± 2.0, and 87 ± 1.5% viability, respectively, whereas cells exposed to simulated ischemia demonstrated 81 ± 5.4% viability (not significantly different from controls).
nuclei/hpf). Cell viability in this treatment group was not significantly different from controls at 78 ± 4%.

Effect of PKC on HSP72 expression and heat stress-induced ischemic tolerance. PKC inhibition with either 2 μM chelerythrine chloride or 2 μM bisindolylmaleimide abolished thermal induction of HSP72 (Fig. 1) and prevented heat stress-induced ischemic tolerance. Cells treated with either 2 μM chelerythrine chloride or 2 μM bisindolylmaleimide before heat stress demonstrated a significant degree of apoptosis in response to simulated ischemia (78 ± 9 and 64 ± 9 apoptotic nuclei/hpf, respectively, P < 0.05 vs. control, Figs. 2D and 3), whereas cell viability was not significantly different from controls (81 ± 5.8 and 77 ± 7.2%).

Effect of simulated ischemia, thermal preconditioning, and PKC inhibition on Bcl2 expression. Simulated ischemia reduced Bcl2 expression compared with control samples (Fig. 4). Although Bcl2 expression approached control levels on cell exposure to heat stress and simulated ischemia, PKC inhibition with either 2 μM chelerythrine chloride or 2 μM bisindolylmaleimide abolished this response.

**DISCUSSION**

Thermal preconditioning confers cellular resistance against a variety of adverse conditions, in part, through the production of HSPs. HSP72, a highly stress-inducible HSP, is known to protect a variety of cells from thermal injury, toxins, and ischemia (37, 44, 45). In renal tubular cells, HSP72 is protective against thermal injury and toxins; however, its cytoprotective role in ischemia has been unclear in both animal and in vitro models (15, 17, 31, 37, 41, 42). Although overexpression of HSP72 (via transfection) in LLC-PK1 cells has been demonstrated to protect against oxidative cellular injury (H2O2 exposure) (17), and heat stress induction of HSP72 (43°C × 1 h) has been demonstrated to protect against ATP depletion-induced apoptosis (opossum kidney tubular cells) (41, 42), transfected HSP72 has not been shown to protect against hypoxic injury in LLC-PK1 cells, as measured by loss of intracellular luciferase activity (37). To clarify the cytoprotective role of heat stress in renal tubular cell ischemia, we investigated the effect of heat stress induction of HSP72 on ischemia-induced apoptosis in LLC-PK1 cells using an oil immersion model of simulated ischemia. This model uniquely simulates in vivo renal cell I/R injury by restricting cell exposure to oxygen and nutrients and preventing metabolite washout. In addition, as the mechanisms of renal HSP72 induction remain unclear, we investigated the role of PKC in HSP72 induction. PKC has been implicated as a mediator of HSP72-induced cytoprotection in the heart; however, its role in renal cell HSP72 induction has not been evaluated. This study therefore constitutes the initial demonstration that heat stress prevents simulated ischemia-induced apoptosis in LLC-PK1 renal tubular cells and distinguishes PKC as an important mediator of this process.

HSP72 induction after heat stress was assessed by exposing renal tubular cells (LLC-PK1) to a 43°C water bath for 1 h. Twenty-four hours later, cells were subjected to Western blot analysis, and a marked increase in HSP72 production was observed. The effect of heat shock on ischemia-induced apoptosis was then evaluated. Simulated ischemia was induced by immersing the entire cellular monolayer in mineral oil, a technique demonstrated to deprive cells of nutrients and restrict metabolite washout (12, 38). Apoptosis was quantified after cell exposure to 1 h of simulated ischemia and 24 h of substrate replacement in the presence or absence of heat preconditioning. The degree of apoptosis was significantly less in cells previously exposed to heat shock. Furthermore, expression of the anti-apoptotic factor Bcl2 (18, 19) decreased in response to simulated ischemia, whereas prior heat preconditioning ameliorated this response. Turman and Rosenfeld (37) suggest that transfected inducible HSP70 does not protect these cells from hypoxic injury (loss of cotransfected luciferase activity); however, our results clearly indicate that heat preconditioning prevents ischemia-induced apoptosis and support previous observations of ischemic cytoprotection (31, 41). The discrepancy between these two studies may be related to differences in the model of injury, the assessment of injury, and/or the expression HSP72 (transfection vs. heat induction).

The mechanisms of HSP72 induction were subsequently investigated by exposing the cells to a PKC inhibitor before thermal stress. PKC inhibition abolished thermal induction of HSP72, and further, prevented the ischemic cytoprotective effect of heat preconditioning. Indeed, cells exposed to PKC inhibition before heat stress demonstrated the same degree of apoptosis as cells subjected to simulated ischemia alone. These findings provide an additional link between HSP72 and cellular protection as well as indicate that PKC is an important mediator of HSP72 induction in renal tubular cells.

The mechanisms by which HSP72 prevents apoptosis appear to be multifactorial. In nonrenal cells, HSP72 has been shown to inhibit early events in the apoptotic cascade, including nuclear factor kB activation (9), p38 mitogen-activated protein kinase activation (10), and tumor necrosis factor-α-mediated cytotoxicity (14). In renal cells, evidence suggests that novel interactions between HSP72 and the caspase inhibitor Bcl2 may attenuate ischemia-induced apoptosis (41).
Renal tubular cell apoptosis is increasingly recognized as an important mechanism of cell death after ischemia (23, 34). The data presented in this study indicate that thermal induction of HSP72 protects renal tubular cells from ischemia-induced apoptosis through a PKC-dependent mechanism. As we develop a greater understanding of stress proteins, such as HSP72, and their interaction with signal events in the apoptotic cascade, effective strategies to limit or prevent ischemic renal injury may be realized.

**Perspectives**

Ischemia-induced acute renal failure (ARF) remains an important cause of patient morbidity and mortality. The loss of renal tubular epithelium associated with ARF is attributed to both apoptotic and necrotic cell death, and patient recovery depends, in part, on the protection of renal tubular cells from death. Apoptosis, as a gene-driven process, is uniquely suited to molecular modulation and possible inhibition. As we develop a greater understanding of the intracellular signals that protect against apoptosis, these signals may be manipulated to prevent renal compromise and improve patient outcome. HSPs are a large family of rapidly inducible proteins that provide cellular protection against a variety of noxious stimuli. In this study, we have demonstrated that heat stress and the subsequent induction of HSP72 protect renal tubular cells from ischemia-induced apoptosis. Although the contributions of HSP72 to renal cellular protection are significant, other members of the HSP family, including HSP27, are likely involved. The further elucidation of this family of protective mediators and their intracellular signaling mechanisms will advance the development of therapeutic strategies for the treatment and prevention of ARF.

**REFERENCES**


