Heat Shock–Induced Protection of Renal Proximal Tubular Epithelial Cells from Cold Storage and Rewarming Injury

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Cold storage and reperfusion injury to transplanted kidneys contributes to increased incidence of delayed graft function and may have a negative impact on graft survival. This study examined the mechanisms by which previous heat shock protects against cell death in an in vitro model of kidney storage. Cold storage is mimicked by incubating human renal proximal tubular epithelial (HK-2) cells in University of Wisconsin solution at 4°C with and without subsequent rewarming. Heat shock was induced by incubation of cells at 42°C for 1 h. Altered protein expression was measured by Western blot, and cell viability and apoptosis were measured by propidium iodide DNA staining using flow cytometry. The specific role of heat-shock protein 70 (HSP-70) was determined both by siRNA knockdown and by stable overexpression approaches. Cold storage and rewarming-induced cell death was associated with decreased expression of HSP-70, HSP-90, HSP-27, and Bcl-2. Previous heat shock significantly reduced HK-2 cell death after cold storage and rewarming and was associated with the maintenance of HSP-70, HSP-27, and Bcl-2 protein levels. Blocking heat stress–induced HSP-70 with siRNA did not significantly block the protective effect of heat stress against cold storage and rewarming cell death; however, overexpression of HSP-70 protected HK-2 cells from this stress. It is concluded that previous heat shock protects HK-2 cells from cold storage and rewarming injury. siRNA inhibition of HSP-70 induction did not block the protective effect of heat shock, indicating that HSP-70 is not essential to the heat stress–induced protective effect reported in this study.

increase the tolerance of organs to cold ischemia is exposure to short-term sublethal hyperthermia to induce the expression of heat-shock protein (HSP) (16). The HSP are a family of stress proteins that are expressed in cells as a protective mechanism to cellular damage caused by many different stresses (17). There is evidence to support a role for HSP in protection against transplantation-associated stresses in in vitro (18–20) and in vivo (21,22) models of kidney transplantation, although an exact mechanism of action has not been determined. This study describes the protective effects of heat shock against cell death in an in vitro model of kidney cold storage and rewarming and relates this to the expression of HSP during the cold storage period. In addition, we assessed the effects of heat shock and cold storage on Bcl-2 expression levels, because altered Bcl-2 expression is associated with cold storage–induced cell death and correlates with graft function posttransplantation (23).

Materials and Methods

HK-2 cells were purchased from LGC Promochem (Teddington, UK). Mouse anti-human HSP-27, HSP-60, HSP-70, HSP-90, and Bcl-2 antibodies were purchased from StressGen (Victoria, Canada). UW solution was supplied by Barr Laboratories (Pomona, NY). Scrambled and HSP-70–directed siRNA products were purchased from Ambion Inc. (Cambridge, UK). Fugene 6 transfection reagent was purchased from Roche Diagnostics (Basel, Switzerland). PIRESpuro3 vector was purchased from Clontech (Palo Alto, CA). DMEM/F12 cell culture medium, l-glutamine, penicillin-streptomycin solution, and Lipofectamine 2000 transfection reagent were purchased from Invitrogen Life Technologies (Paisley, UK). All cell culture materials were from Greiner Bio-One BioScience (Kremsmunster, Austria). Unless otherwise stated, all other chemicals were from Sigma-Aldrich (Gillingham, UK).

Cell Culture

HK-2 cells were cultured in 75-cm² culture flasks with DMEM/F12 that contained 5% (vol/vol) FCS, 2 mM glutamine, 2.5 ng/ml EGF, and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Induction of Heat Stress

HK-2 cells were heated to 42 ± 0.2°C for 60 min in a temperature-regulated incubator, followed by incubation at 37°C for 2 to 3 h. These conditions have previously been shown to result in optimal expression of HSP-70 in HK-2 cells (24).

Quantification of Apoptosis

Apoptosis was quantified as the proportion of cells with hypodiploid DNA, as assessed by propidium iodide (PI) incorporation as described previously (25), and analysis using an Epics XL-MCL Coulter Elite flow cytometer (Coulter, Fullerton, CA). A minimum of 5000 events were recorded and analyzed. Apoptotic nuclei were distinguished from normal nuclei by their hypodiploid DNA. Cellular debris was excluded from analysis by raising the forward threshold. All measurements were performed under the same instrument settings.

Quantification of Necrosis

PI exclusion assays were performed to distinguish intact cell membranes from the disrupted membranes of necrotic cells. Briefly, cells were centrifuged at 1300 rpm for 5 min and resuspended in 300 μl of hypotonic fluorochrome solution (50 μg/ml PI in PBS) and analyzed using an Epics XL-MCL Coulter Elite flow cytometer (Coulter). A minimum of 5000 events were recorded and analyzed. Membrane disruption was quantified as the proportion of cells with increased PI fluorescence.

RNA Extraction, cDNA Synthesis, and Reverse Transcription—PCR

Total RNA was isolated from HK-2 cells using TRIZOL reagent (Life Technologies Inc., Rockville, MD). Chromosomal DNA was removed from total RNA using DNase I (Life Technologies). First-strand cDNA was generated by reverse transcription using a SuperScript First-Strand Synthesis System for PCR (Invitrogen Life Technologies) according to the manufacturer’s instructions. The cDNA were used as a template in the subsequent PCR analysis. Real-time PCR TaqMan assay was used to quantify the relative gene expression levels of HSP-72 on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primers and probe for HSP-72 were designed using Primer Express version 2.0 (Applied Biosystems) and were supplied as a Pre-Developed Assay Reagent (PDAR kit; Applied Biosystems). Probes were labeled with 5′-FAM and with 3′-TAMRA as quencher, with the exception of the ribosomal probe, which was labeled with 5′-VIC to facilitate multiplexing. Cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C for enzyme activation, and then 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension. Values were normalized to 18S rRNA quantity for each sample.

Western Blot Analysis

Cell lysates that contained 40 μg of protein were loaded onto 12% SDS polyacrylamide gradient gels. Resolved proteins were electrophotochemically transferred to Immobilon-P (Millipore, Bedford, MA) membranes. Membranes were incubated in blocking buffer (5% BSA in PBS plus 0.1% Tween) for 1 h at room temperature and then incubated in primary antibody (1:1000 HSP-70, 1:1000 HSP-27, 1:100 HSP-90, 1:1000 Bcl-2) for an additional 1 h at room temperature. After washing for 5 min × 5 in PBS-0.1% Tween, membranes were incubated for 1 h at room temperature in horseradish peroxidase–conjugated anti-mouse IgG at 1:5000 dilution (Transduction Laboratories, Lexington, KY). Blots were washed for an additional 5 min × 5 in PBS-Tween and developed using an ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK) enhanced chemiluminescence substrate system.

siRNA Inhibition of HSP-70 Production

Incorporation of siRNA targeted against HSP-70 was achieved using Lipofectamine 2000 according to the manufacturer’s instructions. Initial experiments used fluorescence siRNA (siGLO) to optimize transfection efficiency. The cells were transfected for 4 h before replacement of the transfection medium with DMEM/F12 medium.

Generation of Stable Cell Line Overexpressing HSP-70

The HSP-70 gene was supplied by the group of A. Catapano (University of Milano, Milan, Italy). The gene was subcloned into the pIRE8puro3 bicistronic vector using BamHI and NotI restriction enzymes. Cells were grown in 75-cm² flasks to 70% confluence before transfection. Cells were transfected with 6 μg of plasmid DNA in the presence of Fugene 6 reagent for 24 h at 37°C (ratio of Fugene 6:DNA was 6:1). Stable transfectants were selected with 1.0 μg/ml puromycin for approximately 10 d followed by continued growth in the presence of 0.5 μg/ml puromycin.
Statistical Analyses
All values are expressed as mean ± SD unless specified. Statistical analysis among three or fewer groups was determined using a t test (SPSS Inc., Chicago, IL). ANOVA was used to determine statistical differences among four or more groups. Further analysis was performed using the Student-Newman-Keuls post hoc test. \( P < 0.05 \) was considered statistically significant.

Results
Effect of Cold Storage and Rewarming on HK-2 Apoptosis and Membrane Integrity
Previous studies in our laboratory demonstrated that cells that are stored in medium at 4°C or UW solution at 37°C undergo significant apoptosis and necrosis within 24 h (unpublished data). However, when HK-2 cells were stored in UW solution at 4°C, there was no significant (\( P = 0.09 \)) increase in apoptosis (Figure 1A) up to 24 h, but there was a significant increase (\( P = 0.008 \)) in cell necrosis (Figure 1B). Rewarming of the cells after 8 or 16 h of cold storage significantly increased apoptosis (\( P = 0.02 \)) as well as necrosis (\( P = 0.002 \)) at 24 h (Figure 1A). Furthermore, 16 h of cold storage followed by 8 h of rewarming resulted in increased necrosis (\( P = 0.03 \)) compared with 8 h of cold storage followed by 16 h of rewarming (Figure 1B). These results indicate that reperfusion of renal tubular epithelial cells after cold storage was associated with a significant increase in apoptosis in the presence of ongoing membrane disruption compared with control cells or those that were cold-stored for 24 h without a rewarming period.

Effect of Cold Storage and Rewarming on HK-2 Intracellular Stress Protein and Bcl-2 Expression Levels
Short-term cold storage (4 h) did not significantly alter the expression of HSP-70, HSP-90, HSP-7, or Bcl-2 (Figure 2). After 8 h of cold storage, intracellular HSP-90 levels were lower than control levels, with no change in levels of any other proteins examined (Figure 2). However, upon extension of the cold storage period to 16 h, there was a clear decrease in the levels of intracellular HSP-70, HSP-90, HSP-27, and Bcl-2 compared with control levels (Figure 2). After 16 h of cold storage, it was found that subsequent rewarming of the HK-2 cells at 37°C for 6 h led to a significant increase in the expression of all HSP and Bcl-2 to levels similar to or above those of control cells and those that had been cold-stored alone (Figure 2).

Figure 1. The effect of cold storage and recovery on HK-2 apoptosis and viability. Cells were grown in 24-well plates to 80% confluence. After varying periods of cold storage (4°C) in University of Wisconsin (UW) solution, the cells were incubated under normal cell culture conditions in warm (37°C) DMEM/F12 for varying times as indicated. (A) Apoptosis was determined as the percentage of cells with hypodiploid DNA on propidium iodide (PI) staining using flow cytometry. (B) The percentage of cells with disrupted membranes was assessed by PI exclusion using flow cytometry (\( n = 6 \)). Statistical analysis was carried out using ANOVA, using Student-Newman-Keuls post hoc test. \( *P = 0.02 \) versus control; \( **P < 0.05 \) versus all other data points at 24 h.

Figure 2. The effect of cold storage and rewarming on protein expression in HK-2 cells. Cells were grown in 24-well plates to 80% confluence and cold-stored (4°C) in UW solution with or without a subsequent reperfusion period under normal cell culture conditions at 37°C. Total cellular protein was extracted at the times indicated for Western blot analysis. Blots shown are a representative of at least three independent experiments.
Effect of Previous Heat Shock on Cold Storage and Rewarming-Induced HK-2 Cell Death

HK-2 cells were either cultured at 37°C (control) or heat shocked (42°C for 1 h) and allowed to recover for 2 h at 37°C. The cells then were cold-stored for 16 h, at which point they were either harvested for analysis of cell death or rewarmed with DMEM/F12 at 37°C for 6 h and then analyzed for cell death. Previous heat shock significantly reduced the rate of apoptosis (Figure 3A) and necrosis (Figure 3B) after cold storage and rewarmed compared with non-heat-shocked cells.

Effect of Previous Heat Shock on Cold Storage and Rewarming-Induced Changes in HK-2 Protein Expression

Transient heat shock of HK-2 cells was shown previously to induce increased HSP-70 (24). In this study, we confirmed that heat shock for 1 h at 42°C followed by 2 to 3 h of recovery at 37°C results in increased expression of HSP-70 with no change in the expression of HSP-27 or HSP-90 compared with control levels (Figure 4, A through C, lane 1 versus 2 for each protein). In addition, we report that heat shock also results in an up-regulation of Bcl-2 (Figure 4D, lane 1 versus 2).

We examined the effect of previous heat shock on the altered expression of HSP and Bcl-2 described in Figure 2. We found that the expression levels of HSP-70, HSP-27, and Bcl-2 in heat-shocked cells were maintained above those in unshocked cells that were subjected to similar cold storage periods (Figure 4, A, C, and D, lane 5 versus 6). It is interesting that the levels of these proteins in heat-shocked cells that were stored in the cold for 16 h did not fall below control levels (Figure 4, A, C, and D, lane 1 versus 6). Previous heat shock did not maintain HSP-90 levels above those of unshocked cells during cold storage (Figure 4B). Levels of HSP-70 and HSP-90 expression were upregulated upon rewarmin to a greater extent in heat-shocked compared with unshocked cells (Figure 4, A and B, lane 7 versus 8), with no differences seen in postwarming HSP-27 and Bcl-2 levels (Figure 4, C and D, lane 7 versus 8).

Effect of siRNA Directed against HSP-70 on the Protective Effect of Heat-Shock against Cold Storage and Rewarming Injury

To determine whether the specific increase in HSP-70 expression was involved in the heat shock-induced protective re-

Figure 3. The effect of previous heat shock on cold storage and recovery-induced HK-2 cell death. Cells were grown in 24-well plates to 80% confluence. Cells were treated with or without heat shock at 42°C for 1 h and allowed to recover for 2 h at 37°C. Cells then were cold-stored (4°C) in UW solution for 18 h and returned to optimum culture conditions for 6 h. (A) Apoptosis then was determined as the percentage of cells with hypodiploid DNA on PI staining using flow cytometry. *P < 0.05 versus unshocked cells. (B) The percentage of cells with disrupted membranes was assessed by PI incorporation using flow cytometry. *P < 0.001 versus unshocked cells. Each data point represents n = 9 from three independent experiments.

Figure 4. The effect of previous heat shock on protein expression levels in HK-2 cells after cold storage and rewarmin. Cells were grown in 24-well plates to 80% confluence and either maintained at 37°C or subjected to heat-shock treatment at 42°C for 1 h followed by recovery for 2 h at 37°C. Cells then were cold-stored (4°C) in UW solution with or without a subsequent rewarmin period under normal cell culture conditions at 37°C. Cells were harvested, and protein was extracted for Western blot analysis. Western blots shown are representative of the results of at least three independent experiments.
sponse, we used siRNA directed against HSP-70. HSP-70–di-
rected siRNA (5 nM) led to a significant decrease in heat
shock–induced levels of HSP-70 mRNA (Figure 5A) and pro-
tein in HK-2 cells (Figure 5B). In contrast, the same concentra-
tion of scrambled siRNA had no effect (Figure 5, A and B).
Despite blocking the increase in HSP-70 after heat shock with
siRNA, there was no significant reversal of the protective effect
of heat shock against cold storage and rewarming-induced
apoptosis (Figure 5C) or necrosis (Figure 5D) of HK-2 cells.

Effect of Overexpression of HSP-70 on Cold Storage and
Rewarming Injury in HK-2 Cells

We next wanted to determine whether overexpression of
HSP-70 could protect HK-2 cells from cold storage and reper-
fusion injury. We generated a HK-2–derived cell line stably
overexpressing HSP-70 using the bicistronic pIRESpuro-3 vec-
tor. Stably transfected HK-2 cells expressed higher levels of
HSP-70 than control or mock-transfected cells (Figure 6A).
Transfected cells seemed to grow normally, exhibited basal
rates of apoptosis and necrosis similar to control cells (Figure 6,
B and C), and maintained their responsiveness to heat shock as
indicated by the fact that levels of HSP-70 were higher in
heat-shocked transfected cells compared with unshocked trans-
fected cells (data not shown). After 16 h of cold storage and 6 h
of rewarming, apoptotic rates were lower for the HSP-70–
overexpressing cells compared with control cells or mock-
transfected cells (Figure 6B), whereas there was no change in
the rate of necrosis observed (Figure 6C).

Discussion

In vitro cold storage of human renal tubular epithelial HK-2
cells in UW solution was used as a model of kidney storage.
HK-2 cells were used because tubular epithelial cells bear the
brunt of ischemia and reperfusion injury and undergo ATN,
leading to the pathology of DGF and acute renal failure. In our
model, we subjected cells to 16 h of cold storage, which is
comparable to cold storage times experienced by cadaveric
kidneys (1). We found that cell death that was caused by cold
storage was almost exclusively necrotic in nature, but rewarm-
ing induced both apoptosis and necrosis. Similar findings were
shown in primary human renal tubular epithelial cells, in
which cold storage induced necrosis and rewarming induced
significant apoptosis (26). The degree of cell death was signif-
icantly greater after a period of at least 8 h of cold storage
followed by rewarming compared with cold storage alone,
indicating that cold storage may prime kidney cells for cell
death during rewarming. An extrapolation between our in vitro
cell death findings and the situation in the intact kidney is
made difficult by a number of factors. First, the rapid and
efficient clearance of apoptotic cells makes apoptosis extremely
difficult to detect in tissue sections, and the incidence of apo-
pitosis may generally be underestimated because of this phe-
nomenon. Also, there is a scarcity of knowledge regarding the
relative contributions of cold storage alone versus cold storage
with subsequent reperfusion on cell death in human kidneys.
Only two studies have compared the effects of cold storage
with and without subsequent reperfusion on cell death in hu-
human kidney biopsies (27,28), and further research is required to determine the contribution of cold storage to kidney damage before transplantation.

All organisms respond to stresses by the preferential production of stress proteins. However, little is known about whether human or animal cells can mount a stress response, including production of stress proteins, during cold storage. It was reported previously that cold storage induces increased HSP-70 expression in human umbilical vein endothelial cells (29) and human neutrophils (30), whereas other studies have demonstrated no effect of cold on HSP expression in a rat model (31). In this study, we examined the effects of prolonged cold storage on stress protein expression. In addition, because the cold storage and rewarming injury in this study is likely to proceed via mitochondrial-derived increases in oxidative stress (10,11), we also examined Bcl-2 expression, which has been correlated with cadaveric graft survival (23). Only one previous study examined the effect of cold storage in isolation on Bcl-2 expression, with no effect being reported (11). We report that whereas a short period of cold storage (4 h) does not alter the expression levels of any of the HSP (HSP-70, HSP-90, or HSP-27) or Bcl-2 (Figure 2), 16 h significantly decreased their expression compared with controls (Figure 2). We had expected that the stress of cold storage would induce increased expression of stress proteins such as HSP-70, and the decreased expression seen may be due to a number of possibilities, such as the enhanced degradation or decreased synthesis of HSP protein as a result of altered transcriptional or translational activity in HK-2 cells at low temperatures. The timing of the changes in protein expression caused by cold storage described above may explain the pattern of cell death induced by the stresses seen in Figure 1. For instance, 4 h of cold storage does not alter the expression levels of any of the HSP or Bcl-2, explaining the lack of cell death in Figure 1. However, on increasing the time of cold storage there is a loss of HSP and Bcl-2 with a corresponding sensitivity to rewarming-induced apoptosis, despite the increased expression of these proteins upon rewarming (Figure 2). The increased expression of such pro-survival proteins during rewarming may represent an inadequate and somewhat belated attempt by the cells to respond to the cold storage and reperfusion insult. It is clear that there is a lack of understanding of the effects of cold storage on cell stress responses. Further studies are required to elucidate why pro-survival proteins such as HSP and Bcl-2 are lost during cold storage so that these changes may be manipulated for therapeutic intervention to improve organ graft survival.

Preconditioning of cells or organs with exposure to short-term sublethal hyperthermia induces the expression of HSP and may prove to be a promising strategy to increase the tolerance of organs to cold ischemia and reperfusion injury. We were interested to determine whether such preconditioning heat stress could protect HK-2 cells from cold storage and rewarming-induced cell death, particularly because we have already demonstrated that such cell death is associated with decreased expression of HSP during the cold storage period. We demonstrated that previous heat shock significantly reduced cold storage and rewarming-induced apoptotic and necrotic cell death in HK-2 cells. The heat-shock effect was associated with increased expression of HSP-70 without affecting the levels of HSP-27 or HSP-90, as previously reported by Power et al. (24). It is interesting that heat-shocked HK-2 cells that were subjected to subsequent cold storage were found to maintain their HSP-70 and HSP-27 content far above levels in nonshocked cells that were subjected to 16 h of cold storage. Upon rewarming, HSP-70 and HSP-90 expression was significantly increased in heat-shocked cells to a level greater than that seen for nonshocked cells. To our knowledge, this is the first study to report the maintenance of HSP expression levels during cold storage and rewarming by previous heat shock and
helps to explain the protective effects of heat shock reported in this study. HSP-70, HSP-27, and HSP-90 all are known to promote cell survival through various mechanisms within cells, including stress kinase inhibition (32,33), protection of cells from DNA damage (34,35), modulation of mitochondrial reactive oxygen species production (36), and inhibition of caspase activation (37,38). Thus, maintenance of HSP levels during cold storage may help cells to cope better with reperfusion-associated increases in oxidative stress, mitochondrial disruption, and activation of caspase pathways.

As already described, Bcl-2 levels were dramatically decreased after 16 h of cold storage compared with control levels. It is interesting that previous heat shock was associated with an increased Bcl-2 expression. Bcl-2 upregulation after heat shock may be linked to increased HSP levels, because HSP-90 has been shown to upregulate Bcl-2 expression after vascular endothelial growth factor addition (39). HSP-70 has also been shown to activate Bcl-2 via a direct link made by a nucleotide exchange factor for HSP-70 known as BAG (40). We speculate that heat shock–induced upregulation and maintenance of Bcl-2 levels during cold storage may contribute to cell survival by blocking the mitochondrial membrane disruption that is likely to be central to cold storage and reperfusion injury in HK-2 cells.

We next focused specifically on whether HSP-70 plays a direct role in the heat-shock effect observed. We generated HK-2 cells that stably overexpress HSP-70 and found them to be more resistant to cold storage and rewarmed-induced apoptosis than control HK-2 cells. This finding indicates that HSP-70 alone in the absence of a stress response is sufficient to confer resistance against cold storage and rewarmed injury in HK-2 cells and is in agreement with other HSP-70 overexpression studies. For example, addition of recombinant HSP-70 has been shown to inhibit cytochrome C/dATP-mediated caspase activation (41) and to block cell death via reduced p38 kinase activation (42). In addition, an overexpression study in human lung adenocarcinoma cells demonstrated a specific role for HSP-70 in blocking hyperoxia-mediated lipid peroxidation and cell death (43). Despite the ability of HSP-70 overexpression to protect HK-2 cells, blocking of HSP-70 upregulation with siRNA did not inhibit the protective effect of heat shock. In agreement with our finding, it was reported previously that antisense inhibition of HSP-70 failed to abolish the heat shock–induced protection of neuroblastoma cells from apoptosis (44). We speculate that the heat-shock protective effect that was seen in this study is a multifactorial response to the complex insult of cold storage and rewarmed and that a degree of redundancy may exist such that blocking induction of a specific protective factor such as HSP-70 will not blunt the overall protective effect. Further research is required to understand fully the stress response mechanisms of organ preconditioning for transplantation.

Conclusion

Our findings indicate that cold storage of HK-2 cells predisposes them to further injury and death after rewarmed. This injury is associated with decreased expression of stress proteins (HSP-70, HSP-90, and HSP-27) and Bcl-2 during the cold storage period. Such injury can be partially blocked by previous heat shock associated with maintenance of HSP and Bcl-2 expression levels during cold storage. HSP-70 overexpression is sufficient to confer protection against cold storage and rewarmed injury. However, the upregulation of HSP-70 is not the only factor involved in heat shock–induced protection against such injury.

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References
