Differential Inhibition of HSP72 and HSP25 Produces Profound Impairment of Cellular Integrity

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Abstract. To test a putative cause and effect relationship between heat-shock protein (HSP) expression and response to renal cell injury, HSP72 and HSP25 were differentially inhibited in LLC-PK1 cells by means of transcription factor decoy and short interference RNA (siRNA). Cellular injury was assessed by solubilization of NaK ATPase (S-NaK). An exonuclease-resistant, ethylene glycol–bridged, circular oligonucleotide decoy for heat-shock transcription factor (HSF)-1, based on the sequence of the porcine heat-shock element, was constructed and validated. It was found that under all experimental conditions, cells had comparable ATP levels; that decoy of unligated or scrambled sequence was ineffective; that HSP72 depletion remains unclear. Studies aimed at establishing a causal link between HSP induction and cytoprotection have been undertaken by means of transcription factor decoy and short interference RNA (siRNA).

In unstressed cells, preformed heat-shock transcription factors (HSF-1 and HSF-2) are inactive in the cytosol (1). HSF-1 is predominantly involved in stress-related responses, whereas HSF-2 appears to be an important developmental regulator. HSF-1 is rapidly released from the constitutively expressed heat-shock protein (HSP) 90 when demand for this chaperone increases as denatured and aggregated proteins accumulate under conditions of cell stress (2). Activated HSF rapidly trimerizes and translocates to the nucleus, where it binds to GAAn repeats that comprise the heat-shock element (HSE). Studies from our laboratory and others have previously demonstrated the activation of HSF-1 by graded reductions in cellular ATP in vivo (3) and in vitro (4–7). Activation of HSF-1 resulted in the induction of HSP72 and HSP25 after either ATP depletion or ischemic injury (5).

The relative importance of HSP72 and HSP25 in the protection or repair of renal epithelial cells exposed to ATP depletion remains unclear. Studies aimed at establishing a causal link between HSP induction and cytoprotection have examined the effects of overexpression of the heat-shock response by means of preconditioning (8–12), pharmacologic induction (13,14), or transfection (15). Preconditioning requires the application of an initial injury to induce HSP, which confounds the interpretation of outcome. Pharmacologic induction and transfection may alter basic molecular and cellular processes in addition to overexpressing HSP.

Attempts to downregulate induction of the heat-shock response have been based on pharmacologic methods (11) or the application of antisense (16–18), both of which may have intrinsic difficulties that could complicate the interpretation of results. Overall, studies aimed at altering the heat-shock response to injury have produced inconclusive results, and the role of individual members of the heat-shock family of proteins in response to renal cell injury requires additional clarification (6,11,19–23). To explore the role of the HSP in the recovery from renal cell injury, specific inhibition of the inducible HSP, HSP72 and HSP25, has been undertaken by means of transcription factor decoy and short interference RNA (siRNA).

The technique of transcription factor decoy is based on the use of multiple copies of short segments of double-stranded DNA sharing sequence homology with the target transcription factor binding site (24,25). The specifically designed decoy is bound in the cytosol by its corresponding transcription factor and thereby prevents attachment to genes that contain the target transcription factor binding site (24–29). In this manner, the transcription factor is “decoyed” and diverted from annealing to genes to which it would normally bind. Thus, oligonucleotide decoys provide a pretranscriptional dampening of protein synthesis controlled by a particular transcription factor. In this study, a decoy designed to match the specific sequence of the HSF-1 binding site (30) within the heat-shock transcription activation in decoy-treated, injured cells was accompanied by a substantially amplified loss of cellular integrity (S-NaK was 85% compared with baseline levels). Specific inhibition of HSP72 that used siRNA directed against an inducible porcine HSP72 gene resulted in complete ablation of injury-induced HSP72. Isolated inhibition of HSP72 was also associated with marked NaK ATPase detachment from the cytoskeleton (S-NaK was 135% compared with baseline levels). These studies suggest that an HSF-1 decoy effectively dampens the HSP72/HSP25 response to injury in renal cells; that HSP72 siRNA ablates injury-induced induction of HSP72; and that dampening of the HSP72/HSP25 response and ablation of the HSP72 response are both associated with impaired restitution of cellular polarity.
element was constructed. This HSF-1 decoy was used to down-regulate the induction of HSP72 and HSP25 in renal cells injured by substrate depletion. The effect on restitution of cellular polarity during recovery was examined.

To establish a discrete effect of HSP72 inhibition on the recovery process, isolated, gene-specific inhibition of HSP72 by means of siRNA was undertaken. siRNA are synthesized short-double stranded RNA duplexes of 21 to 23 bp. Entry into the cell is achieved by formal cell transfection techniques. The precise mechanism of action of siRNA remains incompletely understood. Posttranscriptional gene silencing is achieved by the assembly of siRNA with other proteins into an RNA-induced silencing complex that tags and degrades the corresponding complementary mRNA (31,32). Effects are highly specific; single-base substitutions within the siRNA can diminish or abolish inhibitory activity (33). Activity of an individual siRNA is unpredictable. Accordingly, a panel of potential targets must be screened for gene silencing ability (34).

Materials and Methods

Cell Preparation

LLC-PK1 cells, derived from porcine proximal tubular cells, were grown in α-MEM (Cellgro, Mediatech, Herndon, VA) with 10% FBS at 37°C in 5% CO₂. Cell culture filters (25 mm) (Collaborative/BD Biosciences) coated with 40 µg of collagen IV (Collaborative/BD Biosciences) were plated with 2.8 × 10⁵ cells. Studies were conducted 4 d after passage, with cells having achieved confluence.

Cell Injury Model

Before the application of injury or control media, the upper and lower wells of each cell culture filter were rinsed twice with prewarmed PBS. ATP depletion was induced by incubation with prewarmed substrate-free media and 0.1 mM of the mitochondrial inhibitor antymycin A. Substrate-free media lacked the amino acids normally found in standard α-MEM and contained 5 mg/dl L-glucose to maintain osmolarity. Cells were subjected to 2 h of energy deprivation, followed by a single rinse in PBS and 4 h recovery in standard growth media (α-MEM). Control cells were treated with identical washes and cultured in standard growth media.

Transcription Factor Decoy

Pretranscriptional inhibition of heat-shock genes was achieved by a transcription factor decoy. Multiple copies of a 23-bp oligodeoxynucleotide, of a sequence corresponding to the transcription element of the heat-shock gene (the HSE), were added to the cell culture medium. After absorption by the cells, the decoy provided an alternative—and ineffective—target for binding of activated transcription factor.

Oligodeoxynucleotide design was based on the published sequence of the injury inducible porcine HSP70 gene (NCBI accession no. M69100) (30). HSE consists of a GAAn sequence that is highly conserved and found in the promoter region of all heat-shock genes. The number, orientation, and localization of the HSE varies from gene to gene. In the case of M69100, HSE is found at positions 58 and 179 and identified as HSE II and HSE I, respectively. The design of the decoy was based on the sequence flanking and included HSE I, with additional G/C bases added around the bridges to increase annealing.

The basic sequence of the cyclic ethylene glycol–bridged decoy oligodeoxynucleotide was synthesized via standard phosphoramidite chemistry as a linear strand of complementary nucleotides with ethylene glycol spacers (Spacer 9, Glen Research) inserted at the desired bridging points: gtttcg—Spacer 9—cgaacacctggaatattctagc—Spacer 9—cagaaatattcagPO₄.

After the spontaneous annealing of complementary sequences, the free ends of oligodeoxynucleotide (120 µM/µl) were ligated by incubation with 350 mM N-(3-dimethylaminopropyl)-N-ethylcarbodiimide, 50 mM 2-morpholinoethanesulfonic acid sodium salt, and 20 mM magnesium chloride at 4°C for 60 h while stirring. A 3’ phosphate is essential for the ligation reaction to proceed.

Oligodeoxynucleotides were subsequently removed from the reaction mix by ethanol precipitation and resuspended in 10 mM Tris pH 7.5. Ligation efficiency was approximately 50%, with the balance of the oligonucleotide remaining in a linear form. Ligated and unligated oligonucleotides were not separated from the reaction mix because preliminary studies demonstrated that unligated HSF-1 decoy did not affect HSP72 expression.

Decoy specificity was assessed with a scrambled decoy that shared the same base composition as the HSF-1 decoy but with a random base order: gtgtcatg—Spacer 9—ctaatgaacgctaaactega—Spacer 9—aatgccaggtatcggPO₄.

Decoy Treatment

Decoy oligonucleotides were added to the injury or control media immediately before the beginning of the 2-h injury period. Oligodeoxynucleotides were applied to 0.5 ml of media in the upper well of the of the cell culture filter only; the lower well was filled with 2 ml of the corresponding injury or control media alone. At 2 h, oligonucleotide was added to the 500 µl of recovery media in the upper well to ensure the continued presence of decoy throughout the 4-h recovery period (6-h experimental period). The effects of decoy concentrations of 10, 5, 1, and 0.5 µg/500 µl were examined. Uninjured cells treated with decoy were studied after a total of 6 h exposure to oligonucleotide.

siRNA

A panel of four short double-stranded RNA sequences was constructed. Targets were identified by the Ambion siRNA Target Finder and Design Tool Web site (35). This software scans the mRNA sequence of the gene for adjacent AA dinucleotides and their corresponding 3’ 19 nucleotides; the most effective siRNA found to date are 21-bp dsRNA with 3’ overhangs of thymidine or uridine (TT or UU) (33). Targets were chosen to provide an even distribution along the gene and to give an siRNA G/C content of less than 50%. Potential targets were eliminated if they shared significant homology with important cellular proteins to avoid unwanted cross reaction. Targets showing cross-species homology were preferentially selected. The coding sequences within the inducible porcine gene M69100 corresponding to each target were as follows: target 1, aagacaagaggacctgc; target 2, aacaagactcattcacaac; target 3, aagaaggtctgcaag; and target 4, aattgctctatggatga.

Briefly, sense and antisense DNA templates for each siRNA target were produced by standard phosphoramidite chemistry. Templates were 29 bases long and contained a sequence based on the desired 21-nucleotide siRNA, along with eight bases of a T7 promoter primer. In separate reactions, each DNA oligonucleotide had a complementary strand generated by the Kenlow fragment of DNA polymerase. Transcription of each of the two resulting double-stranded DNA, with an RNA polymerase, produced sense and antisense RNA strands. When mixed, these complementary RNA strands annealed to form short double-stranded RNA (Ambion, Austin, TX).
siRNA Transfection

Cells underwent siRNA transfection the day before achieving confluence. Standard growth medium was removed and, after a single wash, replaced with 500 μl of OptiMEM I (Invitrogen, Carlsbad, CA) media in the upper portion of the filter and 2000 μl in the lower portion. A mixture of 10 nM siRNA and 3 μl siPort Lipid, or siPort Amine (Ambion, Austin, TX), was added to the 500 μl of OptiMEM I in the upper portion of the filter. Cells were incubated in this serum-free environment for 6 h; at the end of this time period, media in the upper well was supplemented with 2 ml of standard growth media; media in the lower well was replaced with 2 ml of standard growth media.

Initial studies focused on the identification of the target siRNA that would produce the lowest levels of HSP72 expression in LLC-PK1 cells 24 h after transfection. Subsequent studies examined cells transfected with this siRNA 24 h before injury. Consequently, in this series of experiments, cells had reduced levels of HSP72 before injury.

Cell Harvest

Injured and uninjured cells were harvested by scraping them into chilled extractions buffer containing 0.10% Triton X-100, 60 mM piperazine-N,N-bis(2-ethanesulfonic acid) pH6.8, 25 mM HEPES, 10 mM EGTA, 2 mM magnesium chloride, 1 mM benzamidine, 2 mM sodium vanadate, and Complete Protease Inhibitor Cocktail (Roche, Germany). Samples for HSP analysis were sonicated and stored at −80°C. Samples for NaK ATPase analysis were centrifuged and stored at −80°C. For ATPlase, samples were centrifuged at 36,000 g for 30 min to separate the Trition-soluble from the insoluble protein fraction. The supernatant was separated from the pellet, and both were stored at −80°C.

Protein Determination and Western Blot Test

Protein concentrations of samples were determined after protein precipitation with the Compat-Able Protein Assay Preparation Reagent Set (Pierce, Rockford, IL), by a bicinchoninic acid method that used BSA as a protein standard (BCA Protein Assay Kit; Pierce).

Equal amounts of protein (15 μg) were mixed with an equal volume of 2X loading buffer containing 100 mM Tris pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 200 mM DTT; heated to 50°C for 5 min; and separated by SDS-PAGE electrophoresis on 4% to 20% gradient gels ( Criterion; Bio-Rad). Proteins were transferred onto nitrocellulose membranes (Biotrace NT, Pall, FL) and nonspecific binding sites blocked with 5% skim milk in 10 mM Tris pH 7.5, 37.5 mM sodium chloride, and 0.5% Tween-20. Membranes were incubated for 1 h with monoclonal antibodies directed against inducible HSP72, HSP25, HSP60, HSC (SPA-810, SPA-801, SPA 806, SPA-815; Stressgen) and NaK ATPase, respectively. After repeated washings, the membranes were incubated with an appropriate species-specific secondary antibody for 1 h. After further washing, immunoreactive antigen was detected with enhanced chemiluminescence. Western blot analysis reagents and protocols were supplied by the manufacturer (Pierce). Chemiluminescence was detected by exposure of photographic film (Kodak X-Omat AR; Kodak, Rochester, NY) and quantified by means of densitometry. Films were exposed, and the resulting images were analyzed by Scion Image software (Scion, Frederick, MD).

Results

ATP Measurement

ATP levels were measured in injured cells treated with HSF decoy, scrambled decoy, or unligated decoy and compared with levels in uninjured and injured, untreated cells. Cellular ATP levels were measured after 2 h of injury. Cells in each group were rinsed and then harvested in cold PBS. Cellular ATP concentration was measured by a modification of the luciferase assay described by Uchida and Endou (36). Briefly, adenine nucleotides were extracted from the cells by addition of an equal volume of 3.6% perchoric acid. ATP content was measured with a liminometer (Lumat LB 9501; Berthold, Wallac, Gaithersburg, MD) and an ATP bioluminescent assay kit (Sigma Chemical, St. Louis, MO). Sample protein concentrations were determined after protein precipitation performed with the Compat-Able Protein Assay Preparation Reagent Set (Pierce) by a bicinchoninic acid method that used BSA as a protein standard (BCA Protein Assay Kit; Pierce). Cellular ATP levels, normalized as nanomoles of ATP per milligram of total cellular protein, were expressed as a percentage of levels observed in uninjured control cells.

Semiquantitative RT-PCR

Aliquots of harvested cells underwent RNA extraction with a kit (RNaseasy; Qiagen, Valencia, CA). Purified RNA was quantified by absorption spectroscopy at 260 nm. Reverse transcription was performed with 1 μg of total RNA from each condition as a template and pdT12–18 as a primer. Equal volumes of the resulting cDNA served as templates for subsequent PCR reactions. Sense and antisense primers for the inducible porcine HSP72 gene (accession no. M69100) consisted of 5’-atggcgaagagcgtggcc-3’ and 5’-ctaatccacctcctcgatgggt-ggggcc-3’, respectively. After PCR, equal volumes of reaction were loaded and run on a 1% agarose gel containing 0.5 μg per milliliter of ethidium bromide. Images of the gel were obtained under ultraviolet exposure, and the intensity of staining was quantified by Scion Image software.

HSF-1 Transcription Factor Decoy

To exert a pathobiological effect, an oligonucleotide decoy must be taken up by the cell, enter the cytoplasm, and bind activated transcription factor. In turn, mRNA production and protein synthesis of a specific gene will decrease. Fluorescein-activated transcription factor. In turn, mRNA production and protein synthesis of a specific gene will decrease. Fluorescein-labeled decoy (10 μg) that shared sequence homology with the HSE was diffusely taken up by LLC-PK1 cells after incubation.

The effect of HSF-1 transcription factor decoy treatment on HSP72 mRNA levels was studied with semiquantitative PCR. Primers for HSP72 were chosen because this protein represented the most abundant synthesis product of the heat-shock response (37). Uninjured, untreated cells demonstrated a baseline level of HSP72 mRNA expression (Figure 1) that was substantially decreased as a consequence of treating uninjured cells with 10 μg of decoy. As expected, cells exposed to 2 h ATP depletion followed by 4 h recovery demonstrated an
increase in HSP72 mRNA levels. Injury and recovery in the presence of 10 μg of decoy significantly dampened the injury-induced increase in HSP72 mRNA abundance.

The effect of decoy on HSP72 protein synthesis was assessed by Western blot test and densitometry. Confluent, uninjured, untreated LLC-PK1 cells growing under baseline conditions expressed low levels of HSP72, similar to other immortalized cell lines. After 6 h of decoy treatment, HSP72 expression was suppressed by approximately 25% compared with baseline levels in uninjured cells (Figure 2).

Cells injured in the presence of decoy demonstrated a substantially muted increase in HSP72 (Figures 3 and 4). Exposure of untreated cells to 2 h ATP depletion and 4 h recovery induced an increase in HSP72 expression to 85% above baseline values, as described previously (Figure 4). The effect of HSF-1 decoy treatment on HSP72 protein levels in injured cells was studied with varying concentrations of decoy (Figure 4). Maximal suppression of HSP72 induction occurred in cells treated with 10 μg of decoy. Levels increased to only 10% above baseline, a 75% inhibition of protein induction. In cells treated with 1 or 5 μg of decoy, injury-induced levels remained significantly suppressed, increasing to only 40% above baseline. In cells treated with 0.5 μg of decoy, HSP72 levels increased to 55% above baseline, a significant inhibition of protein induction.

To establish that the effects of HSF-1 transcription factor decoy on HSP72 expression were specific, a scrambled decoy of identical base composition to the HSF-1 decoy was constructed. In parallel studies, cells were treated with either HSF-1 or scrambled decoy. Treatment with scrambled decoy did not significantly alter HSP72 expression in injured or uninjured cells. Because the ligation mix contained residual

Figure 1. Effect of heat-shock transcription factor (HSF) 1 decoy treatment on heat-shock protein (HSP) 72 mRNA. Confluent LLC-PK cells were injured by ATP depletion for 2 h, followed by 4 h recovery. Treated cells had 10 μg of decoy added to both the injury and recovery media. The upper panel shows product of a semiquantitative PCR reaction run on an ethidium bromide–stained 1% agarose gel. The lower panel shows the relative intensity of each lane as assessed by densitometry.

Figure 2. Effect of heat-shock transcription factor (HSF) decoy treatment on heat-shock protein (HSP) 72 expression. Untreated, uninjured LLC-PK1 cells were incubated with normal growth media. Treated cells had 10 μg of decoy added to normal growth media. Both groups underwent a single PBS wash at 2 h, with replacement of media, and were harvested 4 h later. The graph shows densitometry results for Western blots stained with antibodies directed against HSP72. Protein expression in untreated, uninjured cells is set at 100%.
unligated decoy, it was also necessary to determine whether the presence of unligated decoy would modify the effectiveness of ligated decoy. In cells treated with 10 μg of unligated decoy, no significant effect on HSP72 expression in injured or uninjured cells was detected. To ensure that the presence of decoy did not interfere with the effect of the insult, levels of ATP were measured in injured cells treated with HSF-1 or scrambled decoy. After 2 h of injury, ATP levels decreased to less than 5% of baseline in injured cells with or without decoy treatment.

To assess the effects of HSF-1 decoy treatment on other HSP, the injury-induced synthesis of HSP25, HSP60, and HSC was studied in the presence of 10 μg of decoy. Treatment with decoy did not alter the expression of HSP60 or HSC. After injury, HSP25 levels increased by 30% in untreated cells, as previously documented (5). However, levels increased to only 3% above baseline as a consequence of injury in the presence of decoy (Figure 5).

The effect of HSF-1 decoy treatment on cellular integrity during recovery from ATP depletion was examined. Detergent solubility of NaK ATPase was used as an index of detachment of this integral membrane protein from the cytoskeleton (38,39). As previously reported, NaK ATPase solubility in untreated LLC-PK1 cells 4 h after 2 h of ATP depletion was moderately increased to 33% above baseline (Figure 6). The dampened response to heat-shock activation in cells treated with HSF-1 transcription factor decoy was accompanied by a substantially impeded restitution of cellular integrity. Cells treated with 10 μg of decoy demonstrated an 85% increase in NaK ATPase solubility above baseline that was significantly greater than that in the untreated cells (P < 0.05).

diRNA
The ability of siRNA to inhibit protein synthesis depends on efficient cell entry, binding to target mRNA, and the induction of an mRNA cleavage mechanism that remains incompletely understood. The choice of transfection agent and timing of the procedure can be critical to the success of gene silencing. The effect of two different proprietary siRNA transfection agents was studied in LLC-PK1 cells by using a panel of HSP72 siRNA targets. The effect of transfection agent on target gene expression was assessed by comparing untreated cells with cells treated with transfection agent in the absence of siRNA (TA alone).

LLC-PK1 cells treated with an amine-based transfection agent demonstrated increased HSP72 expression (Figure 7).
This effect was not seen in cells treated with lipid-based transfection agent. The pattern of siRNA inhibition of HSP72 protein synthesis was similar with either lipid or amine transfection (Figure 7). Targets 1, 2, and 3 produced a decrease in HSP72 protein levels; target 4 failed to produce a substantial decrease in either group.

On the basis of the results of these initial screening and optimization experiments, subsequent studies focused on establishing the reproducibility of lipid-based transfection of target 2 siRNA. Four groups were studied 24 h after transfection: uninjured cells treated with transfection agent alone (TA alone); uninjured cells transfected with target 2; injured cells treated with TA alone; and injured cells transfected with target 2.

The effect of target 2 siRNA on HSP72 protein synthesis was assessed by Western blot test and densitometry. Target 2 siRNA treatment produced a 47% decrease in HSP72 expression compared with uninjured cells treated with TA alone (Figure 8). Exposure of cells treated with TA alone to 2 h ATP depletion and 4 h recovery induced an expected increase in HSP72 expression to 57% above baseline values, as described previously (40). This increase was completely ablated in cells injured after treatment with target 2 siRNA. HSP72 levels in this group remained at 37% below baseline. These levels were not statistically different from those seen in uninjured cells treated with target 2 but were considerably lower than constitutively expressed HSP72 levels. Thus, treatment with target 2 appears to have eliminated the induction of HSP72 by ATP depletion and may have rendered cells more vulnerable to injury. Treatment with target 2 siRNA did not significantly
antioxidant, a property associated with cytoprotection (43). In
by the confounding effect of quercetin, acting as a potent
deleterious effects of HSP inhibition may have been obscured
in a similar model. In these studies, the
identify an inhibitory effect; or even conclude that the maneu-
might underestimate the efficacy of the inhibition; fail to
likely occur. Subsequent studies that use such an intervention
target results in cellular injury, then a stress response will
difficult because changes, even at steady state, may prove
increased expression of HSP is protective, then downregulation
key role for HSP in modulating acute cellular injury. If in-
vides important correlative, but not definitive, evidence of a
Figure 7. Effect of short interference RNA (siRNA) directed against
different target areas within an inducible heat-shock protein (HSP) 72
gene (M69100). LLC-PK1 cells were transfected with siRNA 24 h
before harvest. Cells treated with transfection agent alone were used
as a control. The upper panel shows the effect of transfection with a
lipid based agent; the lower panel shows results obtained in an
identical and simultaneous experiment where an amine based agent
was used. Shown are Western blots stained with antibodies directed
against HSP72.

alter the established response of HSP25 (Figure 9) to ATP
depletion (5).
The effect of inhibition of HSP72 on cellular integrity after
recovery from ATP depletion was examined (Figure 10). As
anticipated, NaK ATPase solubility increased in cells treated
with TA alone after injury. In comparison, cells transfected
with target 2 demonstrated a more substantial increase in
detached NaK ATPase, 135% above baseline by densitometry.
Even after 4 h of recovery, the obliteration of injury-induced
HSP72 in cells treated with target 2 siRNA resulted in pro-
found impairment of cellular polarity.

Discussion
Data concerning the cytoprotective effect of HSP induced by
heat (8,11,41), injury (3,4,10,12), or transfection (15,18) pro-
vides important correlative, but not definitive, evidence of a
key role for HSP in modulating acute cellular injury. If in-
creased expression of HSP is protective, then downregulation
should augment cellular injury or impair restitution of cellular
integrity.

Inhibition of putative cellular cytoprotective mechanisms is
difficult because changes, even at steady state, may prove
intrinsically injurious. If an attempt to inhibit a cytoprotective
target results in cellular injury, then a stress response will
likely occur. Subsequent studies that use such an intervention
might underestimate the efficacy of the inhibition; fail to
identify an inhibitory effect; or even conclude that the maneu-
ver is cytoprotective. Attempts to downregulate the heat-shock
response have focused on the use of quercetin or antisense.
Kelly et al. (21) report that the induction of HSP72 and HSP84
mRNA is blocked by pretreatment with quercetin with no
significantly deleterious effect on postischemic renal function.
Kahraman et al. (42) suggest that quercetin may have a cyto-
protective effect in a similar model. In these studies, the
deleterious effects of HSP inhibition may have been obscured
by the confounding effect of quercetin, acting as a potent
antioxidant, a property associated with cytoprotection (43). In
 fact, the more specific inhibition of HSP72 by stable transfection
with a vector expressing antisense DNA resulted in in-
creased sensitivity to curcumin-induced apoptosis in human
colon cancer cells (18).

Transcription factor decoy provides a powerful technique for
pretranscriptional inhibition of inducible genes. Specific inhibi-
tion of groups of genes controlled by a defined common
transcription regulation element can be achieved. Circular,
ethylene glycol–bridged oligonucleotides offer an attractive
method of modulating the molecular consequences of gene
activation because these compounds do not require formal
transfection and are resistant to exonuclease degradation.

An exonuclease-resistant, ethylene glycol–bridged, circular
oligonucleotide decoy for HSP-1, based on the sequence of the
porcine HSE, was constructed. Decoy was validated by both
unligated and scrambled forms of the oligonucleotide. Treat-
ment with unligated or scrambled decoy demonstrated no del-
eterious effects, in terms of cellular stability or induction of an
injury response. Ligated decoy was rapidly taken up by cells
without transfection and did not alter cellular ATP levels after
energy deprivation. Treatment with ligated decoy resulted in an
expected reduction in mRNA levels for HSP72. Thus, the
HSF-1 oligonucleotide decoy used in the present experiment
had no unexpected deleterious effects on the LLC-PK1 cells
and exhibited the anticipated inhibition of the molecular se-
quence of the activation of the targeted gene, dampening of
HSP72 mRNA.

In keeping with the predicted molecular mechanism, cells
treated with ligated decoy demonstrated a consistent reduction
in the expression of HSP under the control of HSF-1. After 6 h
of decoy treatment, HSP72 levels were reduced both at base-
line and after energy deprivation in a dose dependent manner.
In addition, levels of HSP25 were significantly reduced in
response to injury. The transcription site used in the synthesis
of the HSF-1 decoy was based on the published sequence of an
inducible porcine HSP72 gene, centered on the GAAn repeat
common to the HSE of both the HSP72 and HSP25 genes.
Overlapping effects suggest the expression of a simplified form
of the GAAn motif alone may be sufficient to inhibit HSP
synthesis.

In contrast, the levels of two HSP not induced by the
activation of HSF-1 after ATP depletion, HSC and HSP60,
were unaffected by treatment with decoy. Treatment of cells
with scrambled decoy did not alter the heat-shock response.
This sequence of studies demonstrates that the effects of decoy
are sequence dependent and specific. This technique of pre-
transcriptional gene inhibition offers an opportunity to disrupt
the molecular sequence of events occurring in response to
injury without perturbing other cellular mechanisms that may
be involved in the process of injury or recovery.

To clarify the independent role of HSP72 in energy depriva-
tion–induced renal cell injury, gene-specific inhibition of an
inducible HSP72 by means of siRNA was undertaken. The
high specificity of posttranscriptional gene silencing that can
be gained when siRNA is used provides a powerful technique
for the study of protein function. This study has established an
effective protocol for the transfection of LLC-PK1 cells with siRNA.

Preliminary studies examined the effects of a panel of siRNA directed against different parts of an inducible HSP72 gene, M69100 (30). Cells were transfected in parallel with either lipid- or amine-based transfection agents. Cell treated with transfection agent alone were used as a control. Cells were injured by 2 h ATP depletion, followed by 4 h recovery. Shown are Western blots stained with antibodies directed against HSP25.

As expected, the silencing ability of individual siRNA was sequence specific, and HSP72 inhibition varied across the panel of siRNA. Target 2 demonstrated the greatest efficacy in silencing HSP72. This target corresponds with bases 1980 to 2001 in the published sequence of the M69100 inducible porcine HSP72 gene (30). This area of the gene is highly conserved, and potential silencing activity across mouse, rat, and human cell lines was predicted. Consequently, target 2 was chosen for further studies.

Subsequent studies demonstrated the reproducibility of HSP72 silencing by target 2 and examined its specificity. Injured and uninjured cells treated with transfection agent alone 24 h before study were used as controls. In keeping with the predicted molecular mechanism, cells treated with siRNA demonstrated a consistent reduction in the expression of HSP72. Baseline levels of HSP72 normally expressed in immortalized LLC-PK1 cells were reduced by almost 50%. After energy deprivation, HSP72 levels in cells treated with target 2 were similar to those seen in treated, uninjured cells. Thus, the well described induction of HSP72 by ATP depletion (4) was completely obliterated by siRNA treatment. In keeping with the anticipated gene specificity of siRNA, alterations in HSP72 expression in both injured and uninjured cells treated with target 2 were not accompanied by parallel inhibition of HSP25.
Previous studies in our laboratory indicate that recycling of NaK ATPase, rather than de novo synthesis, is the means by which renal epithelia initially repolarize after ischemic injury (44). In LLC-PK1 cells, graded ATP depletion resulted in a stepwise dissociation of NaK ATPase from the cytoskeleton and the activation of HSF (3,4). During recovery, NaK ATPase is restored to its domain-specific location and associated with the cellular distribution of HSP72 and HSP25 (45). In vivo, ischemic preconditioning of rat kidneys resulted in reduced NaK ATPase solubility in response to subsequent ischemic injury (10). Stabilization of NaK ATPase is associated with marked induction and cytoskeletal redistribution of HSP25 and HSP72. Overexpression of HSP27 in LLC-PK1 cells resulted in reduced detachment of NaK ATPase from the cytoskeleton in response to energy deprivation (15).

In the study presented here, differential inhibition of HSP72 and HSP25 by either decoy or siRNA was associated with substantial alterations in cellular polarity after ATP depletion. Inhibition of HSP72 and HSP25 expression was associated with a significant increase in detergent soluble NaK ATPase in HSF-1 transcription factor decoy-treated cells. In siRNA-treated cells, isolated inhibition of HSP72, with consequent elimination of the normal HSP72 response to injury, was associated with a profound increase in detachment of NaK ATPase from the cytoskeleton. The enhanced detachment of NaK ATPase from the cytoskeleton after energy deprivation may reflect impairment in restitution of polarity during recovery or increased susceptibility to injury. Contrasting experimental design may provide some insight into relative contribution of each effect.

In cells treated with HSF-1 decoy, HSP72 levels are unlikely to have been affected before or during injury because decoy is added immediately before injury; because HSF-1 activation occurs during injury; and because synthesis of inducible HSP occurs primarily during recovery. In this sequence of studies, it appears likely that the effects of the reduced levels of HSP72 and HSP25 primarily affected on the process of recovery. Taken together, the previously established pattern of HSP activation, the abundance of these proteins during recovery from injury, and the effect of reduction of these proteins by decoy treatment suggest a prominent role of HSP72 and HSP25 in the repolarization of NaK ATPase, a process essential for the resumption of normal tubular function after insult.

The studies that use siRNA differ from the decoy studies in several important ways. First, target 2 siRNA was transfected 24 h before injury. Because this target siRNA was chosen because it maximally suppressed baseline HSP72 at 24 h after transfection, it is quite possible that these cells were more susceptible to injury than the decoy-treated cells. Second, siRNA treatment resulted in an ablation of the induction of HSP72 after injury. Thereby, the siRNA-treated cells have HSP72 levels during the recovery period that are significantly below baseline values, substantially less than injury-induced levels in cells treated with transfection agent alone and less than that in decoy-treated cells that had a dampened response to ATP depletion. In addition, treatment with siRNA resulted in isolated reduction in HSP72 without any effect on HSP25 as compared with decoy-treated cells, which had diminished abundance of both HSP. Because overexpression of HSP27 (the human analog of HSP25) has been shown to be cytoprotective and to diminish detachment of NaK-ATPase from the cytoskeleton after energy deprivation, one might have expected the solubilized NaK-ATPase to have been less in the siRNA-treated cells than in the decoy-treated cells. However, the effect of siRNA treatment on disruption of cellular integrity 4 h after injury (solubilized Na/K-ATPase 130% above baseline) was greater than that observed in decoy-treated cells (85%). Consequently, the enhanced loss of cellular integrity associated with siRNA treatment may be related to diminished baseline HSP72 levels before injury, to ablation of the induction of a heat-shock response, to a threshold level of HSP72 required for the cytoprotective effect of HSP25, or some combination of these. Interactions between these interrelated mechanisms in this dynamic and complex pathobiologic response cannot be clearly delineated in the study presented here.

In conjunction with studies demonstrating a cytoprotective effect of overexpression of HSP, the study presented here provides compelling support for a fundamental role of inducible HSP in the process of recovery from renal cell injury. Whether this role is to limit the degree of disruption of cytoskeletal and integral membrane protein interactions or whether it is more directly reparative in nature remains to be determined, although it is likely that these effects are interrelated.

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References
7. Benjamin IJ, Horie S, Greenberg ML, Alpern RJ, Williams RS: Induction of stress proteins in cultured myogenic cells: Molec-