

# Prevention of Renal Ischemic Injury by Silencing the Expression of Renal Caspase 3 and Caspase 8

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**Background.** Apoptotic pathways mediated by caspases play a critical role in renal ischemia-reperfusion injury (IRI). Downregulation of the caspase cascade, using small interfering RNA (siRNA) to silence the expression of caspase 3 and caspase 8, may have substantial therapeutic potential for limiting renal injury.

**Methods.** IRI was induced in mice by clamping of the renal vein and artery for 25 or 35 min at 37°C. Caspase 3 and caspase 8 (caspase 3/8) siRNA was administered by hydrodynamic injection. Quantitative polymerase chain reaction (PCR) and immunohistochemistry were used to analyze the gene silencing efficacy, and the therapeutic effects of siRNA were evaluated by renal function analysis, histological examination, and overall survival of mice suffering from IRI.

**Results.** In this study, we have shown, using quantitative PCR, that IRI is associated with increased levels of renal caspase 3/8 mRNA. Mice treated with caspase 3/8 siRNA showed a significant down-regulation in kidney expression of caspase 3/8 at both, transcriptional and protein levels. Kidney function in IRI was protected by siRNA therapy, as levels of blood urea nitrogen and creatinine were significantly reduced in mice treated with siRNA. Histological examination demonstrated that tissue injury caused by IRI was significantly reduced as a result of caspase 3/8 siRNA treatment. Furthermore, survival data showed that more than 70% of mice in siRNA-treated groups survived until the end of the eight-day observation period.

**Conclusion.** Herein, we have demonstrated the therapeutic potential of using siRNA to knock down the expression of caspases and prevent acute renal injury.

**Keywords:** Kidney, Ischemia reperfusion injury, Caspase 3, Caspase 8, Gene silencing, Small interfering RNA.

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Renal ischemia-reperfusion injury (IRI) contributes to acute renal failure in native kidneys, as well as rejection injury in kidney transplants (1). Acute renal failure is the most costly of renal diseases because it is not uncommon and often requires hospitalization. Ischemic acute renal failure, traditionally called acute tubular necrosis (ATN) (2), has a poor prognosis in critically ill patients, and has an average mortality rate of 50% (3). In spite of its description as necrotic in nature, ATN following ischemia/reperfusion (I/R) is mediated largely by apoptosis (4). Although many inhibitors of apoptosis have been used in the attempt to reduce organ damage, the results have not been encouraging, showing little potential in the meaningful reduction of mortality rates following renal failure.

Small-interfering RNA (siRNA) is a powerful tool that can be used to silence gene expression in mammalian cells.

X. Zheng and X. Zhang equally contributed to this study.

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We have previously used siRNA to successfully silence immune-associated genes and to induce immune tolerance following transplantation (5–7). Recent studies have shown that systemic administration of siRNA can prevent local gene expression (8). For example, systemic administration of caspase 8 siRNA inhibits the expression of caspase 8 in the mouse liver, and is also capable of preventing Fas (CD95)-mediated apoptosis of hepatocytes (9). Intraportal administration of caspase-3 and caspase-8 (caspase 3/8) siRNA can inhibit the expression of these two proteins and attenuate warm liver IRI (10). Furthermore, Fas-specific siRNA has been shown to protect mice against renal IRI (11). It may be beneficial to inhibit multiple levels of caspase activation in order to block this cascade more effectively from receptor-dependent (i.e., Fas/FasL) and -independent activation, following IRI. However, preventing kidney IRI by simultaneously silencing caspase 3/8 genes using siRNA has not yet been reported.

In this study, we have established for the first time that efficient, *in vivo* silencing of caspase 3/8 using siRNA-expressing vectors can block renal IRI and effectively protect mice from death caused by acute ischemia induced renal failure.

## MATERIALS AND METHODS

### Caspase 3 and Caspase 8 siRNA Design

Target sequences of the caspase 3 and caspase 8 genes were selected. The oligonucleotides contained target-specific sequences (italic) and restriction enzyme sites at the end of the strands. The sequences were: 1) targeting the caspase 3 gene: 5'-GATCCCAACTACTGTCCAGATAGATCCTTCA-AGAGAGGATCTATCTGGACAGTAGTTTTTTTTTCCAAA-3' (sense), 5'-AGCTTTTGGAAAAA AACTACTGTCCA-GATAGATC CTCTCTTGAAGGATCTATCTGGACAGTA-

GTT GG-3' (antisense); and 2) targeting the caspase 8 gene: 5'-GATCCGACCTTAAAGGAGCTTCATTTCAAGAGAATGAAGCTCCTTAAAGGTCTTTTTTCCAAA-3' (sense), 5'-AGCTTTTGGAAAAAGACCTTAAAGGA GCTTCAT-TCTCTTGAA ATGAAGCTCCTTAAAGGTGG-3' (antisense). The oligonucleotides were synthesized, annealed, and inserted into the PRNATU6.1/Neo siRNA expression vector (Genescript, Piscataway, NJ) to generate shRNA.

### In Vitro Silencing of the Caspase 3/8 Genes

L929 cells were transfected with caspase 3/8 siRNA using lipofectamine 2000. The vector vehicle alone and scrambled (nonsense) siRNA were used as negative controls. Briefly, cells were plated into 24-well plates ( $1 \times 10^5$  of cells per well) and allowed to grow overnight to reach 90% confluence. Cells were transfected with 2  $\mu$ g of caspase 3/8 siRNA or negative control siRNA plasmids in serum-reduced medium for five hr. The cells were then incubated in complete medium for 24 hr. All RNA was extracted from the transfected cells using Trizol (Invitrogen, Burlington, CA) and prepared for subsequent analysis (following the manufacturer's protocol).

### Quantitative Polymerase Chain Reaction

RNA was extracted from kidneys and cells using Trizol (Invitrogen, Burlington, CA). cDNA was synthesized using oligo(dT) primer and reverse transcriptase (Invitrogen). The following paired primers were used to detect glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-TGATGACATCAAGAA GGTGGTGAA-3' (forward) and 5'-TGGGATGAAATTGTGAGG GAGAT-3' (reverse); Caspase 3: 5'-CGGGGTACGGAGCTGGACTGT-3' (forward) and 5'-AATTCGGTTGC CACCTT CCTGT-3' (reverse); Caspase 8 5'-TCGCCCGAGCTGGAGTTGTGA-3' (forward) and 5'-CTCGGTTGCAGTCTAGGAAGTTGA-3' (reverse). Real-time polymerase chain reactions (PCRs) were performed using SYBR Green PCR Master mix (Stratagene, La Jolla, CA) and 100 nM of gene-specific primers. The PCR reaction conditions were 95°C for 10 min, 95°C for 30 sec, 58°C for one min, and 72°C for 30 sec (40 cycles).

### Renal IRI model

Six- to eight-week-old CD1 mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and placed on a heating pad to maintain their body temperature during surgery. Following abdominal incisions, renal pedicles were bluntly dissected and a microvascular clamp (Roboz Surgical Instrument, Washington, DC) was placed on the left renal pedicle for 25 min. During the procedure, animals were kept well hydrated with warm saline and at 37°C. After 25 min of ischemia, the clamps were removed and the right kidney was excised.

### In Vivo Gene Silencing and siRNA Treatment

In vivo gene silencing was performed based on a previously reported "hydrodynamic" injection method (12). In brief, 48 hr prior to IRI experiments, 50  $\mu$ g of caspase 3/8 siRNA plasmid DNA was diluted in 1 ml of phosphate buffered saline (PBS) and intravenously injected into mice over a short time period of 10 seconds.

### Assessment of Renal Function

Blood samples were obtained from the inferior vena cava at 24 hr postischemia. To monitor renal function, levels of serum creatinine and blood urea nitrogen (BUN) were measured by the core laboratory at the London Health Sciences Center.

### Histopathology and Immunohistochemistry Examination

For histopathological examination, kidneys were collected, fixed in 10% formalin, sectioned, and stained with hemolysin and eosin (H&E).

For immunohistochemistry, kidneys were snap-frozen in OCT (Sakura, Fineck, Torrance, CA) and 5- $\mu$ m frozen sections were obtained. Sections were fixed and incubated with a 1:10 dilution of primary rabbit antimouse antibody against caspase 3 or caspase 8 (Santa Cruz Biotechnology). After washing with PBS, the slides were incubated with rabbit EnVison<sup>+</sup> HRP (DAKO, Carpinteria, CA) and developed with ready-to-use 3,3'-Diaminobenzidine (DAB) for one to three min and counterstained with hematoxylin (DAKO, Carpinteria, CA). Both antibody incubations lasted for 30 min at room temperature.

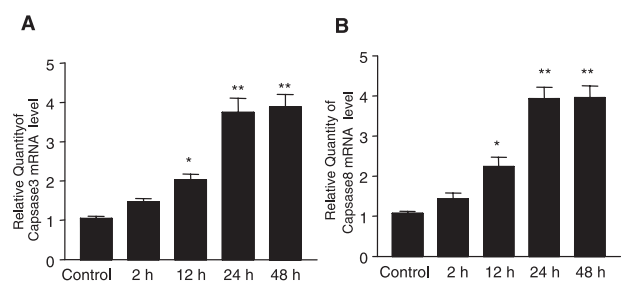
### Statistical Analysis

Data are expressed as means  $\pm$  SEM. Statistical comparisons among groups were performed using one-way analysis of variance followed by the Newman-Keuls Test and rank-log test for survival statistical analysis. Statistical significance was determined as  $P < 0.05$ .

## RESULTS

### Upregulation of Caspase 3/8 in Kidney IRI

As a proof of principle, we first analyzed the expression of caspase 3/8 using a kidney IRI model, in which renal arterial flow was blocked by clamping for 25 min. Renal expression of caspase 3/8 was determined at the mRNA level by quantitative PCR (Fig. 1, A and B). Results showed detectable expression of caspase 3 and caspase 8 transcripts in kidneys of healthy control mice. Following induction of renal IRI, caspase 3 mRNA level had started to increase at two hr and



**FIGURE 1.** Increased expression of caspase 3/8 in kidney IRI. Left kidney was clamped for 25 min, as described in Materials and Methods. Then 2–48 hr following clamping, kidney was harvested and total RNA was extracted. (A and B) Caspase 3/8 expression was detected by quantitative PCR. Transcription was amplified using specific primers to caspase 3 (A) and caspase 8 (B), and GAPDH genes. Data represents experiments performed on five animals per group. (\* $P < 0.05$ , \*\* $P < 0.001$  vs. normal control mice).

was significantly elevated after 12 hr (Fig. 1A). Similar increases of caspase 8 mRNA levels were observed in renal IRI (Fig. 1B).

### Silencing Caspase 3/8 In Vitro and In Vivo Using siRNA

In previous work, we have successfully silenced gene expression using synthesized siRNA (5). In the current study, we designed siRNA-expressing vectors for the purpose of in vivo treatment. In order to assess the gene silencing ability of caspase 3/8 siRNA vectors, we transfected L929 cells, which express high levels of caspase 3/8 (data not shown), with caspase 3/8 siRNA. Effective gene silencing was observed using caspase 3/8 siRNA vectors (data not shown).

We next examined the in vivo gene silencing efficacy of the caspase 3/8 siRNA vectors. In vivo gene silencing has been previously demonstrated in the kidney by an independent group, which used a “hydrodynamic” injection method to administer siRNA (12). Accordingly, we used the same method for in vivo delivery of caspase 3/8 siRNA to the kidney. The incorporation of green fluorescent protein-expressing, caspase 3/8 siRNA vectors was tracked using epifluorescence microscopy (data not shown), and silencing efficacy was examined at the level of mRNA, using quantitative PCR. As shown in Figure 2A and B, the relative quantity of caspase 3 and caspase 8 mRNA, respectively, was significantly reduced relative to control groups that were treated with a blank vector or PBS.

To further confirm the gene silencing efficacy, immunohistochemistry was used to analyze the inhibition of caspase 3 and caspase 8 at the protein level. Positive staining

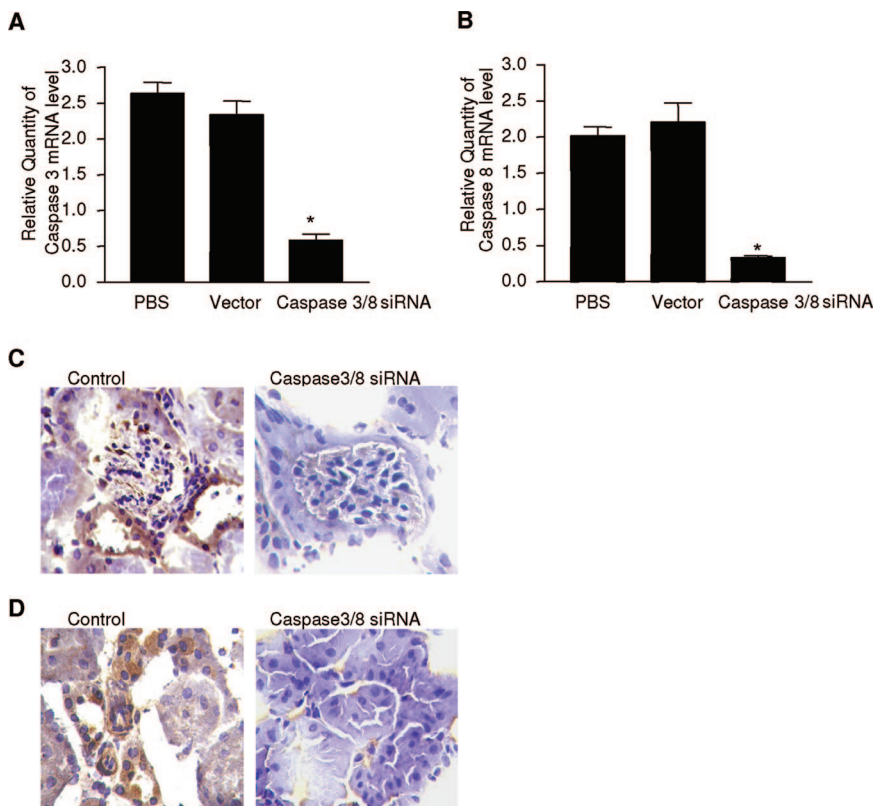
of caspase 3 and caspase 8 was observed in glomeruli and tubules of nonsilenced IRI kidneys. In contrast, mice that were treated with caspase 3/8 siRNA showed significant reductions in protein levels of both caspase 3 and caspase 8 (Fig. 2, C and D).

### Caspase 3/8 siRNA Protects from Renal Dysfunction in IRI

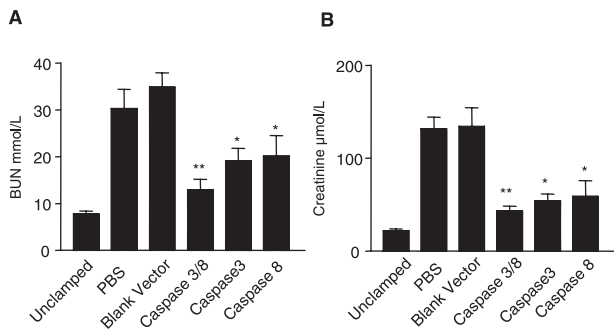
We hypothesized that caspase 3/8 siRNA would also prevent the renal damage caused by apoptosis in ischemia-reperfusion. In order to assess the degree of renal dysfunction exhibited by siRNA-treated versus untreated mice, we measured levels of BUN and creatinine in serum that was collected from the mice, 24 hr following reperfusion. After clamping, the levels of BUN (Fig. 3A) and creatinine (Fig. 3B) significantly increased relative to unclamped controls. However, treatment with caspase 3/8 prior to induction of IRI significantly prevented increase of BUN (12.9 mmol/L) and serum creatinine values (43.4  $\mu$ mol/L) 24 hr following IRI injury. Hence, treatment with caspase 3/8-siRNA significantly attenuated IRI-induced renal damage.

### Caspase 3/8 siRNA Protects against Lethal Kidney Ischemia

The above results, in which caspase-siRNA treatment prevented renal dysfunction, prompted us to further explore whether caspase 3/8 treatment could protect against lethal kidney damage in IRI. We therefore evaluated the survival rate of treated versus untreated mice, following 35 min of ischemia followed by reperfusion. Because most mice with renal failure died by the fourth day, long-term survival was



**FIGURE 2.** Silencing caspase 3/8 in vivo. Mice were injected intravenously with 50  $\mu$ g of caspase 3/8 siRNA-expressing vectors. Forty-eight hours subsequent to siRNA treatment, kidneys were clamped for 25 min. Twenty-four hours following clamping, kidneys were harvested and gene silencing of caspase 3 and caspase 8 was assessed by quantitative PCR (A and B, respectively) and immunohistochemistry (C and D, respectively). Control groups included treatment with PBS or a blank vector that does not encode siRNA. Data are expressed as means  $\pm$  SEM, statistical significance as compared with PBS-treatment and blank vector control group (\* $P$  < 0.001).



**FIGURE 3.** siRNA protects renal function in IRI. Renal pedicles were clamped for 25 min. Blood was collected 24 hr after IR to determine levels of BUN (A) and serum creatinine (B). Data shown are means ± SEM; statistical significance as compared with PBS or blank vector treated group (\* $P < 0.05$ , \*\* $P < 0.001$ ).

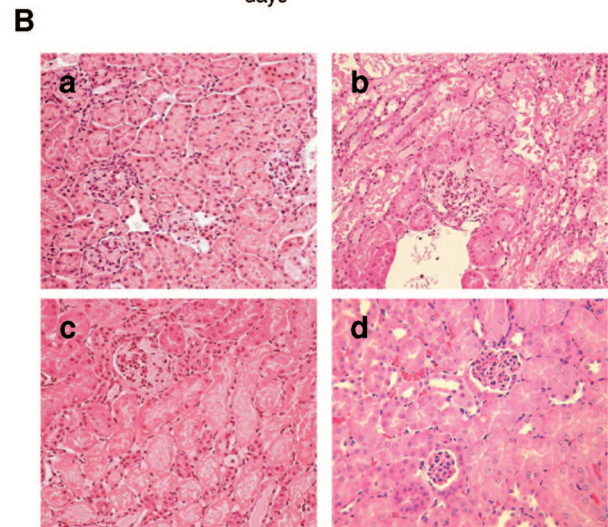
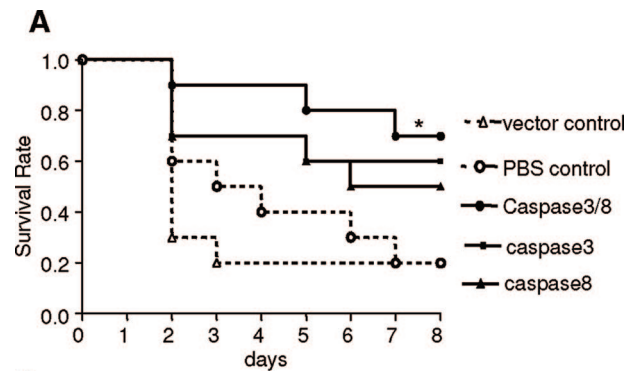
defined as surviving past eight days. As can be seen in Figure 4A, single treatment with caspase 3 or caspase 8 siRNA improved survival, but without significant difference ( $P > 0.05$ ) when compared to PBS- or vector-treated control groups. In contrast, the combinational treatment of caspase 3 and caspase 8 siRNA had statistical significance in protection against lethal IRI ( $P < 0.05$ ).

To confirm the beneficial effects of siRNA treatment, we further examined histopathological changes in the I/R injured kidneys. Kidneys from mice that were treated with PBS or empty vectors were severely damaged by ischemia and reperfusion (Fig. 4B), which was observed from presence of widespread tubular necrosis, vacuolization, and neutrophil infiltration. In contrast, kidneys from mice that were pre-treated with the combinational therapy of caspase 3/8 siRNA were protected from IRI and seemed almost normal, exhibiting mild renal congestion.

**DISCUSSION**

Renal IRI is a common cause of acute renal failure (ARF) and contributes to loss of function and long-term allograft changes. Following an ischemic episode, the initial damage is caused by tubular epithelial cell (TEC) apoptosis and necrosis. This is followed by endothelial cell injury, which further exacerbates the continued ischemia of tubular epithelium, as well as the inflammatory response (13). To date, there is no specific therapy available to improve the clinical outcome of IRI, which only relies on nutritional and supportive care. Therefore, a new therapeutic strategy for patients with ARF and other forms of kidney failure is desperately needed.

Although acute tubular necrosis (ATN) is described as following IRI, the process is actually mediated largely by apoptotic pathways and caspase activation (4). Blocking caspase-associated activation of apoptotic pathways from surface death receptors, such as Fas or tumor necrosis factor-R1, may therefore improve organ survival (14–16). The loss of Fas, a transmembrane protein, has been shown to attenuate ischemia reperfusion injury (17). In addition, Bax (18) and BCL-X(s) (19) are mitochondrial proteins associated with induction of renal cell damage, which induce the ultimate activation of caspase 3 and irreversible cell injury. All



**FIGURE 4.** siRNA protects against kidney ischemia. (A) siRNA protects against lethal kidney ischemia. CD1 mice (n = 10 per group) were intravenously treated with 50 µg of caspase 3 and 8-siRNA vectors, blank vectors, or PBS, following clamping for 35 min. Survival of siRNA-treated, blank vector-treated, or PBS-treated mice was observed over eight days (\* $P < 0.05$ , Caspase 3/8-siRNA treated vs. PBS or blank vector treated mice). (B) Histopathological examination. Normal or IRI kidney tissue from (A) were harvested, sectioned and stained with H&E. (a) Normal unclamped kidney shows no signs of tubule necrosis, vacuolization, and neutrophil infiltration. (b) PBS-treated I/R kidney exhibits widespread tubule necrosis, severe vacuolization, and neutrophil infiltration. (c) Empty vectors-treated I/R kidneys show similar histopathological changes to (b). (d) Caspase 3 siRNA and caspase 8 siRNA-treated I/R kidney exhibits a mild kidney congestion.

these suggest that caspases 3 and 8 could be potential therapeutic targets for prevention of renal IRI.

Although many inhibitors of apoptosis have been used for preventing IRI, there has been little practical translation of these approaches to preclinical levels. Specific silencing of genes using siRNA is a newly developed method that is 1,000-fold more potent than using antisense oligonucleotides (20), indicating therapeutic potential of this technique (7). An siRNA approach has been applied to prevent kidney IRI by silencing complement genes, which are centrally involved in early ischemic injury (21).

The rapidity of apoptosis following IRI suggests that caspase expression and function occur mainly via preformed

proteins, and this process is not largely controlled at the transcriptional level. However with IRI, genes for caspases 3 and 8 are upregulated and attenuation of renal injury using siRNA suggests that a gene transcription significantly contributes to ongoing IRI. We have had similar results when blocking complement 3 expression with siRNA (21), and other groups have had success in silencing Fas expression using siRNA, using an IRI model (C. Du and A. Jevnikar, University of Western Ontario, personal communication). Nonetheless, additional strategies to block protein function using caspase inhibitors may further improve protection from IRI and could also be added to organ preservation solutions.

In summary, we have established that systemic administration of caspase 3/8-specific siRNA is capable of inhibiting renal caspase 3/8 in kidney in IRI. We further proved that the systemic application of caspase 3/8 siRNA can prevent renal ischemia-reperfusion injury. Consequently, the use of systemically-delivered caspase 3/8-siRNA may represent a novel approach to preventing renal apoptotic damage in IRI, and may become therapeutically useful, both in the setting of renal transplantation and for other conditions associated with IRI in native and transplanted kidneys.

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