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Protection against cold storage induced renal tubular cell apoptosis

Swati Jain^{*,1}, Daniel Keys^{*,1}, Danica Ljubanovic³, Charles L Edelstein^{1,2}, and Alkesh Jani^{1,2}

¹University of Colorado, Denver, Colorado, USA

²Denver Veterans Affairs Medical Center, Denver, Colorado, USA

³University Zagreb SOM, Zagreb University Hospital Dubrava, Zagreb, Croatia

Abstract

Background—Prolonged cold storage of donor kidneys is associated with tubular cell apoptosis and caspase-3 activation. We have previously shown that pan-caspase inhibition prevents cold storage associated tubular apoptosis. Due to the non-specific nature of pan-caspase inhibitors, which block all caspases including pro-inflammatory caspase-1, the effect of specific caspase-3 inhibition during cold storage is unknown. X-linked inhibitor of apoptosis (XIAP) is the most potent naturally occurring specific inhibitor of caspase-3. We hypothesized that prolonged cold storage would decrease XIAP, while upregulation of XIAP with the novel compound UCF-101, would protect against caspase-3 activation and tubular cell apoptosis.

Methods—LLC-PK1 tubular cells and whole kidneys from C57BL/6 mice were subjected to prolonged cold storage with or without UCF-101, and examined for XIAP, caspase-3, and tubular apoptosis.

Results—Tubular cells subjected to prolonged cold storage in vitro demonstrated significantly decreased XIAP, and significantly increased apoptosis, caspase-3 protein and activity. UCF-101 treatment significantly increased XIAP, significantly decreased caspase-3 protein and activity, and protected against apoptosis. To determine the therapeutic significance, whole kidneys were subjected to prolonged cold storage with UCF-101. UCF-101 significantly increased XIAP in donor kidneys and protected against apoptosis.

Conclusions—Prolonged cold storage of tubular cells in vitro and whole mouse kidneys ex vivo is associated with loss of XIAP and subsequent tubular cell apoptosis. UCF-101 protects against

Address for Correspondence: Alkesh Jani, University of Colorado Denver Division of Renal Diseases and Hypertension, 12700 East 19th Avenue, C281, Aurora, Colorado 80045; Tel: 303 724 4852; Fax: (303) 724-4831; Alkesh.jani@ucdenver.edu.

*Authors contributed equally to the study.

Swati Jain (swati.jain@ucdenver.edu) participated in performance of research, data analysis and writing of paper. No conflicts of interest.

Daniel Keys (daniel.keys@ucdenver.edu) participated in performance of research, data analysis and writing of paper. No conflicts of interest.

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the loss of XIAP during prolonged cold storage both in vitro and ex vivo, and is associated with significantly reduced tubular cell apoptosis. UCF-101 may represent an attractive approach to improve organ preservation.

INTRODUCTION

Both human and animal studies suggest that the adverse impact of cold storage of organs is associated with caspase-3 activation and tubular cell apoptosis (1) (2) (3). Caspase-3 is the “executioner caspase” that is centrally important in apoptotic cell death in vivo (4). Apoptosis rate has been shown to correlate significantly with cold-ischemia time in human cadaveric renal transplants (1). Biopsies of human donor kidneys which subsequently develop post-operative acute tubular necrosis (ATN) demonstrate an increase in apoptosis in renal tubular epithelial cells (2). Our previous work demonstrates that prolonged cold storage results in caspase-3 activation, tubular injury and tubular cell apoptosis in a model of cold storage in mice (3). Treatment of cold stored kidneys with a pan-caspase inhibitor prevented cold-storage associated tubular cell apoptosis and brush border injury. Due to the non-specific nature of pancaspase inhibitors, which block all caspases including pro-inflammatory caspase-1, the effect of specific caspase-3 inhibition on cold stored donor kidneys was not defined.

XIAP (X-linked inhibitor of apoptosis), a naturally occurring, specific inhibitor of caspase-3 (5), belongs to the Inhibitor of apoptosis protein (IAP) families, whose member's binds and inhibit caspases 3, 7, and/or 9, but not caspase 8 (6). XIAP is inhibited by HtrA2 (High temperature requirement protein A2), a mitochondrial serine protease released during mitochondrial injury (7). Mitochondrial injury is known to occur during cold storage (8). UCF-101 is a novel compound that specifically inhibits the proteolytic activity of HtrA2, leading to less degradation of XIAP, and thus prevents apoptosis. UCF-101 has been shown to prevent apoptotic cell death during myocardial ischemia and reperfusion (9), treatment with TNF α (10) and staurosporine (7), and AKI due to cisplatin (11, 12).

We hypothesized that prolonged cold storage would lead to decreased XIAP protein expression and an increase in caspase-3 protein, activity and tubular cell apoptosis during cold storage of both tubular cells in vitro and whole mouse kidneys ex vivo. Furthermore, we hypothesized that upregulation of XIAP with UCF-101 would lead to protection from tubular cell apoptosis during prolonged cold storage.

The purpose of this study was to evaluate UCF-101 as a novel therapy to prevent tubular cell apoptosis during in vitro and ex vivo cold storage.

MATERIALS AND METHODS

In vitro cold storage model

LLC-PK1 (ATCC® CL-101™) cells were cultured in Dulbecco's modified Eagles medium (DMEM)/F-12 50/50 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100µg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂, and fed with fresh medium at intervals of 48 hours. LLC-PK1 cells were incubated at

70% confluence in UW solution for 24 hours at 4°C with or without UCF-101 (Calbiochem, 496150) at a final concentration of 50 µM. The concentrations of UCF-101 employed were selected as previously described (12) and per the manufacturer's instructions. Does of Control cells were kept at 37 degrees and were treated with vehicle, dimethyl sulfoxide (DMSO).

Ex vivo cold storage model

Experiments were performed with C57BL/6 mice. Mice were used at 12 weeks of age weighing 20-25g. After anesthesia with avertin, cervical dislocation was performed. Sternotomy was performed with administration of 10 cc of cold UW solution through the left ventricle with venting of blood out of the right atrium. The left kidney underwent nephrectomy and storage in UW solution at 4°C for 24 hours with or without UCF-101 at final concentration of 100 µM. The right kidney was processed immediately as control. Hemi-section of the kidney was used for protein extraction and one half for histology.

Apoptosis scoring

Paraformaldehyde (4%)-fixed and paraffin-embedded kidneys are sectioned at 4 µm and stained with hematoxylin-eosin and periodic acid-Schiff (PAS) by standard methods. Morphologic criteria are used to count apoptotic tubular epithelial cells on hematoxylin-eosin and PAS staining as previously described (3, 13, 14). These characteristics included cellular rounding and shrinkage, nuclear chromatin compaction and formation of apoptotic bodies. Apoptotic tubular epithelial cells are quantitatively assessed per high-power field (×400) by a renal pathologist in a blinded fashion. At least 10 high-power fields per slide were counted.

Immunoblotting

Protein concentration for whole kidney extracts and cell lysates prepared in RIPA buffer was performed using BCA protein ascertainment. Twelve percent polyacrylamide gel was used for protein separation run for 2 hours at 100 V. Proteins were transferred to a PVDF membrane, which was blocked with 5% milk, and then incubated with antibodies against XIAP (Cell signaling #2042) and cleaved caspase-3 (Cell signaling #9661) overnight in 1:1000 concentration in 2% milk. Donkey anti-rabbit with HRP at 1:1000 concentration in 2% milk was used as a secondary antibody.

Caspase-3 Activity

Protein (100 µg) from cell culture lysate and whole kidney extract (100 µg) was used to determine caspase-3 activity. Caspase-3 activity was assayed using a colorimetric kit, (Biovision, K106-100) according to the manufacturer's directions.

Flow cytometry

Dead Cell Apoptosis Kit (Life Technologies, V13241) with Alexa® Fluor 488 annexin V and PI was used to quantify apoptotic cell death in LLC-PK1 cells, with and without UCF-101 treatment, during cold storage,. Briefly, cells were washed twice with cold PBS and then resuspended in 1X Annexin V binding buffer at a density of 1×10^6 cells/ml. Alexa

Fluor® 488 annexin V and propidium iodide (PI) were added to the suspended cells, and the reaction was incubated in the dark for 10 minutes. Samples were immediately analyzed using the core flow cytometry service at the University of Colorado Cancer Center (Aurora, CO). Untreated cells were used as a negative control. Results were obtained from six independent experiments.

Statistical Analysis

The data are representative of at least 4 independent studies with reproducible results. Values are presented as mean $\pm$ SEM. Data were analyzed by analysis of variance (ANOVA) using GraphPad prism software version 5.01 (California, USA). Differences between groups were considered to be significant at $p < 0.05$.

RESULTS

In vitro model of cold storage

Apoptosis (flow cytometry)—LLC-PK1 tubular cells subjected to prolonged in vitro cold storage in UW solution at 4°C demonstrated a significant increase in apoptosis detected by flow cytometry. Treatment of LLC-PK1 with UCF-101 during prolonged cold storage significantly reduced the percentage of apoptotic tubular cells (Figure 1). PI positive necrotic cells were not significantly increased by cold storage (Figure 1).

Caspase-3—Caspase-3 is the major mediator of apoptotic cell death. Increased tubular cell apoptosis during prolonged cold storage was associated with a significant increase in cleaved caspase-3 activity. Caspase-3 activity in cells subjected to cold storage was significantly increased versus control LLC-PK1 cells (Figure 2A). Treatment of cold stored cells with UCF-101 significantly reduced caspase-3 activity to levels similar to control values (Figure 2A). Immunoblotting was performed to detect the processed (active) form of caspase-3 (~17 kDa) (Figure 2B and 2C). Active caspase-3 was absent in the control cells, but was significantly increased in cells subjected to cold storage, corresponding to the significant increase in apoptosis during cold storage (Figure 1). Treatment of cold stored cells with UCF-101 significantly reduced active caspase 3 (Figure 2B and 2C).

XIAP—Immunoblotting was performed to determine whether the anti-apoptotic effect of UCF-101 was associated with less XIAP (~53 kDa). XIAP protein was present in the control cells, but significantly decreased in cold stored cells, corresponding to the significant increase in active caspase-3. Treatment of cold stored cells with UCF-101 significantly increased XIAP (Figure 3).

Ex vivo model of cold storage

Apoptosis (histologically)—To determine the possible therapeutic effect of UCF-101, ex vivo experiments were performed. Whole kidneys from C57BL6 mice were subjected to cold storage in UW solution at 4°C for 24 hours with or without UCF-101. Apoptotic tubular cells were significantly increased in cold stored kidneys whereas cold stored kidneys treated with UCF-101 had significantly less apoptotic tubular cells (Figure 4).

Caspase-3—Increased tubular cell apoptosis during prolonged ex vivo cold storage was associated with a significant increase in cleaved caspase-3 activity. Caspase-3 activity in cold-stored cells was significantly increased over control kidneys (Figure 5A). Treatment of cold stored kidneys with UCF-101 significantly reduced caspase-3 activity to levels similar to control values (Figure 5A). Immunoblotting revealed a significant increase in active caspase-3 in cold stored kidneys compared to controls (Figure 5B and 5C) corresponding to the increase in casapse-3 activity (Figure 5A) and tubular cell apoptosis in cold stored kidneys (Figure 4). Treatment of cold stored kidneys with UCF-101 significantly reduced active caspase 3 protein expression (Figure 5B and 5C).

XIAP—Immunoblotting revealed a significant decrease in XIAP in cold stored kidneys compared to controls (Figure 6) corresponding to the increase in casapse-3 activity (Figure 5A) and the increase in tubular cell apoptosis in cold stored kidneys (Figure 4). Treatment of cold stored kidneys with UCF-101 significantly increased XIAP protein expression (Figure 6).

DISCUSSION

Prolonged cold storage of organs is a known risk factor for the development of delayed graft function (DGF) (15-17), and is associated with tubular cell apoptosis (2, 3). The mechanism by which prolonged cold storage leads to apoptosis is not known. Here we provide novel evidence that prolonged cold storage may sensitize cells to apoptosis by inducing decreased expression of X-linked inhibitor of apoptosis (XIAP) protein. To our knowledge, these studies are the first to examine the role of XIAP in prolonged cold storage -associated apoptosis.

Our previous studies demonstrated that pancaspase inhibition with QVD-OPH could reduce tubular cell apoptosis and brush border injury of cold stored kidneys. QVD-OPH, a carboxy terminal phenoxy group conjugated to the amino acids valine and aspartate, is a broad spectrum pancaspase inhibitor that inhibits several apoptotic pathways, including those involving caspases 3,8,9,10 and 12 (18, 19). In addition, pancaspase inhibitors also inhibit pro-inflammatory caspase-1 (20, 21). In the current study, we endeavored to understand the specific effect of caspase-3 inhibition during prolonged cold storage.

XIAP belongs to the inhibitor of apoptosis protein (IAP) family, whose members are classified according to the presence of baculovirus IAP repeat (BIR) domains (22). Class 1 IAPs, including XIAP, contain a RING finger domain and homologous BIR domains. (22). XIAP is the most potent, naturally occurring inhibitor of caspase-3 (23) via its BIR 2 binding domain (24). Cerebral and myocardial warm ischemia/reperfusion injury leads to degradation of XIAP protein and increased apoptosis (9, 25, 26). Loss of XIAP is implicated in cisplatin induced tubular cells apoptosis (12) and apoptotic tubular cells death during urinary tract obstruction (23).

We observed significantly decreased XIAP protein expression in LLC-PK1 tubular cells subjected to cold storage in vitro. Loss of XIAP in LLC-PK1 cells in vitro was associated with increased caspase-3 activity, increased caspase-3 protein and tubular cell apoptosis. To

determine the therapeutic significance of the in vitro findings, we subjected mouse whole kidneys to ex vivo cold storage in a manner analogous to human donor kidney cold storage. Prolonged ex vivo cold storage of whole mouse kidneys also reduced XIAP protein expression, and was associated with tubular cell apoptosis, thus corroborating the in vitro findings.

We therefore treated LLC-PK1 tubular cells with UCF-101 in vitro, to assess the effect of increasing XIAP protein expression. UCF-101 is highly specific for HtrA2, with little activity against other serine proteases (27). Increased XIAP expression in UCF-101 treated cells was associated with a significant decrease in caspase-3 activity, protein and tubular cell apoptosis. To assess the therapeutic potential of UCF-101, we treated whole kidneys subjected to cold storage with UCF-101. We observed an increase in XIAP protein expression that was associated with decreased caspase-3 protein and tubular cell apoptosis, in agreement with the in vitro findings.

The finding of preserved XIAP protein in UCF-101 treated cells implicates HtrA2 in the pathogenesis of cold storage associated tubular cells apoptosis. XIAP is regulated by HtrA2 (high temperature-regulated A2), a mitochondrial serine protease that promotes apoptosis by inhibiting XIAP (9). HtrA2 is released along with cytochrome c during mitochondrial disruption (28). Mitochondrial dysfunction is associated with prolonged cold ischemia (8), but the mechanism by which prolonged cold storage and mitochondrial dysfunction leads to tubular cell apoptosis is not known. Our findings suggest that prolonged cold storage disrupts mitochondrial membrane integrity, leading to the release of HtrA2, with subsequent inhibition of XIAP.

It is not known whether inhibition of HtrA2 or tubular cell apoptosis in donor kidneys subjected to prolonged cold storage translates into improved post-transplant renal function. DGF is associated with tubular cell apoptosis (1, 2) and tubular cell apoptosis of donor organs predicts the occurrence of DGF post-transplant (2). Future studies examining whether inhibition of apoptosis in donor kidneys prevents post-transplant renal dysfunction will contribute to our understanding of the pathogenesis of DGF.

In summary, we have shown that prolonged cold storage of tubular cells in vitro and whole mouse kidneys ex vivo is associated with loss of XIAP protein, and subsequent increase in caspase-3 protein, caspase-3 activity and tubular cell apoptosis. Use of the novel HtrA2 inhibitor, UCF-101 prevented the loss of XIAP during prolonged cold storage both in vitro and ex vivo, and was associated with significantly reduced tubular cell apoptosis. The relevance of these studies to organ preservation and the subsequent risk of DGF are substantial. UCF-101 may represent an attractive approach to improving organ preservation, and to potentially preventing DGF.

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ABBREVIATIONS

ATN	Acute tubular necrosis
BIR	Baculovirus IAP repeat
CS	Cold storage
DGF	Delayed Graft Function
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulfoxide
HtrA2	High temperature requirement protein A2
IAP	Inhibitor of apoptosis protein
PAS	Periodic acid-Schiff
PI	Propidium iodide
XIAP	X-linked inhibitor of apoptosis

Figure 1. UCF-101 significantly decreases apoptotic tubular cells in culture during cold storage

The percentage of apoptotic and necrotic cells were assessed by Alexa® Fluor 488 annexin V and PI binding by flow cytometry (n=6). **A:** Apoptotic cells (annexin V positive and PI negative) were increased in cold stored tubular cells (CS) compared to controls (Cont). Apoptotic cells were decreased by treatment with UCF-101 during cold storage (CS +UCF-101) (*p < 0.001 vs. Cont and CS+UCF-101, n=6). **B:** Representative flow cytometry experiment of 6 independent experiments of LLC-PK1 cells exposed to cold storage (CS) with and without treatment with UCF-101 is shown. Apoptotic cells (annexin V positive and PI negative) are shown in quadrant A4. PI positive necrotic cells (quadrant A1) were not significantly increased by cold storage.

Figure 2. UCF-101 significantly decreases caspase-3 activity and cleaved caspase-3 expression in tubular cells in culture during cold storage

A: Caspase-3 activity was increased in cold storage (CS) vs. controls (Cont) but was decreased by treatment with UCF-101 treatment (CS+UCF-101). (* $p < 0.001$ vs. cont and * $p < 0.01$ Cold+UCF-101, $n=5$). **B and C:** Cleaved caspase-3 was increased in cold storage (CS) vs. control (Cont). Treatment with UCF-101 decreased cleaved caspase-3 during cold storage (CS+UCF-101). (* $p < 0.001$ vs. Cont and * $p < 0.05$ vs. Cold+UCF-101, # $p < 0.05$ vs. Cont, $n=4$). The densitometry values were adjusted to loading control (β -actin) and are representative of at least 4 independent studies with reproducible results.

Figures 3. UCF-101 prevents degradation of XIAP during cold storage of tubular cells in culture

A and B: XIAP was decreased in tubular cells exposed to cold storage (CS) vs. control (Cont). XIAP protein was restored by treatment with UCF-101 during cold storage (CS + UCF-101). (* $p < 0.01$ vs. Cont and * $p < 0.001$ vs. Cold+UCF-101, $n=4$). The densitometry values were adjusted to loading control (β -actin) and are representative of at least 4 independent studies with reproducible results.

Figure 4. Tubular apoptosis was significantly decreased by UCF-101 treatment in whole kidneys during cold storage

A: Apoptotic cells were increased in whole kidneys during cold storage (CS) compared to controls (Cont). Apoptotic cells were decreased in kidneys during cold storage with UCF-101 (CS+UCF-101) (* $p < 0.001$ vs. Cont and * $p < 0.01$ vs. CS+UCF-101, $n=5$ animals in each group). **B-D:** Representative periodic acid-Schiff (PAS; X400) stained sections of control kidney and kidney stored for 24 hours of cold with or without UCF-101 demonstrating decreased apoptosis with UCF-101 treatment during cold storage.

Figure 5. UCF-101 significantly decreased caspase-3 activity and cleaved caspase-3 expression in whole kidneys during cold storage

A: Caspase-3 activity was increased in kidneys exposed to cold storage (CS) vs. controls (Cont) but was decreased by treatment with UCF-101 (CS+UCF-101) (* $p < 0.001$ vs. Cont and * $p < 0.01$ vs. CS+UCF-101, $n=5$ animals in each group). **B and C:** Cleaved caspase-3 was increased in kidneys during cold storage (CS) vs. control (Cont). UCF-101 treatment decreased cleaved caspase-3 in kidneys during cold storage (CS+UCF-101). (* $p < 0.01$ vs. Cont, * $p < 0.05$ vs. CS+UCF-101, $n=5$). The densitometry values were adjusted to loading control (β -actin) and are representative of at least 5 independent studies with reproducible results.

Figures 6. UCF-101 prevents degradation of XIAP in whole kidney during cold storage
A and B: XIAP was decreased in kidneys exposed to cold storage (CS) vs. control (Cont). XIAP was restored by preservation of donor kidneys with UCF-101 during cold storage (CS +UCF-101). (* $p < 0.05$ vs. Cont, * $p < 0.01$ vs. CS+UCF-101, $n=5$). The densitometry values were adjusted to loading control (β -actin) and are representative of at least 5 independent studies with reproducible results.