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ORIGINAL ARTICLE

Inhibition of autophagy increases apoptosis during re-warming after cold storage in renal tubular epithelial cells

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Summary

Prolonged cold storage and re-warming (CS/REW) of kidneys are risk factors for delayed graft function (DGF). Studies in renal tubular epithelial cells (RTECs) have determined apoptosis and autophagy in models of either cold storage (CS) or re-warming alone. The effect of both cold storage and re-warming on apoptosis and autophagy, in RTECS is not known and is relevant to DGF as the kidney is subjected to both CS and re-warming. We hypothesized that CS/REW of RTECs would induce autophagy that protects against apoptosis. In CS/REW, there was increased autophagic flux of RTECs. Autophagy inhibition using an Atg5 siRNA resulted in increased cleaved caspase-3 and increased apoptotic cells (on both morphology and annexin V staining) during CS/REW. The effect of autophagy inhibition on necrosis in RTECs is unknown. There were increased necrosis and caspase-1, a mediator of necrosis, during CS/REW, and the Atg5 siRNA had no effect on necrosis and caspase-1. In a kidney transplant model, there was an increase in LC3 II, a marker of autophagy, in kidneys transplanted after cold storage. In summary, autophagic flux is increased during CS/REW. Autophagy inhibition resulted in increased cleaved caspase-3 and increased apoptosis during CS/REW without an effect on necrosis or caspase-1. In conclusion, autophagy inhibition in RTECs after CS/REW induces apoptotic cell death and may be deleterious as a therapy to decrease DGF.

Introduction

Prolonged cold storage and re-warming (CS/REW) of kidneys are risk factors for delayed graft function [1]. Delayed graft function (DGF) occurs in up to 42% of primary deceased-donor renal transplants in the United States and independently predicts reduced 1- and 5-year kidney transplant survival [2,3]. Our previous study in cold ischemic kidneys suggested that autophagic flux inhibition with Bafilomycin-A1 resulted in less apoptotic cell death and that autophagy inhibition may be a potential therapy to reduce DGF [4].

Autophagy is a highly conserved pathway that involves cell degradation of unnecessary or dysfunctional cellular components and is rapidly upregulated during starvation or cell stress [5,6]. Cold ischemia during organ preservation and re-warming during transplantation represents cellular stresses and nutrient deprivation that may induce autophagy. Inhibition of autophagy during cell stress may result in apoptosis [7–9]. The relationship between apoptosis and autophagy and the effect of specific autophagy inhibition on renal tubular epithelial cells (RTECs) during CS/REW is not known. Cold preservation alone causes apoptosis and autophagy in kidneys [4] and autophagy in hepatocytes [10]. Nonspecific autophagy inhibition using Bafilomycin-A1 during cold preservation in kidneys or wortmannin during cold preservation in hepatocytes resulted in less cell death [4,11]. During warm ischemia–reperfusion, autophagy inhibition results in impaired kidney function and increased tubular cell apoptosis suggesting that autophagy prevents cell death during warm ischemia–reperfusion [12]. Thus, most previous studies have focused on the effect

of either cold preservation or warm ischemia–reperfusion on apoptosis and autophagy. The effect of both CS/REW on apoptosis and autophagy in RTECs is not known.

Many previous studies of autophagy inhibition have been limited by the use of chemical drugs, for example,. Bafilomycin-A1 that do not specifically target autophagy and inhibit a broad spectrum of biological functions in addition to autophagy [12]. For example, autophagy suppression with the phosphatidylinositol 3 kinase (PI3K) inhibitor, wortmannin, resulted in less hepatocyte injury after CS/REW [11]. In contrast, tubule-specific ATG7-null mice are more sensitive to warm ischemia–reperfusion injury to kidney [13,14]. The effect of specific autophagy inhibition using an Atg5 siRNA on apoptosis, necrosis, and autophagy in RTECs is not known. It is important to determine the effect of specific autophagy inhibition on CS/REW injury in RTECs as autophagy suppression has been suggested as a potential therapy to decrease CS/REW injury [11]. The aim of the study was to determine the effect of specific autophagy inhibition, using an Atg5 siRNA that specifically targets autophagy, on apoptosis, and necrosis in RTECs exposed to CS/REW.

Materials and methods

Reagents and antibodies

Bafilomycin-A1 and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to cleaved caspase-3, LC3B, Atg5, β-actin, and RIPA buffer were purchased from Cell Signaling Technology (Boston, MA, USA). Caspase-1 activity assay kit was purchased from BioVision (Milpitas, CA, USA). DeadEnd™ Colorimetric TUNEL assay kit was purchased from Promega (Madison, WI, USA). Atg5 siRNA (Catalog ID: 4390771), siRNA scrambled control (Catalog ID: 4390843), Lipofectamine2000™ reagent, and Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit with Alexa® Fluor 488 annexin V and PI for Flow Cytometry were purchased from Life Technologies (Grand Island, NY, USA).

Cell culture

The porcine-derived renal proximal tubular cell line LLC-PK1 (ATCC® CL-101™) was 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 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2 and fed with fresh medium at intervals of 48 h. Experiments were performed with cells grown to 70–80% confluence. To stimulate cold storage, DMEM media containing FBS was replaced with University of Wisconsin (UW) solution and cells were subjected to cold storage for 24 h at 4 °C in a humidified atmosphere with 5% CO_2. To stimulate re-warming after cold storage (CS/REW), UW solution was replaced with DMEM containing FBS and the cells were again incubated at 37 °C incubator for 24 h. Control cells were kept at 37 degrees.

Bafilomycin-A1 was added to the cells 1 h prior to induction of cold storage and CS/REW. The final concentration of Bafilomycin-A1 used was 150 nM. Control cells were treated with vehicle, dimethyl sulfoxide (DMSO) 1 h prior to cold storage and CS/REW to serve as the respective control.

Autophagic flux inhibition

RNA interference was used to inhibit Atg5. To check that the Atg5 siRNA was functioning, the LLC-PK1 cells were starved in saline to induce autophagy and then treated with Atg5 siRNA to knockdown Atg5 expression as described below.

One day before transfection, LLC-PK1 cells were plated in 60 mm dishes in growth medium without antibiotics such that they will be 60–70% confluent at the time of transfection. Five microlitre of Lipofectamine 2000 was mixed with 100 pmol of Atg5 siRNA or scrambled control siRNA in 250 μl of Opti-MEM reduced serum medium and incubated for 20 min at room temperature. The growth medium of cells was replaced with Opti-MEM, and cells were transfected with the above Lipofectamine 2000 mixture. After 6 h, the medium containing transfection mixture was replaced with the complete growth medium and cells were further incubated for 18 h at 37 °C in CO_2 incubator. On day 2, the medium of cells was replaced with fresh growth medium and incubated for another 24 h. On Day 3, cells were made cold in UW solution and incubated for 24 h in cold. *On* day 4, re-warming was simulated by the replacement of UW solution with complete growth medium. RTEC were then incubated for 24 h at 37 °C in CO2 incubator to complete re-warming. On day 5, cells were harvested and analyzed for the expression of proteins.

Cell lysis and immunoblot analysis

Total cellular extracts were prepared by washing cells in ice cold phosphate buffer saline (PBS) twice and then lysing cells in RIPA buffer containing protease inhibitor cocktail. The same amount of protein was loaded in each lane, separated by 8–15% SDS-PAGE, and then transferred onto a nitrocellulose membrane. The membrane was incubated with the designated primary antibodies, washed, and incubated with secondary HRP-labeled antibodies. Bound antibodies were detected using ECL. Each membrane was stripped and re-probed with anti-β-actin antibody, to ensure equal protein loading.

LC3B was detected using a rabbit polyclonal antibody to LC3B (Cell Signaling Technology, Catalogue Number 2775). The protein Light chain 3 (LC3) serves as a marker for autophagy. There are three human isoforms of LC3 – LC3A, LC3B, and LC3C – that undergo post-translational modifications during autophagy. The conversion of LC3B (microtubule-associated protein 1 light chain 3) from LC3B I (free form) to LC3B II (phosphatidylethanolamine-conjugated form) represents a key step in autophagosome formation. The presence of LC3B in autophagosomes as well as the conversion of LC3B to the lower migrating form LC3-II is used as indicators of autophagy.

Flow cytometry

To quantify cold storage and CS/REW-induced apoptotic and necrotic cell death in LLC-PK1 cells, annexin V and propidium iodide (PI) staining was performed followed by flow cytometry. For staining of cells, the Dead Cell Apoptosis Kit with Alexa® Fluor 488 annexin V and PI was used following the step-by-step protocol provided by the manufacturer. Briefly, after treatment (Bafilomycin-A1 or Atg5 siRNA) both floating and attached cells were pooled and washed and re-suspended in the Annexin V binding buffer. Alexa Fluor® 488 annexin V and PI were added to the suspended cells, and the reaction was incubated in the dark for 10 min. Flow cytometric analysis was performed by analyzing 20 000 gated cells utilizing, the core service of the University of Colorado Cancer Center (Aurora, CO, USA). Untreated cells were taken as negative control. All experiments were independently conducted at least four times.

TUNEL assay

Cells were grown in chamber slides to perform TUNEL assay. The TUNEL assay was performed using the DeadEndTM Colorimetric TUNEL assay kit according to manufacturer's directions. Apoptotic cells, that is, cells with apoptosis morphology (cellular rounding and shrinkage, pyknotic nuclei, and formation of apoptotic bodies) that stained positive with the TUNEL assay were counted in seven power fields in a blinded fashion using a light microscope at 40X.

Caspase-1 activity assay

Cells were washed twice with PBS, and cell lysate was prepared as per Caspase-1 activity assay kit manufacturer's directions. The protein concentration was measured using Bradford assay. Briefly, 50 μl of reaction buffer and 5 μl of YVAD-*p*NA (substrate specific for caspase-1 activity) was added to100 μg of protein samples. After incubating the samples with substrate for 2 h at 37 °C, samples were read at 405 nm in a microplate reader. Increased caspase-1 like activity is determined by comparing the results of treated samples with the level of the untreated control. Background readings from cell lysates and buffers were subtracted from both treated and untreated samples before calculating the increase in caspase-1 like activity.

Cold storage and mouse kidney transplant

Experiments were performed on inbred male C57BLl/6 mice weighing 20–25 g at the University of Colorado, Barbara Davis Center Animal Facility. The study protocol was approved by the University of Colorado Denver Animal Care and Use Committee.

Cold storage

Mice were anesthetized with pentobarbital (60 mg/kg IP). Following cervical dislocation, a thoracotomy was performed and the left ventricle of the beating heart was identified. The kidneys were perfused via puncture of the left ventricle with 5 ml of cold heparinized normal saline (100 units/ml). Adequate perfusion was determined by observing the development of a pale color to both kidneys. The left and right kidneys were then removed. The left kidney, not subjected to cold storage, was used as the control. The right kidney was stored in cold heparinized normal saline at 4 °C for 24 h (cold storage group).

Kidney transplant

In separate experiments, kidney transplants were performed as previously described [15]. Briefly, the donor kidney, vessels, and ureter were removed, perfused with heparinized saline, and stored in heparinized saline at 4 °C for 24 h until transplantation. At the end of the cold storage period, the recipient mouse was prepared. Following right recipient nephrectomy, the donor kidney was placed in the right flank and the arterial and venous cuffs were anastomosed to the recipient aorta and inferior vena cava, respectively. The recipient bladder was prepared and the ureters were anastomosed to the bladder according the procedure developed by Han *et al.* [15]. Total implant time was approximately 30–40 min.

On day seven post-transplant, a left recipient nephrectomy was performed leaving the recipient with only the transplanted kidney. On day eight post-transplant, the transplanted kidney was removed and processed for immunoblotting (cold storage plus transplant group).

Statistical analysis

The data are representative of at least three to four independent studies with reproducible results. Values are

presented as mean ± SEM. Data were analyzed by analysis of variance (ANOVA) or unpaired *t*-test using GRAPHPAD PRISM software version 5.01 from GraphPad Software, Inc. (La Jolla, CA, USA). Differences between groups were considered to be significant at $P < 0.05$.

Results

Increased "autophagic flux" during CS/REW

Figure 1a and b shows LC3 II protein expression was significantly increased during CS/REW compared with control and cold storage. Detection of LC3 II by Western blot in tissue or cells treated with the lysosomal inhibitor (Bafilomycin-A1) to prevent further degradation of LC3 II is a standard method to evaluate "autophagic flux". Figure 1a and b shows LC3 II expression was further increased significantly with Bafilomycin-A1 treatment during CS/REW, confirming the increase in autophagic flux during CS/REW compared with control and cold storage.

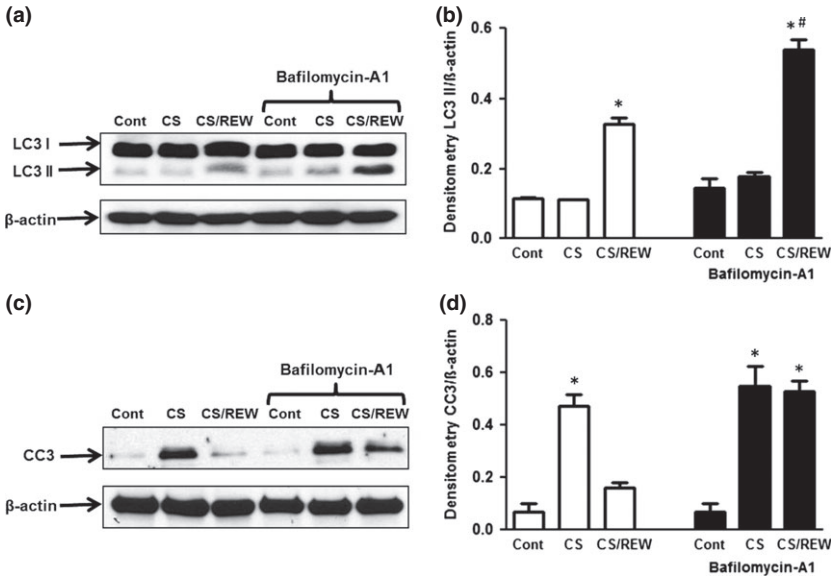
Cleaved caspase-3 is the major mediator of apoptosis [16]. Figure 1c and d shows cleaved caspase-3 expression was increased in cold storage compared with control and CS/REW. Bafilomycin-A1 had no effect on cleaved caspase-3 during cold storage. However, Bafilomycin-A1 significantly increased cleaved caspase-3 protein expression during CS/REW. As Bafilomycin-A1 is not a specific inhibitor of autophagy, Atg5 siRNA was used to specifically inhibit autophagy during CS/REW.

Autophagic flux inhibition using an ATG5 siRNA increases cleaved caspase-3 during CS/REW

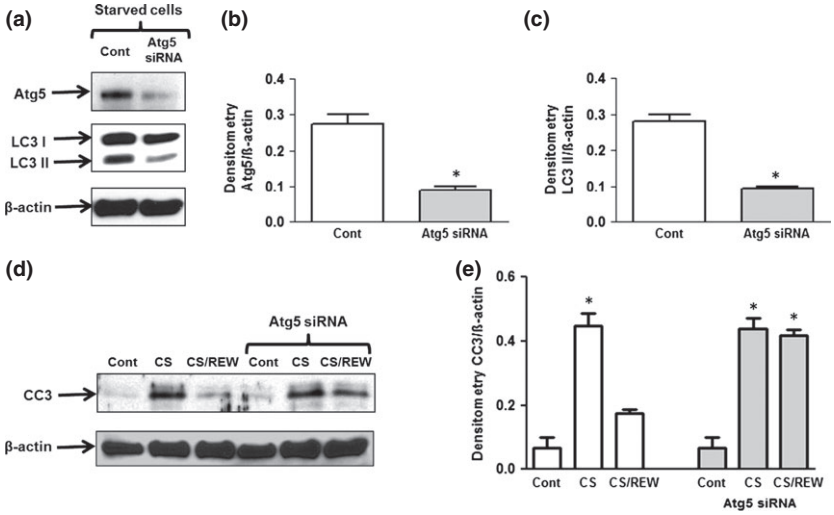
Atg5 is necessary for autophagy due to its role in autophagosome elongation and for LC3 I conjugation to Phosphatidylethanolamine to form LC3II. An siRNA against Atg5 was used to inhibit the expression of Atg5 protein. To confirm the effectiveness of the siRNA in suppressing Atg5 gene expression, LLC-PK1 cells were starved in saline to induce autophagy and then treated with Atg5 siRNA. Figure 2a, b and c demonstrates that the Atg5 siRNA significantly decreases expression of Atg5 and LC3 II protein. The Atg5 siRNA was then used to knockdown the expression of Atg5 during cold storage and CS/REW. Figure 2d and e shows that the ATG5 siRNA had no significant effect on cleaved caspase-3 during CS alone. However, cleaved caspase-3 was significantly increased in Atg5 siRNA treated cells during CS/REW.

Bafilomycin-A1 or Atg5 siRNA results in an increase in apoptotic cells during CS/REW – flow cytometry

The effect of Bafilomycin-A1 or Atg5 siRNA on apoptotic and necrotic cell death during cold storage alone and CS/



Figures 1 Bafilomycin-A1 increases LC3 II and cleaved caspase-3 (CC3) during cold storage followed by re-warming (CS/REW). (a and b) LC3 II was increased in CS/REW versus control (cont) and cold storage (CS). LC3 II was further increased in Bafilomycin-A1 treated cells during CS/REW (*$P < 0.001$ versus cont, CS, cont + Bafilomycin-A1 and CS + Bafilomycin-A1, #$P < 0.01$ versus CS/REW). (c and d) CC3 was increased in CS versus respective controls and CS/REW but was not changed in CS with Bafilomycin-A1 treatment. CC3 was increased in Bafilomycin-A1 treated cells during CS/REW (*$P < 0.001$ versus cont and CS/REW and cont+Bafilomycin-A1). The densitometry values were adjusted to loading control (β-actin) and are representative of at least 3–4 independent studies with reproducible results.



Figures 2 Atg5 siRNA increases cleaved caspase-3 (CC3) during cold storage followed by re-warming (CS/REW). (a, b, and c) Cells were starved in saline to induce autophagy and then treated with Atg5 siRNA. Atg5 and LC3 II expression was decreased in Atg5 siRNA treated cells compared with scrambled siRNA treated starved cells (*$P < 0.05$ versus control starved). (d and e) CC3 was increased in cold storage (CS) with or without Atg5 siRNA treatment. CC3 was increased in Atg5 siRNA treated cells during CS/REW (*$P < 0.001$ versus cont and CS/REW and cont + Atg5 siRNA). The densitometry values were adjusted to loading control (β-actin) and are representative of at least 3–4 independent studies with reproducible results.

REW was studied. There was a significant increase in apoptotic cells (annexin V positive and PI negative) in cold storage and CS/REW compared with control. Bafilomycin-A1 or Atg5 siRNA treatment did not result in a further increase in apoptotic cells during cold storage but resulted in a significant further increase in apoptotic cells during CS/REW (Fig. 3a).

Necrotic cells (annexin V negative and PI positive) were not increased in cold storage, but were significantly increased in CS/REW. There was no significant change in necrotic cells during cold storage or CS/REW with Bafilomycin-A1 or Atg5 siRNA treatment (Fig. 3b).

A representative experiment of 4 separate experiments of annexin V and PI staining by flow cytometry is shown in Fig. 3c.

Bafilomycin-A1 or Atg5 siRNA results in an increase in apoptotic cells during CS/REW – TUNEL staining

To confirm the effect of Bafilomycin-A1 or the Atg5 siRNA on apoptosis seen on flow cytometry, TUNEL staining was performed. Apoptosis was characterized by both TUNEL positivity and morphology (cells with round, condensed, and pyknotic nuclei). Figure 4a and b shows that apoptotic cells were significantly increased in cold storage. Inhibition of autophagic flux with Bafilomycin-A1 or Atg5 siRNA had no effect on apoptotic cells during cold storage, but resulted in a further significant increase in apoptotic cells during CS/REW.

Effect of autophagic flux inhibition on caspase-1 activity

Caspase-1 is a known mediator of RTEC necrosis during warm ischemia–reperfusion [17,18]. Caspase-1 activity was not increased during cold storage when there is predominantly apoptosis of RTECs (Figs 3 and 4). Figure 5 shows caspase-1 activity was significantly increased during CS/REW, a time when there is increased necrosis (Fig. 3). Bafilomycin-A1 or the Atg5 siRNA had no effect on caspase-1 during CS/REW.

Increase in cleaved caspase-3 (CC3) after cold storage and increase in LC3-II in kidneys transplanted after cold storage

Cleaved caspase-3 was increased in kidneys after cold storage alone and in kidneys transplanted after cold storage compared with control kidneys (Fig. 6a). LC3 II was increased in kidneys transplanted after cold storage compared with control kidneys and cold storage alone (Fig. 6b).

Discussion

Delayed graft function occurs in close to half of primary deceased-donor renal transplants in the United States and independently predicts reduced 1- and 5-year kidney transplant survival [2,3]. The discovery of novel therapies that reduce DGF may result in improved kidney transplant survival. For example, the effect of machine perfusion or

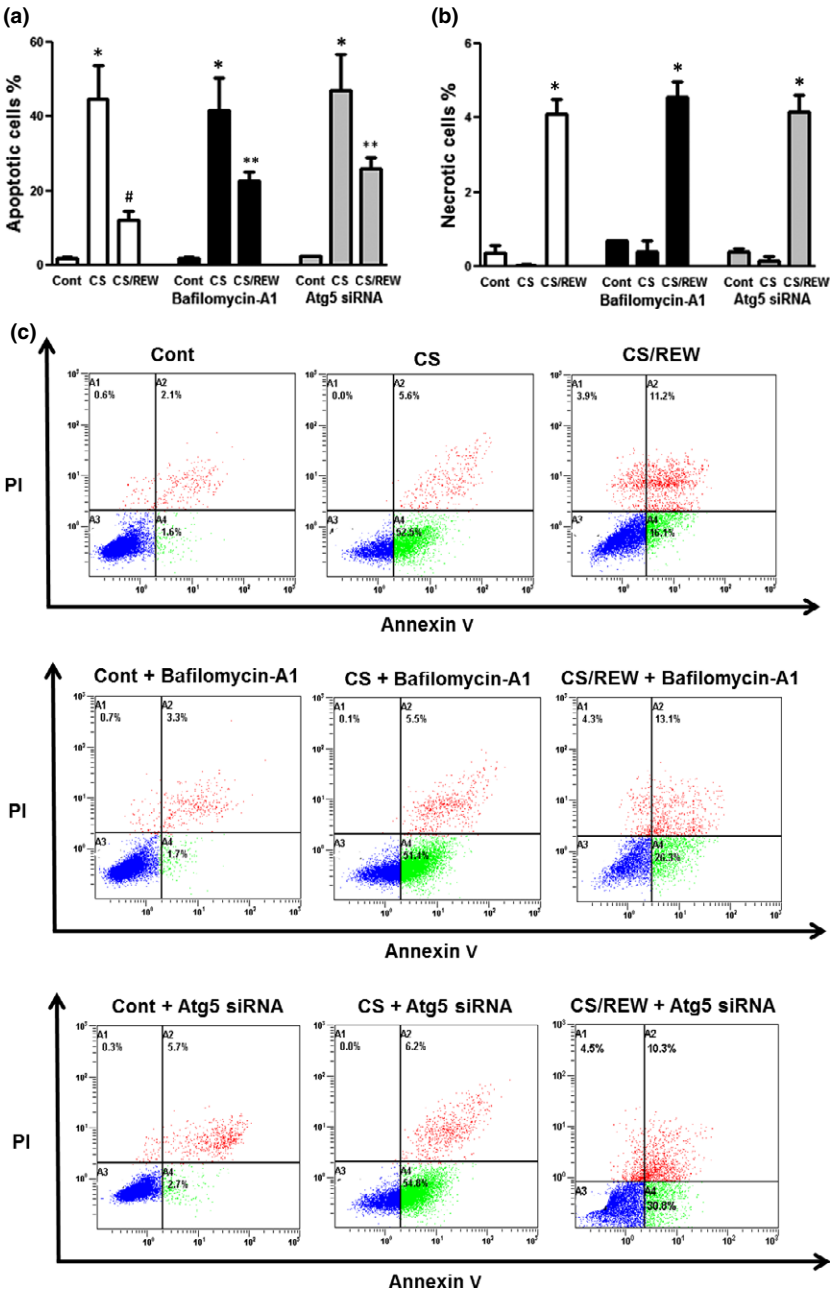


Figure 3 Apoptotic cells during cold storage followed by re-warming (CS/REW) were increased by Bafilomycin-A1 and Atg5 siRNA. The percentage of apoptotic and necrotic cells were assessed by Alexa® Fluor 488 annexin V and PI binding by flow cytometry ($n = 4$). (a) Apoptotic cells (annexin V positive and PI negative) were increased in cold storage (CS) and CS/REW cells compared with controls (cont). Apoptotic cells were further increased with Bafilomycin-A1 or Atg5 siRNA during CS/REW (*$P < 0.01$ versus cont, cont + Bafilomycin-A1, cont + Atg5 siRNA, CS/REW, CS/REW + Bafilomycin-A1 and CS/REW+Atg5 siRNA, **$P < 0.01$ versus cont, cont + Bafilomycin-A1, cont+Atg5 siRNA, **$P < 0.05$ versus CS/REW alone; $^{\#}P < 0.01$ versus cont). (b) Necrotic cells (annexin V negative and PI positive) were increased in CS/REW cells compared with cont and CS (*$P < 0.001$ versus cont and CS, cont and CS+Bafilomycin-A1 and cont and CS + Atg5 siRNA). But necrotic cells were not changed during cold storage or CS/REW with Bafilomycin-A1 or Atg5 siRNA treatment. (c): Representative flow cytometry experiment of 4 independent experiments of LLC-PK1 cells exposed to cold storage (CS) and cold storage followed by re-warming (CS/REW) with and without treatment with Bafilomycin-A1 or Atg5 siRNA is shown. Apoptotic cells (annexin V positive and PI negative) are shown in quadrant A4. Necrotic cells (annexin V negative and PI positive) are shown in quadrant A1.

abdominal regional perfusion to reduce DGF is being tested in kidneys from expanded criteria donors [19,20]. Drugs that interfere with apoptosis or autophagy during CS/REW are also potential therapies for DGF.

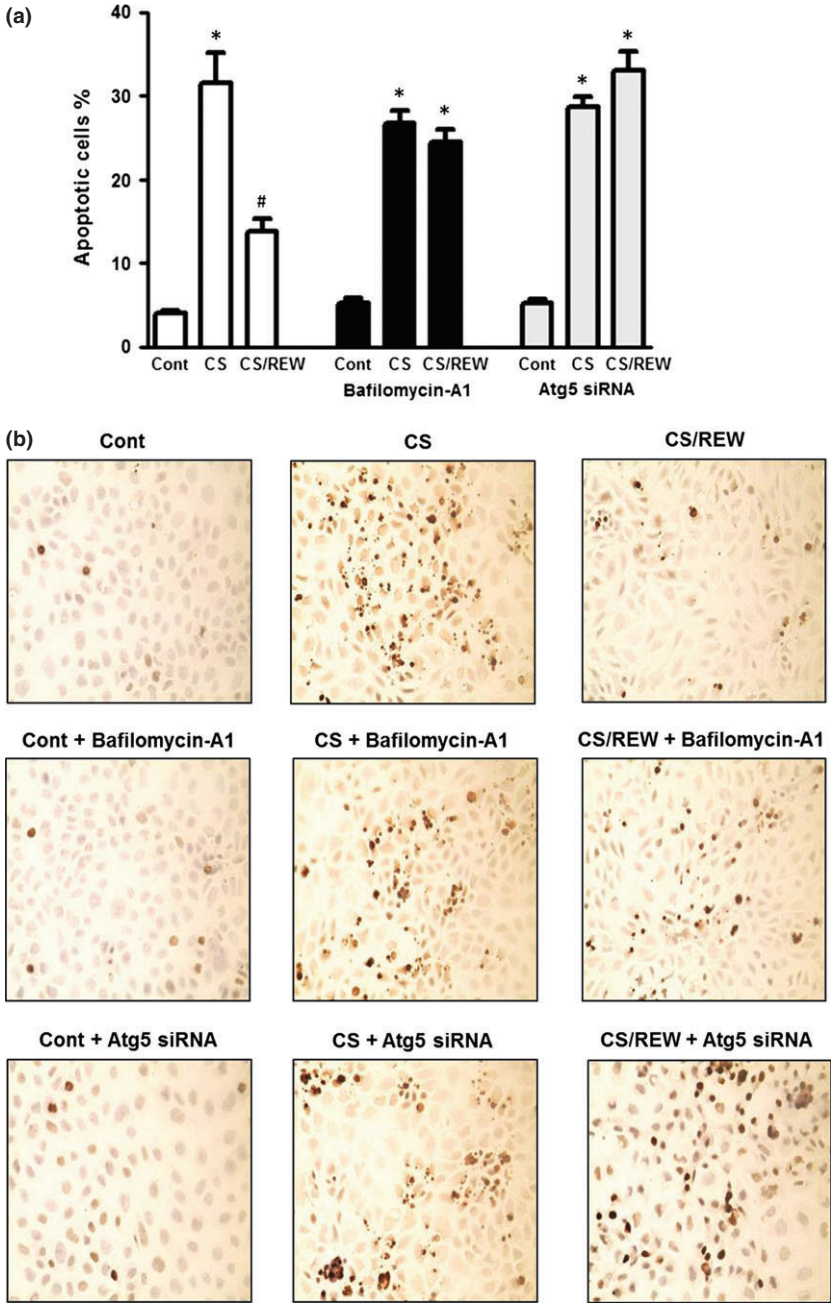


Figure 4 TUNEL-positive apoptotic cells in cold storage followed by re-warming (CS/REW) were significantly increased by Bafilomycin-A1 or Atg5 siRNA. (a) TUNEL-positive apoptotic cells were increased in cold storage (CS) versus controls (cont) and were not significantly affected by Bafilomycin-A1 or Atg5 siRNA treatment during CS. TUNEL-positive apoptotic cells in CS/REW were significantly increased by Bafilomycin-A1 or Atg5 siRNA versus untreated CS/REW (*$P < 0.001$ versus cont, cont + Bafilomycin-A1, cont + Atg5 siRNA and CS/REW, $^{\#}P < 0.05$ versus cont, cont + Bafilomycin-A1, cont + Atg5 siRNA). (b) Representative TUNEL staining images of LLC-PK1 cells exposed to CS and CS/REW with and without treatment with Bafilomycin-A1 or Atg5 siRNA are shown. TUNEL-positive cells with apoptosis morphology (condensed pyknotic nuclei) stain dark brown. The number of apoptotic cells is increased during CS with or without Bafilomycin A1 or Atg5 siRNA. During CS/REW, Bafilomycin-A1 and the Atg5 siRNA result in increased apoptotic cells compared with CS/REW alone.

Human kidney biopsies of patients with DGF demonstrate both RTEC necrosis and apoptosis [21]. CS/REW of RTECs results in both apoptosis and necrosis [22]. To examine the role of autophagy inhibition in CS/REW of RTECs, we employed a cell model of CS/REW to simulate donor kidney preservation and transplantation. The cell

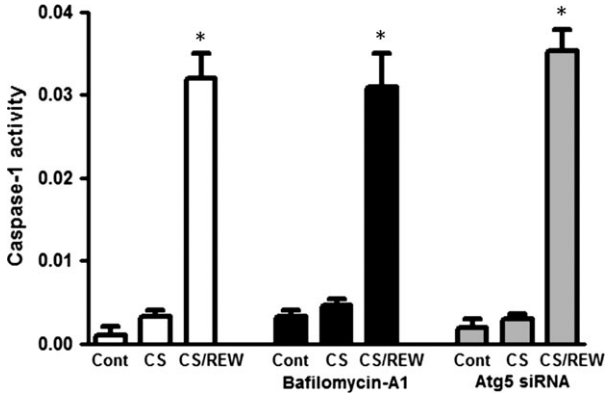


Figure 5 Caspase-1 like activity was significantly increased in cold storage followed by re-warming (CS/REW). Caspase-1 like activity was significantly increased in CS/REW versus cold storage (CS) and controls (cont) but was not affected by Bafilomycin-A1 or Atg5 siRNA treatment (*$P < 0.001$ versus cont and CS, cont and CS+Bafilomycin-A1 and cont and CS+Atg5 siRNA).

model employed allows the study of the individual effects of cold preservation or re-warming as well as the effect of both CS/REW on autophagy, apoptosis, and necrosis. The rationale for performing the studies was that previous studies in RTECs have determined the effect of either cold storage alone on apoptosis [23–26] or warm ischemia–reperfusion alone on autophagy [14,27]. The effect of combined CS/REW of RTECs and specific autophagy inhibition on apoptosis and autophagy is not known.

The first goal of the study was to determine the effect of CS/REW of RTECs on autophagic flux. The detection of autophagy is complex [28]. The amount of LC3 II at a specific time may represent either increased autophagy or suppression of downstream steps, for example, autophagosome–lysosome fusion. "Autophagic flux" is a term used to describe the dynamic process of autophagosome synthesis, delivery of autophagosomes to the lysosome and degradation of autophagosomes in the lysosome [28]. Bafilomycin-A1 inhibits the fusion of autophagosomes and lysosomes and results in LC3 II aggregation on the autophagosomes. If autophagic flux is increased, Bafilomycin-A1 inhibits distal events during autophagy [28]. If the increase in LC3 II during CS/REW is due to increased production, then it would be expected that Bafilomycin-A1 would further increase LC3 II. Alternatively, if the observed increase in LC3 II is due to a lysosomal defect, then Bafilomycin-A1 would not affect LC3 II. The difference in LC3 II between samples in the presence and absence of Bafilomycin-A1 represents the amount of LC3 II that is delivered to the lysosome for degradation [28]. The results demonstrate that increased "autophagic flux" occurs during CS/REW rather than after cold storage alone.

Next the effect of autophagy inhibition on apoptosis was determined. Previous studies in cold storage or CS/REW

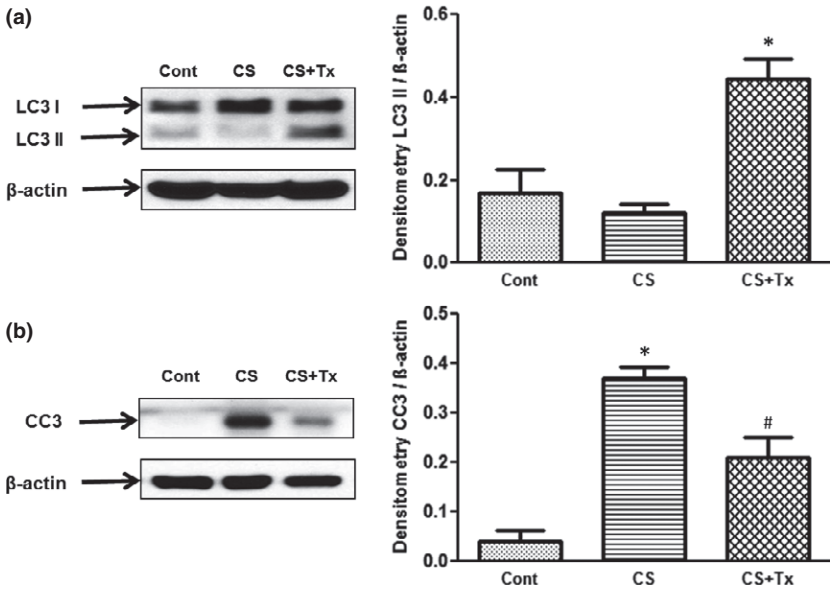


Figure 6 Increase in cleaved caspase-3 (CC3) after cold storage and increase in LC3-II in kidneys transplanted after cold storage. (a) CC3 was increased in kidneys after cold storage alone (CS) and in kidneys transplanted after cold storage (CS + Tx) compared with control kidneys (cont). *$P < 0.001$ versus cont and $^{\#}P < 0.05$ versus cont and CS). (b) LC3 II was increased in kidneys transplanted after cold storage (CS + Tx) versus control (cont) and cold storage alone (CS) *$P < 0.01$ versus cont and CS. The densitometry values were adjusted to loading control (β-actin) and are representative of at least $n = 4$ animals per group.

have used chemical inhibitors of autophagy like Bafilomycin-A1 or wortmannin [4]. However, the chemical approach for autophagy inhibition has limitations because chemical drugs do not always specifically target autophagy and may have off-target effects [12]. Atg5 is an E3 ubiquitin ligase which is necessary for autophagy due to its role in autophagosome elongation [28]. The use of Atg5 knockdown has been used to more definitively define the role of autophagy in the kidney [12]. In the present study, specific autophagy inhibition using an Atg5 siRNA resulted in increased cleaved caspase-3 and increased apoptosis, on both flow cytometry and TUNEL staining, during CS/REW rather than cold storage alone. The increase in apoptosis with autophagy inhibition in the present study suggests that autophagy inhibition may not have therapeutic potential in preventing DGF.

Next the effect of autophagy inhibition on RTEC necrosis during CS/REW was determined. Cold storage is characterized by apoptosis of RTECs [29] while in warm ischemia–reperfusion of the kidney acute tubular necrosis (ATN) predominates over tubular apoptosis [17,18]. Human kidney biopsies of patients with DGF demonstrate both RTEC necrosis and apoptosis [21]. In the present study, there was a small increase in necrosis of RTECs during CS/REW and autophagy inhibition did not affect the necrosis. This study demonstrates that autophagy inhibition increases apoptosis rather than necrosis during CS/REW. The increase in RTEC apoptosis with autophagy inhibition is unlikely to be beneficial even in the absence of increased necrosis.

Next, caspase-1, a known mediator of RTEC necrosis during warm ischemia–reperfusion was measured [17,18]. Caspase-1 activity was not increased during cold storage when there is predominantly apoptosis of RTECs. Caspase-1 activity was significantly increased during CS/REW, a time when there is increased necrosis. Bafilomycin-A1 or the Atg5 siRNA had no effect on caspase-1 during CS/REW suggesting that caspase-1 and necrosis are independent of autophagy during CS/REW.

Morphology is the gold standard for detection of apoptosis [30–32]. Morphologic criteria of apoptosis include cellular rounding and shrinkage, nuclear chromatin compaction, and formation of apoptotic bodies [30–32]. In the present study, cells that were TUNEL positive and demonstrated the morphologic criteria of apoptosis were counted as apoptotic cells. To confirm apoptosis, annexin V staining by flow cytometry was performed. Annexin V staining is the most widely used cytometric method to detect apoptosis [16]. However, annexin V staining may detect damaged nonapoptotic cells that have a damaged cell membrane [16]. Cultured cells may be damaged by mechanical or enzymatic removal from culture plates [16]. This may explain the increase in annexin V staining compared with TUNEL plus morphology in the present study. However, TUNEL plus morphology and annexin V staining demonstrated the same pattern of changes in the present study.

To determine the effect of cold storage alone and CS/REW on cleaved caspase-3 and LC3-II *in vivo*, kidney transplants were performed. The effect of cold storage alone and kidney transplant after cold storage, the equivalent of CS/REW *in vitro*, was determined. The increase in cleaved caspase-3 after cold preservation confirms our previous study [29]. The present study demonstrates that LC3 II was increased in kidneys transplanted after cold storage. While autophagy in the liver has been demonstrated after transplantation [10,11], to our knowledge, our data are the first description of increased LC3 II in a kidney transplant model.

In summary, LC3 II protein expression did not increase during cold storage in renal tubular epithelial cells, but significantly increased after CS/REW. Treatment with Bafilomycin-A1 further increased LC3 II protein expression in cells exposed to CS/REW suggesting an increase in autophagic flux was responsible for the increase in LC3 II protein expression. Inhibition of autophagic flux using an Atg5 siRNA significantly increased cleaved caspase-3 and apoptotic cell death after CS/REW, but had no effect on necrotic cell death. Thus, autophagy during CS/REW appears to protect from apoptosis, but does not prevent necrosis.

Authorship

SJ: performed study, analyzed data. DK: performed study. TN and RJP: performed kidney transplants. CLE: designed study, analyzed data, wrote the paper. AJ: designed study, analyzed data, wrote the paper.

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