Original Contribution

Role of mitochondrial-derived oxidants in renal tubular cell cold-storage injury

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Cold storage (CS) is regarded as a necessary procedure during donation of a deceased-donor kidney that helps to optimize organ viability. Increased oxidant generation during CS as well as during the reperfusion (or rewarming/CS.RW) phase has been suggested to be a major contributor to renal injury, although the source of and/or biochemical pathways involved in oxidant production remain unclear. The purpose of this study was to determine if renal tubular mitochondrial superoxide is capable of inducing oxidant production and mitochondrial damage in response to a CS.RW insult. To test the role of mitochondrial superoxide in CS.RW injury, we used rat renal proximal tubular (NRK) cells overexpressing manganese superoxide dismutase (MnSOD), the major mitochondrial antioxidant. Oxidant production, mitochondrial membrane potential, respiratory complex function, and cell death were all altered after exposure of NRK cells to CS.RW. MnSOD overexpression or inhibition of nitric oxide synthase provided significant protection against oxidant generation, respiratory complex inactivation, and cell death. These findings implicate mitochondrial superoxide, nitric oxide, and their reaction product, peroxynitrite, as key signaling molecules involved in CS.RW injury of renal tubular cells and suggest that therapeutic inhibition of these pathways may protect the donor kidney.

Cold storage (CS) is regarded as a critical step in the process of transplanting a deceased-donor kidney and is used to lower cellular metabolism, maintain organ viability, and provide time for tissue matching between donor and recipient [1]. Despite its advantages and the design of preservation solutions to enhance organ viability, CS and the subsequent reperfusion phase that follows tissue transplantation are associated with renal injury [2–4]. The injury caused by CS/reperfusion may partially explain why long-term renal function is compromised in kidneys harvested from deceased donors compared to kidneys transplanted from living donors, either related or unrelated, which avoid the cold preservation process [5–7].

“Static” CS consists of harvesting a kidney from a donor, flushing it with precooled preservation solution, and then storing it in preservation solution at −4 °C until the transplantation surgery. The University of Wisconsin/Viaspan solution has become the standard CS solution for renal preservation, and its development by Belzer and Southard [8] has allowed deceased-donor kidneys to be preserved for up to 72 h [9–11], although most are transplanted by 24 h [12–14].

Despite this advancement, several studies have revealed that donor kidneys exposed to CS for longer periods have more severe kidney injury after transplantation [14–16]. Unfortunately, the cellular mechanisms leading to CS/reperfusion injury are poorly understood, so rational strategies to minimize these detrimental outcomes are needed. CS-induced renal cell injury has been shown to be dependent on oxygen, because studies using hypoxic proximal tubular cells have documented less cellular damage compared to normoxic cells placed in CS [17,18]. These findings confirm the need to better understand the role that oxygen radicals play in CS-mediated injury. Mitochondrial superoxide is a free radical that is thought to be continually generated by misfires (~1 to 2% of total oxygen consumption under nonphysiological conditions) in the electron transport chain [19,20]. Superoxide can also be generated from other sources, such as its production by NADPH oxidase (or NOX) during the “respiratory burst” noted in activated phagocytes and from other cell types containing a form of NOX [21–25]. Superoxide is the proximal oxidant species involved in the formation of other oxidants, including the hydroxyl radical, hydrogen peroxide, and peroxynitrite. Thus, it is strategically positioned to unleash several oxidant cascades associated with tissue injury. Manganese superoxide dismutase (MnSOD) is a nuclear-encoded protein localized to the mitochondria where it dismutates mitochondrial superoxide to hydrogen peroxide within the mitochondrial matrix; hydrogen peroxide is then efficiently detoxified by catalase or glutathione peroxidase.

The goal of this study was to elucidate the oxidant signaling pathway that is activated during cold storage and rewarming (CS.RW)
to trigger renal tubular cellular and mitochondrial injury. Our hypothesis was that mitochondrial superoxide contributes to oxidant production and mitochondrial damage during renal cell CS. We used an in vitro model of rat renal proximal tubular (NRK) cells to permit us to accurately monitor mitochondrial oxidant production using fluorescence imaging and also to have the ability to transiently transfect cells with MnSOD to lower available mitochondrial superoxide levels during CS.RW. Using this experimental strategy, our results provide initial evidence that mitochondrial superoxide is generated in NRK cells in response to CS, and the resulting oxidant production is associated with mitochondrial dysfunction and cell death.

Materials and methods

Cold storage ±rewarming cell model

Normal rat kidney proximal tubular cells (NRK-52E; American Type Culture Collection No. CRL-1571, Manassas, VA, USA) were maintained in six-well, 100-mm, or 150-mm size plates in a humidified incubator gassed with 5% CO₂, 95% air at 37 °C in Dulbecco’s modified Eagle medium (DMEM) containing 5% fetal calf serum (FCS). Cells were grown to 60% confluency and then divided into three treatment groups: (1) untreated, (2) CS, (3) CS.RW. Untreated cells remained in DMEM containing 5% FCS for 24 or 48 h at 37 °C. CS (groups 2 and 3) was initiated by washing cells with cold phosphate-buffered saline (PBS) twice and storing them in University of Wisconsin/Viaspan (UW) solution for 24 h at 4 °C. To simulate RW (group 3), the UW solution was replaced after 24 h of CS with DMEM containing 5% FCS for 6 h at 37 °C.

MnSOD overexpression

NRK cells grown to 60% confluency were transiently transfected with the pMnSOD plasmid (10 μg), encoding the human MnSOD gene, using Lipofectamine (Invitrogen, San Diego, CA, USA) in OPTI-MEM (Invitrogen) for 6 h at 37 °C; nontransfected cells were incubated in OPTI-MEM alone [26,27]. After 6 h, all cells were treated with DMEM containing 5% FCS overnight at 37 °C. The next day, cells were placed in one of the three treatment groups (group 1, 2, or 3) mentioned above. MnSOD enzymatic activity was measured in cellular extracts using the cytochrome c reduction method [28] and was determined to have a twofold increase in activity compared to nontransfected cells (data not shown). In addition, transfection efficiency was determined to be 93% using a MnGFP plasmid containing the same vector used for MnSOD overexpression except green fluorescent protein was also expressed (data not shown). Efficiency was calculated as the ratio of the number of cells displaying green fluorescence by the total number of cells in a field times 100 (field size: 40×).

Mitochondrial superoxide production

MitoSOX red (Molecular Probes, Eugene, OR, USA) was used to detect mitochondrial superoxide production in groups 1, 2, and 3. This modified cationic dihydroethidium dye is localized to the mitochondria where it is oxidized by superoxide to generate a bright red fluorescence [29]. Briefly, cells were preloaded in the dark with MitoSOX red (5 μM for 10 min) before treatment. Fluorescence was visualized using a Nikon Eclipse E800 microscope with a rhodamine filter using a water immersion objective (60×). All images were captured with equal exposure times. Fluorescence images were quantified for each sample by averaging the mean fluorescence intensity of five random fields in three different fields using Nikon Nis Elements software. Additionally, cells were grown on coverslips, preloaded with MitoSOX red before treatment (group 1, 2, or 3), and evaluated for mitochondrial superoxide generation using a Hitachi spectrophotometer equipped with a coverslip holder using two excitation wavelengths, 396 and 510 nm, with the emission measured at 580 nm as previously described [27,29].

Nitric oxide measurement

Available nitric oxide levels were determined for all three groups by loading NRK cells with diaminorhodamine-4M-AM (DAR; 5 μM; Calbiochem, San Diego, CA, USA) 30 min before the end of treatment in the dark. DAR is a nonfluorescent compound that in the presence of nitric oxide and oxygen forms a fluorescent triazolohomidine analog [30]. Fluorescence was evaluated using a Nikon Eclipse E800 microscope with a rhodamine filter using a water immersion objective (60×). All images were captured with equal exposure times. Fluorescence images were quantified for each sample by averaging the mean fluorescence intensity of five random cells in three different fields using Nikon Nis Elements software.

Nitrotyrosine immunocytochemistry

NRK cells from groups 1, 2, and 3 were washed with cold PBS, fixed for 15 min with 4% formalin, washed with PBS, and permeabilized with 0.1% Triton X-100/0.1% sodium citrate for 2 min on ice. Cells were then washed with PBS and blocked with 3% bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA) in PBS for 1 h, followed by overnight incubation at 4 °C with the rabbit polyclonal nitrotyrosine antibody (1:200; Millipore, Billerica, MA, USA). The following day, the cells were washed with PBS–Tween 0.1% and then PBS and incubated with the goat anti-rabbit IgG Alexa-594 antibody (1:100; Invitrogen) for 30 min in the dark at room temperature (RT). Cells were rinsed with PBS–Tween 0.1% and nuclear counterstaining was initiated using DAPI (1:100; Invitrogen) for 10 min at RT. Subsequently, cells were washed and coverslipped with ProLong Gold antifade reagent with DAPI (Invitrogen). Nitrotyrosine staining was evaluated with a Nikon Eclipse 800 microscope (40× oil). All images were captured with equal exposure times. NRK cells treated with peroxynitrite (0.8 mM) in PBS for 5 min at RT served as positive controls. The negative controls were NRK cells treated with peroxynitrite but the nitrotyrosine antibody was preincubated with excess 3-nitrotyrosine (10 mM; Sigma–Aldrich) before being added to permeabilized cells. In separate experiments, cells were pretreated with the following inhibitors at 37 °C to evaluate the role of specific oxidants during CS: diphenyleneiodonium chloride (DPI; 1 μM for 1 h; Sigma–Aldrich) to inhibit NADPH oxidase, catalase (2000 units/ml for 30 min; Sigma–Aldrich) to block hydrogen peroxide, and N-[4-methylmethyl]-L-ornithine, citrate (L-NMMA; 100 μM for 30 min; Cayman Chemicals, Ann Arbor, MI, USA) to inhibit nitric oxide synthases (NOS). Inhibitors were also included for the duration of CS exposure.

Mitochondrial membrane potential assessment

The relative mitochondrial membrane potential was determined using a lipophilic cationic probe, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes). All treatment groups were incubated in the dark with JC-1 (7.5 μM for 30 min). Fluorescence was observed using a Nikon Eclipse 800 microscope with a 60× water immersion objective equipped with a dual filter for fluorescein and rhodamine. Green staining is indicative of the monomeric form of JC-1 (i.e., lower membrane potential) and the red staining corresponds to the aggregate form (i.e., higher membrane potential). Thus, yellow staining indicates relatively normal membrane potential (combined aggregate and monomer). Average mean fluorescence intensity was calculated using the ratio of JC-1 aggregate/monomer (590 nm/530 nm) using the Nikon Nis Elements software. All images were captured with equal exposure times.
Measurement of respiratory complex activity

NRK cell mitochondria were isolated by centrifugation on a sucrose density gradient, and the activity of mitochondrial complexes I thru IV was assayed spectrophotometrically at 30 °C [31]. Complex I (NADH:ubiquinone oxidoreductase) activity was measured by the oxidation of NADH (Sigma–Aldrich) at 340 nm. Complex II (succinate:ubiquinone oxidoreductase) activity was determined by the reduction of 2, 6-dichloroindophenol (Sigma–Aldrich) at 595 nm. Complex III (ubiquinol:cytochrome c oxidoreductase) activity was evaluated by the reduction of cytochrome c (Sigma–Aldrich) at 550 nm. Complex IV (cytochrome c oxidase) activity was assessed by following the oxidation of reduced cytochrome c (Sigma–Aldrich) at 550 nm. The changes in absorbance in blank samples (containing no mitochondria) were recorded for all assays.

Cell cytotoxicity assessment

NRK cytotoxicity was determined using the LDH-Cytotoxicity Assay Kit II (Biovission Research Products, Mountain View, CA, USA). This kit uses water-soluble tetrazolium salt reagent to react with NADH produced by lactate from lactate dehydrogenase (LDH) to give an intense yellow color directly correlating with the amount of LDH in the medium. Absorbance was measured at 450 nm using a SpectraMax 190 microplate reader using SpectraMax software (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Results are presented as means± standard error of the mean (SEM). Means were obtained from at least three independent experiments. One-way analysis of variance was used to compare the mean values among the untreated and the treated groups, followed by Tukey’s test to compare differences in mean between two groups at 95% level of confidence using the Origin 6.0 statistical software. Differences with a P value less than 0.05 were considered statistically significant.

Results and discussion

MnSOD overexpression decreases measurable superoxide during CS and RW of renal tubular cells

Because superoxide plays a central role in oxidant generation, we initially evaluated the role of mitochondrial superoxide in a cell model of CS-induced injury and RW in which proximal tubular NRK cells were exposed to 24 h of CS with or without 6 h of RW. For these studies, we used MitoSOX red, a dye that is directly targeted to the mitochondria to detect available mitochondrial superoxide. Using this strategy, we observed that 24 h of CS significantly increased the MitoSOX red signal associated with mitochondrial superoxide compared to untreated (Untx) control cells (CS alone 885.7±47.1 vs Untx 68.4±15.1 mean fluorescence intensity; Figs. 1A and B). However, available mitochondrial superoxide levels decreased significantly after 6 h of RW (CS.RW 130.2±6.9 mean fluorescence intensity; Figs. 1A and B).

MitoSOX red can yield two distinct fluorescent species upon oxidation by superoxide, Mito-hydroxyethidium and Mito-ethidium, and their fluorescence emission at 580 nm can be selectively measured by using distinct excitation wavelengths, 396 and 510 nm, respectively [29]. Fluorescence microscopy cannot discriminate between these species; however, a spectrofluorimetric-based assay using cells grown on coverslips can be used to distinguish between the two distinct MitoSOX red fluorescent species [27]. NRK cells exposed to CS showed a significant increase in MitoSOX red fluorescence at the 396-nm excitation wavelength (Mito-hydroxyethidium; CS 2.2±0.1 vs Untx 1.0±0 mean fold change in arbitrary fluorescence units; Fig. 1C), but no increase in MitoSOX red staining was observed using the 510-nm excitation wavelength (Mito-ethidium; CS 1.1±0.1 vs Untx 1.0±0 mean fold change in arbitrary fluorescence units; Fig. 1D). Consistent with the fluorescence microscopy results, MitoSOX red staining was decreased after RW (15 min or 6 h; CS.RW (15 min) 1.2±0.2 vs CS.RW (6 h) 1.1±0.1 vs Untx 1.0±0 mean fold change in arbitrary fluorescence units; Fig. 1C).

These observations extended an earlier finding by Salahudeen et al. [32] that demonstrated that superoxide is generated by human proximal tubular cells during 24 h of CS. However, the cytochrome c reduction method used in this study failed to delineate the origin of superoxide generation, which we now hypothesize is from the mitochondria. The reduction in measurable superoxide during RW presumably reflects superoxide interacting with other oxidants and/or less mitochondrial generation of superoxide at the time of measurement. To verify that the changes in superoxide observed in NRK cells during CS and RW originated in the mitochondria, cells were transiently transfected with MnSOD, the major mitochondrial antioxidant responsible for scavenging superoxide. The CS-induced elevation of superoxide in MnSOD-overexpressing cells was ~50% less than in nontransfected cells (CS + MnSOD 450.7±31.3 vs CS alone 885.7±47.1 mean fluorescence intensity; Fig. 1B). However, MnSOD overexpression did not affect superoxide levels during RW, because it was already restored to basal levels (CS.RW + MnSOD 141.8±11.7 vs CS.RW 130.2±6.9 mean fluorescence intensity; Figs. 1A and B). As mentioned previously, superoxide can also be generated by nonmitochondrial sources including NADPH oxidases [33]. We evaluated this possibility by treating NRK cells with DPI (1 μM), a nonselective NADPH oxidase inhibitor, for 1 h before and during CS. DPI treatment did not affect the rise in MitoSOX red signal in response to 24 h of CS (data not shown), suggesting that NADPH oxidase was not involved in superoxide production during CS. These results support our hypothesis that mitochondrial superoxide is increased during CS of renal tubular cells.

Modulation of nitric oxide levels during CS and RW

Nitric oxide is a diffusible, free radical molecule that has many roles in physiology and pathophysiology. It can be synthesized by at least three forms of NOS [34,35]. Nitric oxide and superoxide can react to form the powerful oxidant and nitrating agent peroxynitrite [36]; hence we hypothesized that the increased superoxide produced during CS might lead to decreased nitric oxide levels. Thus, we evaluated available nitric oxide levels in NRK cells after CS and RW using DAR. Nitric oxide levels in NRK cells exposed to 24 h of CS were significantly lower compared to untreated cells (CS 70.9±20.4 vs Untx 450.0±27.0 mean fluorescence intensity; Figs. 2A and B). Subsequent rewarming (CS.RW) partially restored nitric oxide levels toward control values (CS.RW 326.0±10.7 vs Untx 450.0±27.0 mean fluorescence intensity; Figs. 2A and B).

Overexpression of MnSOD did not significantly alter the CS-mediated loss in available nitric oxide levels. However, we unexpectedly observed that NRK cells overexpressing MnSOD had lower basal nitric oxide levels compared to nontransfected cells (MnSOD 319.8±35.4 vs Untx 450.0±27.0 mean fluorescence intensity; Figs. 2A and B). These findings contradict the traditional view that scavenging superoxide (MnSOD overexpression) should increase the amount of available nitric oxide by minimizing nitric oxide–superoxide interaction that results in the formation of peroxynitrite. However, a recent study characterizing the chemical interaction between nitric oxide and pure MnSOD (from Escherichia coli) by Filipović et al. [37] suggests that MnSOD can actually catalyze the decay of nitric oxide under anaerobic and aerobic conditions. The study also showed that reaction of MnSOD with nitric oxide resulted in partial inactivation of MnSOD. These authors speculate that MnSOD-mediated degradation of nitric oxide is a protective mechanism to defend against nitric oxide toxicity. It is important to point out that the studies described above were test tube studies, not performed in cells, thus it remains unknown whether these mechanisms can occur in vitro or in...
vivo. In our study, the mechanism(s) mediating the loss of nitric oxide in MnSOD-overexpressing cells is unknown, but we believe this intriguing finding warrants further experiments because it may reveal a new paradigm for MnSOD–nitric oxide interaction in renal tubular cells. The decline in nitric oxide levels observed in NRK cells exposed to CS, which occurred in the presence or absence of MnSOD transfection, could be due...
to cold temperatures inhibiting NOS activity or, alternatively, the available nitric oxide could be consumed by increased superoxide production thereby forming peroxynitrite.

Nitrotyrosine formation occurs during CS and RW of renal tubular cells and is decreased with MnSOD overexpression or NOS inhibition

Protein-bound nitrotyrosine formation is a “footprint” widely used immunologically to demonstrate nitric oxide-dependent oxidative damage (e.g., peroxynitrite formation). In this study, we used a polyclonal antibody against nitrotyrosine and a secondary antibody conjugated with goat anti-rabbit Alexa-594 to detect nitrotyrosine using immunocytochemistry. Because our earlier findings demonstrated both an increase in superoxide and a decrease in nitric oxide during CS, we expected to see increased peroxynitrite (nitrotyrosine staining). As hypothesized, exposure of NRK cells to 24 h of CS alone or with 6 h of rewarming resulted in increased nitrotyrosine formation (red staining) compared to untreated cells (Fig. 3). MnSOD overexpression decreased nitrotyrosine formation during CS, suggesting less oxidant damage in the presence of enhanced superoxide scavenging. But surprisingly, a difference in nitrotyrosine staining was not readily apparent between nontransfected and MnSOD-overexpressing NRK cells after CS+RW (Fig. 3). The lack of significant protection by MnSOD during RW could reflect partial inactivation of MnSOD by peroxynitrite formation during CS+RW or that NOX leads to nitration of nonmitochondrial targets that are inaccessible to MnSOD scavenging. Further control experiments using the secondary Alexa-594 antibody alone (MnSOD 2′ alone) revealed that this background staining was probably due to nonspecific effects of the secondary antibody used in these studies (Fig. 3). In addition, NRK cells treated with Lipofectamine alone did not increase nitrotyrosine formation (data not shown).

To provide stronger evidence that peroxynitrite is the oxidant likely to be involved in tyrosine nitration during CS, we used the nonselective NOS inhibitor L-NMMA (100 μM for 30 min before and during CS) to block nitric oxide synthesis. Similar to the effect of MnSOD overexpression, nitric oxide blockade prevented the increased tyrosine nitration seen during CS (Fig. 3). To determine whether NOX might contribute to tyrosine nitration (the superoxide component), NRK cells were treated with the NOX inhibitor DPI (1 μM for 1 h before and during CS). DPI treatment did not decrease NRK cell nitrotyrosine formation during CS (Fig. 3). Finally, hydrogen peroxide also seemed to have no role in CS-induced tyrosine nitration because catalase treatment (2000 units/ml for 30 min before and during CS) did not alter nitrotyrosine staining (Fig. 3). The Amplex red assay was also performed and showed no increase in hydrogen peroxide levels...
after CS of NRK cells (data not shown). These findings strongly suggest that peroxynitrite is the primary oxidant leading to nitrotyrosine formation during CS of renal tubular cells.

MnSOD overexpression does not protect against CS-induced mitochondrial membrane depolarization

A few studies have documented mitochondrial abnormalities induced by CS, including mitochondrial swelling, loss of ATP, and mitochondrial-dependent apoptosis with RW [38,39]. However, the “triggers” or signaling pathways that lead to such mitochondrial damage have not been explored in depth. Thus, we sought to determine whether increases in mitochondrial superoxide lead to altered mitochondrial function during CS. One way to assess mitochondrial function is to measure the relative mitochondrial membrane potential, generated by oxidative phosphorylation, using the cationic probe JC-1. NRK cells exposed to CS alone had significantly lower (depolarized) relative mitochondrial membrane potential (green staining) compared to untreated cells, which were depicted by normal red/green/yellow staining (Fig. 4A). The mitochondrial depolarization of NRK cells observed after CS is consistent with other reports showing early mitochondrial structural abnormalities during CS using electron microscopy [38]. Cold temperatures can lead to a rapid decline in ATP levels [40,41], suggesting that cold slows down oxidative phosphorylation drastically. This slowing of oxidative phosphorylation could explain why depolarization of mitochondria occurred during CS of NRK cells. Lipofectamine treatment of NRK cells did not alter the mitochondrial membrane potential (data not shown). Mitochondrial membrane potential was restored to basal levels after RW of NRK cells (CS.RW 1.24 ± 0.07 vs Untx 1.28 ± 0.06 mean fluorescence intensity; Figs. 4A and B). The reason for this restoration of mitochondrial membrane potential is unknown; however, it could be in response to the cells returning to their normal physiological state (37 °C) or that depolarization was a transient event. Hence, it is possible that during the CS-induced depolarization, superoxide is generated, which leads to further injury during RW even though the mitochondrial membrane potential has returned to basal levels during RW.

Overexpression of MnSOD in NRK cells did not significantly alter mitochondrial depolarization during CS (CS + MnSOD 0.18 ± 0.09 vs CS 0.13 ± 0.07 mean fluorescence intensity; Figs. 4A and B). This suggests that mitochondrial depolarization during CS may not be a result of mitochondrial superoxide, or downstream oxidant...
production, but rather an effect of the lowered temperature. However, it remains possible that the CS-induced mitochondrial depolarization may be responsible, in part, for the increased levels of mitochondrial superoxide observed in NRK cells during CS.

MnSOD overexpression or NOS inhibition protects against partial inactivation of mitochondrial respiratory complexes during CS

Disruption of mitochondrial respiratory complexes can lead to generation of oxidants, mitochondrial depolarization, and cell injury [42–44]. We have previously documented that the activity of all four mitochondrial respiratory complexes declined using an in vivo cold I/R model (40 min of cold ischemia followed by 18 h of reperfusion) [45]. Thus, experiments were carried out to determine whether CS alone or with RW alters NRK cell mitochondrial respiratory complex activity. Complexes I and II were significantly inactivated after 24 h of CS alone compared to untreated NRK cells (complex I, CS 58.3 ± 4.0 vs 100% of control; complex II, CS 53.1 ± 1.7 vs 100% of control; Figs. 5A and B). NRK cells exposed to CS.RW showed a significant decline in activity of respiratory complexes I, II, and III compared to untreated cells (complex I, CS.RW 43.1 ± 3.8 vs 100% of control; complex II, CS.RW 41.5 ± 4.8 vs 100% of control; complex III, CS.RW 54.4 ± 6.3 vs 100% of control; Figs. 5A–C). Additional experiments measuring mitochondrial oxygen consumption/respiration during CS were attempted, but NRK cells were not efficient in responding to traditional oxygen consumption substrates, which may be due to NRK cells’ heavy reliance on glycolytic pathways.

Overexpression of MnSOD in NRK cells prevented inactivation of complexes I and II during CS (complex I, CS + MnSOD 80.2 ± 9.8 vs CS 40.0 ± 3.8; complex II, CS + MnSOD 97.3 ± 4.4 vs CS 41.5 ± 9.1%; Figs. 5E and F). This finding suggests that increased mitochondrial superoxide contributes to the partial inactivation of complexes I and II observed during CS. In addition, the contribution of nitric oxide to respiratory complex inactivation during CS was evaluated using the nonselective NOS inhibitor L-NMMA. L-NMMA treatment before and during CS also provided protection against respiratory complex inactivation of complexes I and II during CS (complex I, CS + L-NMMA 91.9 ± 8.7 vs CS 40.0 ± 3.8; complex II, CS + L-NMMA 97.0 ± 11.2 vs CS 41.5 ± 9.1%; Figs. 5E and F). These data demonstrate that both mitochondrial superoxide and nitric oxide (strongly supporting a role for peroxynitrite) contribute to CS-mediated damage to mitochondrial respiratory complexes during CS, which is in agreement with previous studies in other systems [46–48].

Fig. 4. JC-1 (7.5 μM for 30 min) was used to measure the relative mitochondrial membrane potential of NRK cells. (A) Fluorescence microscopic images of (left to right): untreated (Untx) cells, cells exposed to CS, and cells exposed to CS followed by rewarming (CS.RW). Cells in the lower three images were treated similar to the cells at the top, but were transiently transfected with MnSOD. Results are representative of three experiments using different cell cultures. (B) JC-1 fluorescence intensity was quantified using Nis Elements software. Relative mitochondrial membrane potential was calculated using the ratio of JC-1 aggregate/monomer (590 nm/530 nm) of mean fluorescence intensity. Values are expressed as means±SEM (n = 3). *P < 0.05 compared to untreated cells (+MnSOD).
Several studies have suggested that respiratory complexes I and III serve as the major sources for generation of superoxide in the mitochondria [49,50]. Interestingly, respiratory complexes I and III have also been reported as a target for oxidative damage by reactive oxygen species [48,51]. Thus, it appears that mitochondrial complexes could serve as a source or a target for reactive oxygen...
species, depending on the mechanisms governing the etiology of a disease.

**MnSOD overexpression or NOS inhibition protects against cell death during CS and RW of renal tubular cells**

Previous studies have documented that CS.RW of renal proximal tubular cells leads to cell death [32,38,39]. Salahudeen et al. showed that the mode of cell death was primarily apoptotic when cells were exposed to CS.RW; however, CS resulted in minimal cell death [38]. Consistent with these findings, NRK cells exposed to CS showed no significant increase in cell death, but it was significantly increased after RW (Untx 6.1 ± 2.3% vs CS 9.4 ± 0.6% vs CS.RW 38.1 ± 3.9%; Fig. 6). NRK cells overexpressing MnSOD provided partial protection against cell death during CS.RW (CS.RW + MnSOD 25.8 ± 1.3% vs CS.RW 38.1 ± 3.9%; Fig. 6), which revealed that mitochondrial superoxide contributes to cell death during CS.RW. Further studies were performed to evaluate the role of nitric oxide in cell death induced by CS.RW using the nonselective NOS inhibitor L-NMMA. Treatment of NRK cells with L-NMMA alone resulted in a slight, but not significant, increase in cell death compared to untreated cells (L-NMMA 11.3 ± 1.9% vs Untx 6.1 ± 2.3%; Fig. 6).

Interestingly, NRK cells treated with L-NMMA (30 min before and during CS) blunted cell death significantly during CS.RW, implicating nitric oxide in a pivotal role in cell death during CS.RW (CS.RW + L-NMMA 13.6 ± 2.9% vs CS.RW 38.1 ± 3.9%; Fig. 6). These data support the possibility that peroxynitrite is produced during CS and RW and leads to NRK cell death (Fig. 7).

It is known that respiratory complexes can “leak” superoxide to the matrix side of the mitochondria or toward the cytosol/intermembrane space—a location that would preclude protection by the matrix-localized antioxidant, MnSOD [52,53]. This may provide further insight into the data showing that MnSOD overexpression provided a modest but significant protection from cell death after CS.RW, whereas pretreatment with L-NMMA provided a more robust protection. Hence, it is possible that CS.RW causes superoxide flux toward the cytosol, generating peroxynitrite and tyrosine nitration of nonmitochondrial matrix targets, which leads to cell death. This hypothesis is actually supported by our nitrotyrosine staining shown in Fig. 3, in that overexpression of MnSOD failed to lower nitrotyrosine staining during CS.RW.

Many other studies have implicated “reactive oxygen species” in CS-induced damage by using generic oxidant scavengers or nonspecific assays for oxidant detection [32,33,39,54,55]; our results represent the first known assessment of the extent and identity of oxidant production and mitochondrial dysfunction in renal tubular cells after CS alone and with RW. The data presented here provide support for the idea that blocking superoxide generation during CS might lead to prevention of numerous downstream pathways known to elicit renal injury during cold preservation/reperfusion before transplantation (Fig. 7).

Future studies are under way to address the benefit of adding mitochondrial superoxide or broader-spectrum oxidant scavengers to preservation solutions to mitigate injury during CS.RW. Application of these findings could provide healthier donor kidneys, improved graft function, and enhanced quality of life for graft recipients. The data presented in this study can be used later to validate animal models or could be applicable in testing compounds specifically designed to protect against CS-mediated cellular injury.

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