Cystamine prevents ischemia–reperfusion injury by inhibiting polyamination of RhoA

Dong-Myung Shin a,c, Jinmo Kang a,f, Jongwon Ha a,b, Heun-Soo Kang e, Sang-Chul Park c, In-Gyu Kim c, Sang Joon Kim a,b,d,*

a Xenotransplantation Research Center (XRC), Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-799, Republic of Korea
b Department of Surgery, Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-744, Republic of Korea
c Department of Biochemistry and Molecular Biology/Aging and Apoptosis Research Center (AARC), Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-799, Republic of Korea
d Transplantation Research Institute, Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-799, Republic of Korea
e Metabolab Engineering Lab Incorporate, Cancer Research Institute, Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-799, Republic of Korea
f Department of Surgery, Dankook University College of Medicine, Anseo-dong, Cheonan, Chungcheongnam-Do 330-714, Republic of Korea

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Abstract

Transglutaminase2 (TGase2) activates Rho-associated kinase (ROCK), an important mediator of ischemia–reperfusion (IR) injury, through polyamination of RhoA. Cystamine, an oxidized dimer of cysteamine inhibits the transamidation activity of TGase2. We examined whether addition of cystamine to an organ preservation solution protects rat cardiomyocyte cells (H9C2) from cell death in IR injury. H9C2 cells were stored under hypoxic conditions at 4°C in laboratory-made preservation solution (SNU) or SNU solution supplemented with cystamine (SNU-C1), and cell preservation in the two solutions was compared by measuring the release of lactate dehydrogenase. The cells were preserved more effectively in SNU-C1 than in SNU solution. Cystamine inhibited the intracellular activity of TGase2 which increased during cold storage or reoxygenation. The inhibition of TGase2 by cystamine reduced the polyamination of RhoA, the interaction between RhoA and ROCK2, and F-actin formation. Cystamine also prevented the activation of caspases during cold storage. These results suggest that addition of cystamine to the organ preservation solution significantly enhances cardiomyocytes preservation apparently by inhibiting TGase2-mediated RhoA-ROCK pathway and that TGase2 may play an important role in IR injury by regulating ROCK.

Keywords: Cystamine; Ischemia–reperfusion injury; Organ preservation solution; Rho-associated kinase; Transglutaminase2

Ischemia–reperfusion (IR) injury is a major non-immunological cause of graft malfunction that limits the therapeutic value of organ transplantation. The hypothermic storage of organ in preservation solution leads to ATP depletion that causes two characteristics of IR injury, accumulation of intracellular Ca^{2+} and oxidative stress.

The ATP depletion decreases the activities of Na^{+}/K^{+} ATPase in membrane and Ca^{2+} ATPase in endoplasmic reticulum, resulting in increase of intracellular Ca^{2+}. On reperfusion, the influx of Ca^{2+} induces the contraction of actin-myosin and activation of Ca^{2+}-dependent enzymes that result in mitochondrial failure [1]. Moreover, the ATP depletion leads to an increase in hypoxanthine that is used as a substrate for xanthine oxidase producing reactive oxygen species (ROS) on reperfusion [2]. The oxidative stress elicits an inflammatory response and contraction of endothelial cell or vascular smooth muscle cells (VSMC).
The contraction of VSMC and the migration of inflammatory cells are mediated by RhoA-Rho-associated kinase (ROCK)-signaling pathway [3]. ROCK regulates actin cytoskeleton organization by enhancing the phosphorylation of the light chain of myosin through inhibition of myosin phosphatase [4]. Pharmacological inhibitors of ROCK such as fasudil and Y-2763213 protect hepatic and cardiac tissues from IR injury [5–8], suggesting that the aberrant activation of ROCK plays an important role in the pathogenesis of IR injury. However, the precise molecular mechanism that activates the RhoA-ROCK signaling in IR injury has not been elucidated.

Transglutaminase2 (TGase2) is a calcium-dependent enzyme that catalyzes the post-translational modification of proteins, which results in cross-linked, polyamminated or deamidated proteins [9]. Modified RhoA resulting from polyamination or deamination of its glutamine 63 by TGase2 [10], is constitutively activated and interacts with ROCK2 more efficiently, leading to the formation of stress fiber and focal adhesion complex [11]. The expression level and activity of TGase2 in donor organs were shown to correlate with loss of function of renal-allograft in human kidney transplantation [12], suggesting TGase2 may mediate IR injury of renal allograft through the activation of RhoA.

Cystamine, an oxidized dimer of cysteamine inhibits the transamidation activity of TGase2 [13]. Cystamine also inhibits caspase 3 through mixed disulfide exchange reactions of sulfhydryl group at the active site [14] and exhibits anti-oxidant activity. We wondered therefore whether the preservation of organs destined for transplantation, or the viability of their constituent cells, improve when they are stored in the presence of cystamine. We report here that cold storage of cardiomyocytes in a traditional organ preservation solution supplemented with the cystamine protects the cells from cell death from reoxygenation after cold storage.

Materials and methods

Preservation solution. The preservation solution (SNU solution) was prepared in-house, based on University of Wisconsin (UW) solution [15]. Briefly, hydroxethyl starch (30 g, Fresenius Kabi) was dissolved in 500 ml of distilled water and mixed with 100 ml of water containing 35.83 g of lactobionic acid (Sigma), in which pH was adjusted to pH 7.4 with KOH. The mixed solution of hydroxyethyl starch solution and lactobionic acid (Sigma), in which pH was adjusted to pH 7.4 with KOH. was then mixed with a solution containing raffinose (30 mM, Sigma), and lactobionic acid (Sigma), in which pH was adjusted to pH 7.4 with KOH. The mixed solution of hydroxyethyl starch solution and lactobionic acid (Sigma), in which pH was adjusted to pH 7.4 with KOH.

Briefly, hydroxyethyl starch (50 g, Fresenius Kabi) was dissolved in 500 ml of distilled water and mixed with 100 ml of water containing 35.83 g of lactobionic acid. To mimic the environment representing IR injury, we subjected the cells to either cold storage (CS) or subsequent reoxygenation (RO). For CS, the cells were incubated in GasPak Pouch anaerobic system (Becton, Dickinson and company) at 4 °C for the indicated time. For RO, the cold-stored cells were incubated under normal culture media in a humidified atmosphere with 5% CO2 at 37 °C for 3 days, changing the medium once a day (Fig. 1A).

Cell viability assays. Cell viability was assessed by lactate dehydrogenase (LDH) release assay (Promega) and expressed as the ratio of an experimental LDH release to the maximal LDH release. The maximal LDH release represented the amount of LDH released into the culture medium when the cells were treated with 1% Triton X-100. Viable cells were counted after staining with trypan blue.

Caspase assay. Caspase activity was determined using chromogenic substrates, Ac-DEVD-pNA for caspase 3 and Ac-LEVD-pNA for caspase 9 (A.G. Scientific, Inc), respectively. Cell extracts were prepared by a freezing-thawing in the lysis buffer (100 mM Hepes, pH 7.5, 0.1% CHAPS, 0.1% Triton X-100, and 100 mM EDTA), followed by centrifugation (12,000 g, 10 min at 4 °C). The cell extract (30 µg) was added to assay buffer (100 mM Hepes, pH 7.5, 10% sucrose, 0.1% CHAPS, and 10 mM DTT) containing chromogenic substrates (200 µM) and incubated for 4 h at 37 °C. Caspase activity was quantified by measuring the absorbance at 490 nm and was expressed as folds of activation compared with untreated cells.

Intracellular TGase2 activity. Intracellular TGase2 activity was measured as described previously [16]. In brief, cells were incubated with 1 mM biotinylated pentylamine (BP, Pierce) for 1 h prior to harvesting and cell extracts were prepared by sonication, followed by centrifugation (14,000g, 10 min at 4 °C). For solid-phase microtiter plate assay, the cell extract (0.2 mg/ml, 100 µl/well) was diluted with the coating buffer (50 mM Tris-CI, pH 7.5, 150 mM NaCl, 5 mM EGTA, and 5 mM EDTA) to achieve each of 20% well-microtiter plate (Nunc). TGase2 activity was evaluated by determining the BP incorporated into proteins using HRP-conjugated streptavidin (Zymed). Assays were quantified by reaction with o-phenylenediamine dihydrochloride (Sigma) and measuring the absorbance at 490 nm on microplate spectrophotometer (Molecular Devices). For Western blot analysis, proteins incorporated with BP by intracellular TGase2 were visualized by probing with HRP-conjugated streptavidin (Zymed), followed by enhanced chemiluminescence reagents (Pierce). For cytochemical analysis, cells were plated onto glass coverslips placed in a 6-well plate and cultured for 24 h. Cells were exposed to CS or RO and then were incubated with 1 mM BP for 1 h prior to fixation with 4% formaldehyde in PBS for 15 min. After treatment with 0.5% Triton X-100 in PBS for 5 min at room temperature, cells were incubated with 3% BSA in PBS at room temperature for 30 min. BP incorporation and actin organization were assessed by using Texas Red-conjugated streptavidin (Jackson Laboratory) and Texas Red-conjugated pallidolin (Molecular Probe), respectively. The cells were photographed using a confocal laser-scanning microscope (Charles Zeiss, LSM510).

Streptavidin pull-down and immunoprecipitation. Polyanimated RhoA was detected by streptavidin pull-down assay as previously described [11]. In brief, cells were incubated with 1 mM BP as described in transamidation assay. Cell extracts were prepared by disrupting the cells with lysis buffer (50 mM Tris-CI, pH 7.5, 20% Glyceral, 1% Triton X-100, 5 mM dithiothreitol, and protease inhibitor mixtures), followed by centrifugation (14,000g, 10 min at 4 °C). After dialysis with lysis buffer, Immunopure® Immobilized Streptavidin (100 µl, Pierce) was added into dialyzed extracts. The mixture was incubated overnight and the proteins bound to the Immobilized Streptavidin were eluted using SDS-PAGE buffer and boiled for 10 min. For immunoprecipitation experiments, cell extracts were prepared using the lysis buffer. The cell extract (100 µg) was then incubated overnight at 4 °C with Immunopure® Immobilized Protein G beads (Pierce) coupled with antibody specific to RhoA (Santa Cruz). Protein G beads were washed 4 times with lysis buffer. The bound proteins were eluted using the SDS-PAGE buffer and boiled for 10 min. The eluted proteins were resolved in 12% SDS-PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed with antibodies specific for RhoA and ROCK2, respectively (Santa Cruz).

Western blot. The cell extract (30 µg) was resolved in 6–12% SDS-PAGE, and the protein levels were probed using monoclonal antibodies
specific for TGase2 [17], actin (Santa Cruz), RhoA (Santa Cruz), and ROCK2 (Santa Cruz), respectively.

**Results**

**Inhibition of cell death by cystamine during cold storage-reoxygenation**

We investigated whether cystamine added to the preservation solution prevents cell death. H9C2 cells, a rat cardiomyocyte cell line, were incubated in SNU or SNU-C1 solution in a culture condition mimicking IR injury (Fig. 1A). The viability of the cells was evaluated by measuring the amount of LDH released during 2–3 day cold storage (CS) or one day after subsequent reoxygenation (CS-RO). When H9C2 cells were preserved in SNU solution for 2 days (Fig. 1B) or 3 days (Fig. 1C and D) with the repeated change of culture medium, the release of LDH peaked on the first day in CS-RO and diminished thereafter. In this experimental condition employed, SNU-C1 solution prevented the LDH release during CS or CS-RO in H9C2 cells exposed to CS for 3 days and CS-RO (Fig. 1C). These results were confirmed by counting the number of viable cells (Fig. 1D). These results indicate that the supplementation of SNU with cystamine improves the preservation of cardiomyocytes by preventing the cellular damage under conditions mimicking IR injury.

**Activation of intracellular TGase2 in cardiomyocyte by cold storage-reoxygenation**

To explore the mechanism by which cystamine enhances the preservation of the cells and presumably protects them from IR injury, we monitored intracellular activity of TGase2, which is known to be inhibited by cystamine. When the cells in SNU solution were exposed to CS or CS-RO, intracellular TGase2 activity was increased by 1.9- or 2.4-fold, respectively, without changing the TGase2 expression (Fig. 2A and B). However, in similar studies with H9C2 cells incubated in SNU-C1 solution, there was no elevation of TGase2 activity. Immunocytochemical analysis confirmed the inhibitory effect of cystamine in the preservation solution on TGase2 activity (Fig. 2C). We also observed that the activation of TGase2 following exposure to CS was time-dependent, reaching maximum level in CS-RO after 3 days of CS (Fig. 2D). These results indicate that the CS-RO culture conditions induce the activation of TGase2 in cardiomyocyte, which is inhibited by cystamine.

**Activation of RhoA-ROCK signal pathway by TGase2**

It has been reported that TGase2 activates RhoA through polyamine incorporation or deamidation [11]. To determine whether TGase2 activates RhoA-ROCK path-
When H9C2 cells are exposed to the CS-RO conditions, we examined polyamine incorporation into RhoA. The cells were incubated with biotinylated pentylamine (BP), and proteins incorporated with BP were pulled-down with streptavidin. Western blot analysis with anti-RhoA antibody revealed that RhoA was not polyaminated when the H9C2 cells were cultured under the normal conditions. In contrast, polyaminated RhoA was detected in the cells subjected to CS for 3 days, and polyamination was further increased under CS-RO conditions. We then examined the effect of RhoA polyamination on the interaction with ROCK. When H9C2 cells were exposed to the CS, ROCK2 was co-immunoprecipitated with RhoA. We observed that the level of co-immunoprecipitated ROCK2 increased in cells exposed to CS-RO. Treatment of cystamine abrogated the polyamination of RhoA as well as co-immunoprecipitation of ROCK2 with RhoA (Fig. 3A). As RhoA mediates cell motility or vasoconstriction through cytoskeleton reorganization, we tested whether SNU-C1 solution prevents the reorganization of the cytoskeleton. The F-actin was increased, when the cells were exposed to CS or CS-RO in SNU solution, but not in SNU-C1 solution (Fig. 3B) as determined by immunostaining. These results indicate that cystamine prevents the activation of RhoA-ROCK pathway.

Fig. 2. Cold storage-reoxygenation activates TGase2 in H9C2 cells. (A–C) H9C2 cells were stored in SNU or SNU-C1 solution for 3 days at 4 °C (CS) or additionally incubated in 5% CO2 for 1 day at 37 °C (CS-RO). The cells were incubated for 1 h with BP (1 mM). Intracellular TGase2 activity was determined by microtiter-plate assay (A), Western blot analysis (B), and cytochemical analysis (C). (D) H9C2 cells were subjected to CS for indicated days and then to CS-RO for 1 day before the intracellular TGase activity was measured. WB, Western blot; NT, not treated.

Fig. 3. TGase2 activates RhoA-ROCK pathway. (A) H9C2 cells were subjected to CS for 3 days or additionally incubated in 20% O2 for 1 day at 37 °C (CS-RO). The cells were incubated with BP (1 mM) for 1 h. Polyamination of RhoA was evaluated by pull-down with streptavidin (SA), followed by Western blot analysis using RhoA-specific antibody. Interaction between RhoA and ROCK2 was assessed by immunoprecipitation using RhoA-specific antibody and Western blot analysis using ROCK2-specific antibody. (B) The level of F-actin in H9C2 cells, stored in SNU or SNU-C1 solution for CS or CS-RO, for 3 days was determined by cytochemical staining with Texas-Red conjugated pallloidin. PD, pull-down; SA, streptavidin; WB, Western blot.
pathway through abrogation of TGase2-mediated polyamination of RhoA during CS-RO.

Inhibition of apoptosis by cystamine during cold storage-reoxygenation

One might expect cystamine to protect cells from death since it also inhibits the activity of caspase 3. When the cells were stored either in PBS solution or in SNU solution and subjected to CS for 3 days, the caspase 3 activity increased 5 fold or 3.5 fold, respectively, and decreased during CS-RO (Fig. 4A). Caspase 9 exhibited similar activities as caspase 3 did under comparable experimental conditions (Fig. 4B). Interestingly, SNU-C1 solution effectively inhibited both caspase 3 and 9 during CS, but not during CS-RO (Fig. 4). These results indicate that the supplementation of SNU with cystamine also improves the efficacy of preservation solution by preventing apoptosis.

Discussion

Many preservation solutions have been developed to minimize graft tissue/organ deterioration during preservation [18]. However, the current preservation solutions including Euro-Collins solution, Histidine-Tryptophan-Ketoglutarate (HTK) solution, and UW solution allow only 6 h for the heart and 72 h for the kidney mainly due to IR injury [15]. It is difficult to suppress IR injury because of the complex pathogenesis of IR injury. An increase of Ca\textsuperscript{2+} and ROS production caused by ATP depletion has been thought to initiate IR injury by eliciting cellular responses including activation of various signaling pathways, such as p53, NF\textsubscript{κ}B, RhoA, PI3K/Akt, and Erk/JNK/p38 MAPK signaling pathway [19]. In the present study, we showed that preservation solution containing cystamine increases the cell viability of cardiomyocytes when they are subjected to CS-RO.

Cystamine is an oxidized dimer of cysteamine, a thiolamine, synthesized by pantetheinase [20]. Cystamine is thought to regulate enzymes such as TGases, caspase 3, and protein kinase C-\varepsilon through a sulfhydryl-disulfide exchange between sulfhydryl groups of the enzymes and cystamine [21,22]. We have shown that CS-RO activates intracellular TGase2, which modifies RhoA by polyamination. The modified RhoA caused actin reorganization through increased interaction with ROCK2. These results indicate that TGase2 is a mediator that links CS-RO and the activation of RhoA-ROCK2 signaling pathways. Thus, exogenous cystamine could inhibit the ROCK2 through the inhibition of TGase2.

TGase2 catalyzes the irreversible post-translational modification of proteins, forming deamidated or polyamminated proteins [9]. Our data showed that RhoA is a good substrate of TGase2 in cardiomyocytes. This finding is consistent with the previous report that treatment of retinoic acid, a potent inducer for TGase2, leads to the polyamination of RhoA and subsequent activation of ROCK, an important therapeutic target for IR injury [11,23]. ROCK elevates levels of proinflammatory molecules (interleukin-6, monocyte chemoattractant protein-1, macrophage inhibitory factor, and interferon-\gamma), thrombogenic molecules (platelet-activating factor and tissue factor), and fibrogenic molecules such as transforming growth factor \textbeta 1 [24–26]. Moreover, RhoA-ROCK signaling pathway mediates the vascular effects of vasoactive factors such as angiotensin II, serotonin, thrombin, endothelins-1, and urotensin II [27–29]. RhoA-ROCK signaling pathway plays an important role in IR injury because ROCK inhibitors have been shown to prevent IR injury in various models [5–8]. It follows that TGase2 may play an important role in IR injury because it is an upstream regulator for RhoA-ROCK pathway.

In summary, we showed that cystamine inhibits TGase2, ROCK2, caspases 3 and 9 in cardiomyocytes, thereby increasing their viability when exposed to CS or CS-RO. Supplementation of the preservation solution with cystamine significantly improves its preservative function. Additional studies are underway to examine whether supplementation with cystamine (SNU-C1 solution) can also increase the survival and viability of other cell types.
and, more importantly, of tissues, and organs in in vivo transplantation.

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References


