Cryobiology 65 (2012) 60–67

Contents lists available at SciVerse ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Ezrin functionality and hypothermic preservation injury in LLC-PK1 cells ☆

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ARTICLE INFO

Article history:
Received 5 September 2011
Accepted 12 April 2012
Available online 23 April 2012

Keywords:
ERM proteins
Kidney donation
Mitochondria
mPTP

ABSTRACT

Renal epithelial cells from donor kidneys are susceptible to hypothermic preservation injury, which is attenuated when they over express the cytoskeletal linker protein ezrin [34]. This study was designed to characterize the mechanisms of this protection. Renal epithelial cell lines were created from LLC-PK1 cells, which expressed mutant forms of ezrin with site directed alterations in membrane binding functionality. The study used cells expressing wild type ezrin, T567A, and T567D ezrin point mutants. The A and D mutants have constitutively inactive and active membrane binding conformations, respectively. Cells were cold stored (4 °C) for 6–24 h and reperfused for 1 h to simulate transplant preservation injury. Preservation injury was assessed by mitochondrial activity (WST-1) and LDH release. Cells expressing the active ezrin mutant (T567D) showed significantly less preservation injury compared to wild type or the inactive mutant (T567A), while ezrin-specific siRNA knockdown and the inactive mutant potentiated preservation injury. Ezrin was extracted and identified from purified mitochondria. Furthermore, isolated mitochondria specifically bound anti-ezrin antibodies, which were reversed with the addition of exogenous recombinant ezrin. Recombinant wild type ezrin significantly reduced the sensitivity of the mitochondrial permeability transition pore (mPTP) to calcium, suggesting ezrin may modify mitochondrial function. In conclusion, the cytoskeletal linker protein ezrin plays a significant role in hypothermic preservation injury in renal epithelia. The mechanisms appear dependent on the molecule's open configuration (traditional linker functionality) and possibly a novel mitochondrial specific role, which may include modulation of mPTP function or calcium sensitivity.

Introduction

Renal transplantation is the treatment of choice for patients with end stage kidney failure. However, since the demand for donor kidneys outstrip the availability by 4 to 1, many remain on dialysis or die waiting [31]. About half of transplanted kidneys derive from cadaver donors, which subject them to organ donation stress and preservation injury. This typically includes hypothermia, ischemia, and reperfusion injury that occurs during donation, storage, and at transplantation. In order to expand the donor pool to meet the increased demand, kidneys are being accepted from donors that were once considered unacceptable. These kidneys often experience warm ischemia before harvest and donor risk factors such as advanced age and chronic diseases. These additional risk factors potentiate the existing preservation injury [19,29]. This in turn increases the chances for the development of chronic graft failure, late graft loss, and reduces the life expectancy of the graft, which puts further pressure on organ availability as these patients are re-transplanted or go back on dialysis [28,29,35]. Therefore, significant advancements in organ preservation are needed to expand the donor pool to meet the demand [12,20].

While multiple factors are involved in renal preservation injury including tissue edema, ATP depletion, calcium, oxidative stress, inflammation, mediators, etc. [5,11,21,22,30,33]; interventions directed at many of these targets has not greatly improved clinical preservation injury over the last 20 years. This suggests that a more proximal root mechanism may be important in understanding and controlling preservation injury.

Hypothermic preservation injury is also characterized by severe membrane failure with blebbing, loss of microvilli, calcium overload, and phospholipid catabolism [13,15,16,26]. Membrane ultrastructural components are held in place by elements of the sub-lamellar cytoskeletal system and are important for the proper function of epithelial rich organs like the kidney [3,6]. The cytoskeletal protein ezrin, part of the ERM (Ezrin–Radixin–Moesin) family,

☆ *Statement of funding*: This work was supported by a Grant from the National Institutes of Health R01-DK-087737 and by funds from the Medical College of Virginia Foundation (MCVF).

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http://dx.doi.org/10.1016/j.cryobiol.2012.04.003

functions as a cross-linker between the actin cytoskeleton and the plasma membrane [7]. ERM proteins are involved in microvilli formation and breakdown and are also necessary for cell–cell and cell–substrate adhesions [9,18]. Membrane failure, characterized by breakdown of microvilli, increased membrane permeability, and membrane bleb formation, is a common early event in various types of apoptosis [18] and occurs during cold storage preservation [16,23,25,34]. Therefore, it is reasonable to suggest that preservation-induced failure of the cell membrane and events distal to such failure may be caused by primary disengagement of the ezrin system supporting the plasma membrane ultrastructure. Recently, evidence from our lab suggests that disruption of the sub-lamellar cell cytoskeletal system (ezrin and spectrin) occurs during cold preservation and may cause preservation injury [23,34]. Renal tubules, tubular epithelial cells, and livers subjected to cold ischemia show biochemical and morphological signs of cytoskeletal disruption, which is attenuated by maneuvers that strengthen these systems [17,23]. Cold ischemic storage of renal tubules caused an increase in the solubility of both free ezrin and free Na/K ATPase proteins extracted from the cell membranes [23]. This suggests a disassociation of ezrin binding to both the actin cytoskeletal system and to the outer cell membrane [4,8,37,38].

Ezrin is a multifunctional protein and has many control points where site specific phosphorylation result in specific protein functionality. Specific phosphorylation of T567 opens the molecule and exposes both the NH_2 and COOH terminus to specific binding sites on actin and the cell membrane, respectively [2,36]. This activates the cross-linker function that promotes structural integrity of the cell membrane, sub-lamellar space, and microvilli, and establishes cell polarity. Our central hypothesis is that hypothermic ischemia during kidney preservation results in ezrin structural failure of the tubular epithelium that is causally involved in expression of the renal preservation injury phenotype. We have tested this hypothesis using the pig kidney proximal tubular epithelial cell lines LLC-PK1 expressing either a constitutively active ezrin mutant (T567D) or a constitutively inactive ezrin mutant (T567A) in a cell model of hypothermic preservation injury. We have further identified and explored a novel ezrin mechanism that may involve specific binding of ezrin to mitochondria with effects on calcium sensitivity of the permeability transition pore. Previous studies from our lab have shown that ezrin is involved in hypothermic preservation injury in renal epithelial cells [23,34] and this study's objective is to determine the molecular mechanisms of this protection.

Materials and methods

Materials

pEX Ezrin-YFP, an expression plasmid containing mouse wild-type Ezrin cDNA and the pig kidney proximal tubular epithelial cell line LLC-PK1 were purchased from American Type Culture Collection (ATCC). CytoTox 96 non-radioactive cytotoxicity assay kits were ordered from Promega Corp. (Madison, WI). Live/Dead viability/cytotoxicity kits were purchased from Invitrogen (Carlsbad, CA). Cell proliferation reagent WST-1 was ordered from Cayman Chemical (Ann Arbor, Michigan). QuikChange®II site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Rabbit anti-Ezrin antibody (H-276) and HRP conjugated secondary antibodies were ordered from Santa Cruz Biotechnology (Santa Cruz, CA). The primary anti-ezrin antibodies cross reacts with rabbit, mouse, porcine, and human ezrin.

Site-directed mutagenesis

To substitute the COOH-terminal threonine 567 with an alanine (T567A) or an aspartate (T567D), site-directed mutation was done with the QuikChange®II site-directed mutagenesis kit. The oligonucleotides used to introduce the T567D and T567A mutations in ezrin were (5′–3′) CAGGGACAAGTATAAGGATCTGCGGCAAAT CAGGC and CAGGGACAAGTATAAGGCGCTGCGGCAAATCAGGC. Samples were denatured at 95 °C for 30 s and then subjected to 12 cycles of amplification (95 °C, 30 s; 55 °C, 1 min; 68 °C, 12 min) for the T567D and T567A mutations, respectively. The mutations were confirmed by DNA sequencing.

Stably transfected cell lines

Stably transfected LLC-T567A cells and LLC-T567D were created by transfecting LLC-PK1 cells with pEX_Ezrin-T567A or pEX_Ezrin-T567D plasmid. The LLC-PK1 cells (5×10^5 cells/well) were seeded in 6-well plates the day before transfection. Transfection was carried out using Lipofectamine 2000 reagent (Invitrogen) with 2 μg plasmid (containing the neomycin resistance gene) per well in Opti-MEM cell culture media. Six hours after transfection, the medium was changed. Media 199 supplemented with 5% FBS and 600 μg/ml of G418 were added to the cells and incubated for 1 week. The surviving cells were detached by trypsinization (Trypsin 0.25%, EDTA 0.02%, ~15 min) and analyzed on a MoFlo flow cytometer (Beckman Coulter Corporation, Miami, Florida) using the 488 nm argon line of the Enterprise laser and standard optical filters. YFP-positive cells were sorted into 96-well plates, one cell per well, using the manufacturer's Cyclone cell deposition system. Discrete colonies were picked after 1 month, re-grown, and tested for expression of YFP by flow cytometry. A maintenance concentration of 400 μg/ml of G418 was used for the growth of stably transfected clones.

Cell culture and cold storage–reperfusion

LLC-PK1 cells were grown in Media 199 (GIBCO) supplemented with 5% FBS and 50 unit/ml of penicillin–streptomycin and maintained at 37 °C in 5% CO_2. LLC-T567A and LLC-T567D cells were grown in Media 199 supplemented with 5% FBS and 400 μg/ml G418 and maintained at 37 °C in 5% CO_2. For cold preservation experiments, the cell culture plates were put into a sealed box filled with N_2 gas and stored in a cold room (4 °C) for 6–24 h, removed, and rewarmed (''reperfused'') in a conventional CO_2 incubator for 60 min. The 199 media was first purged with nitrogen gas to remove oxygen (pO_2 reduced to about 20 mm Hg). For 6 h preservation studies, the 199 media was devoid of both fetal calf serum and phenol red indicator to promote the detection of the WST-1 dye conversion at reperfusion. Since the fetal calf serum is protective for cold storage injury, the cold storage time was reduced to 6 h in this group. Preservation injury was measured by LDH release, mitochondrial dehydrogenase activity with WST-1 dye, or live-dead fluorescent dye staining assays.

Ezrin knockdown and preservation viability

Cells at 80% confluence were transfected using Lipofectamine 2000 Reagent with [3] Stealth RNAi (Invitrogen) specific for pig ezrin. Of those 3 sequences, only one was found to be effective. The sequences of the small interfering RNA (siRNA) duplex that was used in all subsequent experiments was (5′–3′): CGGACCAGAUAAAGAGCCAAGAGCA. The cells were also transfected with a negative universal RNAi control (Invitrogen). Opti-MEM (a reduced serum medium from Invitrogen) was used to dilute the siRNA duplexes for transfection. After 48 h in cultures, the cells were placed at 4 °C for 24 h followed by 1 h of warm reperfusion at 37 °C. The cell viability was detected using the Live/Dead viability/cytotoxicity kit (Invitrogen, Carlsbad, CA) and analyzed directly under a fluorescence microscope. Live cells stained with calcein AM