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Original Contribution

Mitochondrial permeability transition pore opening induces the initial process of renal calcium crystallization

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ABSTRACT

Renal tubular cell injury induced by oxidative stress via mitochondrial collapse is thought to be the initial process of renal calcium crystallization. Mitochondrial collapse is generally caused by mitochondrial permeability transition pore (mPTP) opening, which can be blocked by cyclosporine A (CsA). Definitive evidence for the involvement of mPTP opening in the initial process of renal calcium crystallization, however, is lacking. In this study, we examined the physiological role of mPTP opening in renal calcium crystallization in vitro and in vivo. In the in vitro study, cultured renal tubular cells were exposed to calcium oxalate monohydrate (COM) crystals and treated with CsA (2 μM). COM crystals induced depolarization of the mitochondrial membrane potential and generated oxidative stress as evaluated by Cu-Zn SOD and 4-HNE. Furthermore, the expression of cytochrome *c* and cleaved caspase 3 was increased and these effects were prevented by CsA. In the in vivo study, Sprague–Dawley rats were administered 1% ethylene glycol (EG) to generate a rat kidney stone model and then treated with CsA (2.5, 5.0, and 10.0 mg/kg/day) for 14 days. EG administration induced renal calcium crystallization, which was prevented by CsA. Mitochondrial collapse was demonstrated by transmission electron microscopy, and oxidative stress was evaluated by measuring Cu-Zn SOD, MDA, and 8-OHdG generated by EG administration, all of which were prevented by CsA. Collectively, our results provide compelling evidence for a role of mPTP opening and its associated mitochondrial collapse, oxidative stress, and activation of the apoptotic pathway in the initial process of renal calcium crystallization.

Environmental and genetic factors are responsible for causing kidney stone disease. However, efficient methods of prophylaxis have not been established despite the increasing prevalence of the disease, mainly because the mechanism of stone formation has not been described in detail [1].

Renal tubular cell injury is regarded as a major risk factor for renal calcium crystallization [2,3], which can be prevented by antioxidants such as citric acid, vitamin E, traditional medicinal herbs, and green tea [4–7]. Cell membrane injury caused by oxidative stress induces the attachment of crystals to renal tubular cells [8–10], which express osteopontin (OPN), a major component of stone matrix protein [11,12]. Its expression and structure could affect experimental renal calcium crystallization [13]. Many current models of calcium oxalate stone formation suggest that the generation of reactive oxygen species (ROS) stored within mitochondria is intimately associated with renal tubular cell injury [14] and the process of renal calcium crystallization [15–17]. Our recent study also indicated that oxidative stress caused by mitochondrial collapse is involved in the early phase of renal calcium crystallization in mice [18].

Mitochondrial collapse is generated by mitochondrial permeability transition pore (mPTP) opening, which plays an important role in the mechanism of cell death through mitochondrial dysfunction. These pores consist of cyclophilin D, which penetrates the inner and outer membranes of mitochondria [19,20]. When the mPTP opens, cytosolic protons (H^+) flow into the mitochondrial matrix and disrupt the membrane potential, causing the swelling and subsequent collapse of mitochondria [21].

The opening of the mPTP depends on the activation of cyclophilin D located in the mitochondrial matrix. When cyclophilin D remains inactive, the mPTP does not open and the mitochondrion remains intact; thus, inactivating cyclophilin D can prevent mitochondrial collapse [22]. Recent reports showed that cyclosporine A (CsA) [23] can block mPTP opening via inactivation of cyclophilin D.

Abbreviations: COM, calcium oxalate monohydrate; CsA, cyclosporine A; EG, ethylene glycol; 4-HNE, 4-hydroxy-2-nonenal; mPTP, mitochondrial permeability transition pore; MDA, malondialdehyde; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; OPN, osteopontin; PLOM, polarized light optical microphotography; ROS, reactive oxygen species; SOD, superoxide dismutase; TEM, transmission electron microscopy; TMRE, tetramethylrhodamine ethyl ester.

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In this study, we have examined the changes in mitochondrial membrane potential and oxidative stress in an in vitro study; additionally, we evaluated mitochondrial structure and renal calcium crystallization in an in vivo study. We demonstrate that depolarization of the mitochondrial membrane potential and oxidative stress were prevented by CsA. We further show that mitochondrial collapse and renal calcium crystallization were significantly decreased with CsA treatment. Together, these results provide compelling evidence for a role of mPTP opening and the associated mitochondrial collapse, oxidative stress, and activation of the apoptotic pathway in the initial process of renal calcium crystallization.

Materials and methods

Preparation of calcium oxalate monohydrate (COM) crystal suspensions

Oxalic acid (200 mM, 0.5 ml) and 200 mM calcium chloride were mixed at room temperature to a final concentration of 10 mM, and the COM crystals that immediately formed in suspension were equilibrated for 3 days. The COM crystals were then washed three times with sodium and chloride-free distilled water saturated with calcium oxalate, resuspended to a final concentration of 2.92 mg/ml, and adjusted to pH 6.8 [24].

Cell culture

The renal proximal tubular cell line NRK-52E (American Type Culture Collection, Rockville, MD, USA) was cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL, Rockville, MD, USA). The cells were routinely seeded at a density of 1×10^5/60-mm culture dish (Nalge Nunc, Naperville, IL, USA) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every third day and the cells were subcultured before forming confluent monolayers.

NRK-52E cells were seeded at a density of 1×10^6/90-mm dish and cultured to 90% confluence. The cells were then treated with or without CsA (2 μM) for 10 min and then with COM crystals (100 μg/cm^2).

Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

Cells were loaded with the membrane potential-sensitive dye tetramethylrhodamine ethyl ester perchlorate (TMRE; 20 nM in Hepes-buffered salt solution; Invitrogen, Carlsbad, CA, USA) [25]. Cells loaded with TMRE were then analyzed using a confocal microscope (LSM5 PASCAL; Carl Zeiss Co. Ltd., Oberkochen, Germany) equipped with ×20 and ×100 oil-immersion, quartz objective lenses. The cells were then treated with or without CsA (2 μM) for 10 min and then with COM crystals (100 μg/cm^2) for 0, 5, 10, 15, and 30 min. As a negative control, untreated NRK-52E cells were observed, and as a positive control, we used carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; 10 μM), an uncoupler that causes mitochondrial depolarization [26]. Mitochondrial fidelity in cells stained with TMRE was quantified by flow cytometry. After three washes with phosphate-buffered saline (PBS) to remove COM crystals, stained cells in each group were detached using 0.05% trypsin–EDTA, washed with PBS, and diluted to 1 ml. A total of 30,000 events were collected from each sample and the data were displayed on a logarithmic scale of increasing red fluorescence intensity using a FACSCalibur HG (Becton–Dickinson, Franklin Lakes, NJ, USA).

Isolation of mitochondria and cytosol

Mitochondria and cytosol were isolated from NRK-52E cells using the Mitochondria Isolation Kit for Cultured Cells (Pierce Biotechnology, Rockford, IL, USA) [27]. Briefly, after Dounce homogenization, lysates were centrifuged at 700 *g* for 10 min to precipitate nuclei and garbage. The supernatants were then centrifuged at 12,000 *g* for 15 min; the pellet contained the isolated mitochondria and the supernatant contained the cytosol fraction. The cytosol fraction was used for Western blotting to detect cytochrome *c*.

Western blotting

NRK-52E cells stored at −20 °C were immersed in 1× lysis buffer and lysed by sonication on ice. The total protein concentration in the supernatant was spectrophotometrically quantified using an Ultrospec 3100 Pro (GE Healthcare, Wallingford, CT, USA). Samples containing 30 μg total protein were mixed with loading buffer (Laemmli sample buffer; Bio-Rad Laboratories, Hercules, CA, USA). Proteins were resolved by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto Immobilon polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Blocking for 1 h at room temperature was followed by an overnight incubation with a polyclonal anti-rat superoxide dismutase (SOD) antibody (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse 4-hydroxy-2-nonenal (4-HNE) monoclonal antibody (dilution 1:4; Nikken Seil, Shizuoka, Japan), and anti-rat cleaved caspase 3 antibody (dilution 1:1000; Cell Signaling Technology, Beverly, MA, USA) at 4 °C. After being washed, the membranes were treated with the corresponding peroxidase-conjugated secondary antibodies for 1 h at room temperature. Proteins were visualized using enhanced chemiluminescence Western blotting analysis kits (Pierce Biotechnology). The membranes were probed with a β-actin antibody as a loading control (Sigma–Aldrich, St. Louis, MO, USA). For Western blotting of cytochrome *c*, a similar procedure was followed except for the use of samples (cytosol fraction isolated from NRK-52E cells) containing 10 μg total protein and anti-rat cytochrome *c* antibody (dilution 1:1000; Cell Signaling Technology). The same membrane was used for each CsA (−) COM (+) and CsA (+) COM (+) group to ensure uniformity under the conditions. The protein expression levels in the bands corresponding to SOD, 4-HNE, cytochrome *c*, and cleaved caspase 3 ($n=5$ each) were quantified using Image Quant LAS 4000 (GE Healthcare Japan, Tokyo, Japan), which is a multipurpose CCD camera system for quantitative imaging of blots developed by Amersham for enhanced chemiluminescence, with standard UV transillumination for ethidium bromide gel visualization.

Experimental animals

All experiments proceeded with the approval of the Animal Care Committee of the Faculty of Medicine, Nagoya City University Graduate School of Medical Sciences. Male Sprague–Dawley (SD) rats (Charles River Japan, Yokohama, Japan), age 7 weeks and weighing 280–320 g, were acclimated at 23 ± 1 °C on a 12-h light/dark cycle for 7 days in metabolic cages before experiments were started. All animals had free access to standard rat food (containing calcium, 1.12 g; phosphorus, 0.9 g; magnesium, 0.26 g; and sodium, 0.21 g/100 g; Oriental Yeast Co., Tokyo, Japan).

Hyperoxaluric rat model and cyclosporine A administration

Forty-eight SD rats were given free access to water containing 1% ethylene glycol (EG) to form stones [28] and then treated two times per day with CsA via a gastric tube. The rats were assigned to one of the following groups ($n=12$ per group) and weighed weekly: one group received EG only (EG group) and three EG and CsA groups also received 2.5, 5, or 10 mg/kg/day CsA (EG + CsA2.5, EG + CsA5.0, and EG + CsA10.0 groups, respectively). At 7 and 14 days after the start of drug therapy, blood was sampled from the inferior vena cava of 6 rats per group. These rats were sacrificed under ether