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Role of subcellular calcium redistribution in regulating apoptosis and autophagy in cadmium-exposed primary rat proximal tubular cells

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ABSTRACT

Ca^{2+} signaling plays a vital role in regulating apoptosis and autophagy. We previously proved that cytosolic Ca^{2+} overload is involved in cadmium (Cd)-induced apoptosis in rat proximal tubular (rPT) cells, but the source of elevated cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) and the effect of potential subcellular Ca^{2+} redistribution on apoptosis and autophagy remain to be elucidated. Firstly, data showed that Cd-induced elevation of $[Ca^{2+}]_c$ was primarily generated intracellularly. Moreover, elevations of $[Ca^{2+}]_c$ and mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mit}$) with depletion of endoplasmic reticulum (ER) Ca^{2+} levels ($[Ca^{2+}]_{ER}$) were revealed in Cd-treated rPT cells, but this subcellular Ca^{2+} redistribution was significantly suppressed by 2-Aminoethoxydiphenyl borate (2-APB). Elevated inositol 1,4,5-trisphosphate (IP_3) levels with up-regulated IP_3 receptor (IP_3R) protein levels were shown in Cd-exposed cells, confirming that IP_3R-mediated ER Ca^{2+} release results in the elevation of $[Ca^{2+}]_c$. Up-regulated sequestosome 1 (p62) protein levels and autophagic flux assay demonstrated that Cd impaired autophagic degradation, while *N*-acetylcysteine (NAC) markedly attenuated Cd-induced p62 and microtubule-associated protein 1 light chain 3-II (LC3-II) accumulation, implying that the inhibition of autophagic flux was due to oxidative stress. Furthermore, pharmacological modulation of $[Ca^{2+}]_c$ with 1,2-Bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) and 2-APB alleviated Cd-mediated apoptosis, inhibition of autophagic degradation and subsequent cytotoxicity, while thapsigargin (TG) had the opposite regulatory effect on them. In summary, cytosolic calcium overload originated from IP_3R-mediated ER Ca^{2+} release has a negative impact on Cd nephrotoxicity through its promotion of apoptosis and inhibition of autophagic flux.

1. Introduction

Cadmium (Cd) is a widespread heavy metal pollutant in the environment due to its extensive use in various anthropogenic and industrial activities. The main routes of human exposure to Cd are via inhalation of Cd-contaminated dust particles (aerosols) or cigarette smoke and ingestion of contaminated water and food [1]. As a nonessential element, it exerts toxic effects on multiple organs in mammals and has been classified as a human carcinogen by the International Agency for Research on Cancer [2]. With the chronic, low-level Cd exposure that are common in humans, kidney is the most sensitive target organs of toxicity [3], where Cd accumulates primarily in the proximal tubule of the nephron [4], resulting in a generalized reabsorptive dysfunction characterized by polyuria and low molecular weight proteinuria [5]. Primary cultures can better represent the live tissue than permanent cell lines, which are ideal for in vitro toxicology studies. Therefore, primary cultures of rPT cells were established to investigate low-level Cd-induced nephrotoxicity in this study.

Autophagy is a self-digesting mechanism responsible for the degradation and recycling of damaged organelles, misfolded proteins, and other macromolecules in lysosomes, which plays a crucial role in cellular homeostasis and adaptation to adverse stress conditions [6]. In mammals, the net amount of LC3-II is a key hallmark for monitoring autophagy; moreover, analysis of autophagic flux is more reliable to represent the dynamic process of autophagy [7]. There is a growing amount of evidence that dysregulation of the autophagic pathway is implicated in the pathogenesis of kidney aging and in several renal diseases such as acute kidney injury, polycystic kidney disease, diabetic nephropathy, obstructive nephropathy, focal and segmental glomerulosclerosis, and potentially other kidney disease [8]. It has also been demonstrated that blockade of the autophagic flux can induce cell death in various types of cells [9]. Proximal tubular is the target site for Cd-induced renal damage, which prompts us to investigate the role of autophagy in Cd-exposed rPT cells.

Generally, mechanism of Cd nephrotoxicity is ascribed to the action of oxidative stress and apoptosis [10–12]. Our research group found that

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oxidative stress-mediated apoptotic death played a key role in Cd-induced nephrotoxicity in vitro; moreover, it is important to note that intracellular Ca^{2+} overload is involved in this process [13]. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) is tightly controlled by intracellular calcium stores such as mitochondria and ER, which are also the main sites of apoptotic and autophagic regulation [14]. Inositol 1,4,5-trisphosphate (IP_3) is an important secondary messenger in cell signaling [15], and cytosolic signals caused by Ca^{2+} release from the ER through IP_3 receptor (IP_3R) Ca^{2+} channels regulate numerous cellular functions [16]. Ca^{2+} release from the ER is known to be apoptogenic, which ultimately leads to $[Ca^{2+}]_c$ elevation, and associated with apoptotic death and autophagy inhibition [14,17]. However, several critical items remain unclear regarding the crosstalk between apoptosis and autophagy in response to $[Ca^{2+}]_c$ elevation during Cd nephrotoxicity. Where does Cd-induced $[Ca^{2+}]_c$ elevation originate from, intracellularly or extracellularly? What is the role of ER Ca^{2+} stores in the process of Cd-induced subcellular calcium redistribution? What are the eventual functions of autophagy in Cd-induced cytotoxicity of rPT cells? Can $[Ca^{2+}]_c$ elevation serve as a link mediating the apoptosis and autophagy in Cd-exposed rPT cells? This study will offer further evidences to clarify these questions and investigate the regulatory effect of $[Ca^{2+}]_c$ elevation on apoptosis and autophagy in Cd-exposed rPT cells.

2. Materials and methods

2.1. Chemicals and antibodies

All chemicals were of highest grade purity available. 2-Aminoethoxydiphenyl borate (2-APB) and 1,2-Bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) were from Tocris Bioscience (Bristol, UK). Thapsigargin (TG) was from Thermo Fisher Scientific Inc. Dihydro-Rhod-2-AM, Mag-Fluo-4-AM and *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) were bought from Molecular Probes (Eugene, OR, USA). Cell Counting Kit-8 (CCK-8, CK04-3000), Pluronic F-127 and Fluo-4-AM were obtained from Dojindo Laboratories (Tokyo, Japan). Annexin V-FITC apoptosis detection kit was from Pharmingen (Becton Dickinson Company, USA). Five primary antibodies were used: inositol 1,4,5-triphosphate receptor 1 (IP_3R-1) antibody (Sigma, SAB5200080), inositol 1,4,5-triphosphate receptor 2 (IP_3R-2) antibody (Novus Biologicals, NB100-2466), anti-p62/SQSTM1 antibody (Sigma, P0067), anti-LC3B (Sigma, L7543) and anti-β-actin antibody (Sigma, A5441). Secondary antibodies were conjugated to horseradish peroxidase (Jackson Immuno Research, 705-505-303 and 111-006-062). Calcium-free medium (CFM) was a 1:1 (v/v) mixture of Ca^{2+}-free HAM's F12 (US Biological, N8542-10) and Ca^{2+}-free DMEM high glucose (Invitrogen, 21068-028) supplemented with 1.5 mM glutamine, 22 mM sodium bicarbonate, 12.5 mM HEPES, 10 μg/mL insulin and 5.5 μg/mL transferin. Bicinchonininc acid (BCA) protein assay kit and enhanced chemiluminescence (ECL) kit were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Cadmium acetate ($CdAc_2$), propidium iodide (PI), DMEM-F_{12} (1:1) medium, Hoechst 33258, *N*-acetylcysteine (NAC), sodium borohydride ($NaBH_4$), chloroquine diphosphate salt (CQ) and all other chemicals were purchased from Sigma-Aldrich, USA.

2.2. Cell culture and Cd treatment

All procedures followed the ethics guidelines and were approved by the Animal Care and Use Committee of Shandong Agricultural University. Isolation, identification and culture of Sprague-Dawley rPT cells were as previously described [18]. Based on the doses of Cd in our previous study [13], 2.5 μM Cd was applied in this study. BAPTA-AM, TG and 2-APB were dissolved in DMSO to make the stock solution, filtered and stored at −20 °C, then diluted to work solution prior to use. The final concentration of DMSO was <0.1% and 0.1% DMSO has no effect on Ca^{2+} signaling and cell viability [19]. $CdAc_2$, CQ and NAC were dissolved in sterile ultrapure water.

2.3. Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$)

Changes of $[Ca^{2+}]_c$ were assessed by flow cytometry. The first subculture was refreshed with CFM (Ca^{2+}-free DMEM-F_{12} as described above) when it reached around 80% confluence. After 20 min adaptation, cells were exposed to 2.5 μM Cd for a time range of 0 h, 1 h and 2 h, respectively. Following the treatment (0 h, 1 h and 2 h), harvested cells were incubated with 0.5 mM TPEN at 37 °C for 10 min, loaded with 1 μM Fluo-4-AM (containing 0.02% Pluronic F-127) for 30 min in dark at 37 °C, and then washed with D-Hank's solution. Intracellular $[Ca^{2+}]_c$ levels were represented with fluorescent intensity (FL-1, 530 nm) of 10,000 cells on flow cytometer. Meanwhile, changes of $[Ca^{2+}]_c$ were determined in 2.5 μM Cd-treated rPT cells cultured in regular DMEM-F_{12} medium (containing Ca^{2+}) using the same detection method.

2.4. Determination of mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mit}$)

Our previous study has confirmed the specificity of dihydro-Rhod-2-AM to detect $[Ca^{2+}]_{mit}$ [20]. In this study, changes of $[Ca^{2+}]_{mit}$ were analyzed by flow cytometry. So Rhod-2-AM was first incubated with particular amounts of $NaBH_4$ for 10 min at 4 °C to produce dihydro-Rhod-2-AM according to the manufacturer's manual as described previously [21]. Cells were treated with TPEN, followed by incubating with 5 μM dihydro-Rhod-2-AM and 0.02% (w/v) Pluronic F-127 for 30 min at 37 °C in dark. $[Ca^{2+}]_{mit}$ was measured by the fluorescence intensity (FL-1, 585 nm) of 10,000 cells on flow cytometer.

2.5. Analysis of endoplasmic reticulum Ca^{2+} levels ($[Ca^{2+}]_{ER}$)

It has been verified that Mag-Fluo-4-AM was selectively labelled on ER, making its specificity for measuring $[Ca^{2+}]_{ER}$ [20]. After corresponding treatment, cells were incubated with 5 μM Mag-Fluo-4-AM and 0.02% (w/v) Pluronic F-127 for 30 min at 37 °C in dark. Cd was removed by treatment of TPEN as described above. 488-nm laser was used to excite Mag-Fluo-4 fluorescence and $[Ca^{2+}]_{ER}$ was calculated by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells on flow cytometer.

2.6. Determination of IP_3

Cells were plated in 100 mm culture dishes, then treated with 2.5 μM Cd when cell cultures reached 80–85% confluence. After 12 h treatment, cells were harvested, washed twice with a glucose-saline, PIPES buffer (pH 7.2, containing 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 0.1% BSA and 1 mM $CaCl_2$) and stimulated at 37 °C as indicated. The amount of IP_3 was determined according to the manufacturer's manual using a specific IP_3 [^{3}H] radioreceptor assay kit (Dupont NEN, Boston, USA). The lipid phase was counted to measure the phosphatidylinositol phosphate (PIP) lipid pool, except that the aqueous phase of the extract was passed through ultrafiltration units to exclude proteoglycans that interfere with the assay. IP_3 was expressed as a relative value of $(IP_3/PIP) \times 10^3$ (arbitrary units) to correct for the variation in the labelling of the lipid pool.

2.7. Western blotting analysis

Total protein lysates were obtained by lysing the cells with ice-cold RIPA buffer supplemented with protease inhibitors cocktail (Merck Millipore, Darmstadt, Germany). After protein quantification with BCA method, samples were subjected to SDS-PAGE gels and transferred to PVDF membranes. After blocking with 5% skim milk for 1 h at room temperature, membranes were incubated overnight at 4 °C with the following primary antibodies: IP_3R-1 (diluted 1:1000), IP_3R-2 (diluted

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