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Redistribution of subcellular calcium and its effect on apoptosis in primary cultures of rat proximal tubular cells exposed to lead

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ABSTRACT

Previous studies have shown that cytosolic Ca^{2+} ($[Ca^{2+}]_c$) overload was involved in Pb-induced apoptosis in primary cultures of rat proximal tubular (rPT) cells, but the source of elevated Ca^{2+} and the effect of potential subcellular Ca^{2+} redistribution on apoptosis are still unknown. In this study, variations of $[Ca^{2+}]_c$ in two culture media (Ca^{2+}-containing and Ca^{2+}- free) were analyzed, indicating that Pb-induced elevation of $[Ca^{2+}]_c$ was primarily generated intracellularly. Fluo-4-AM, dihydro-Rhod-2-AM and Mag-Fluo-4-AM was loaded to Pb-exposed rPT cells to monitor the imaging of Ca^{2+} concentrations in the cytoplasm ($[Ca^{2+}]_c$), mitochondria ($[Ca^{2+}]_{mit}$) and endoplasmic reticulum (ER) ($[Ca^{2+}]_{ER}$), respectively, under the confocal microscope. Data indicate that elevations of $[Ca^{2+}]_c$ and $[Ca^{2+}]_{mit}$ with depletion of $[Ca^{2+}]_{ER}$ were revealed in Pb-treated rPT cells, but this subcellular Ca^{2+} redistribution could be significantly suppressed by 2-APB, a specific inhibitor of inositol 1,4,5-trisphosphate receptor (IP_3R) that functions to release Ca^{2+} from ER stores. Simultaneously, Pb-mediated mitochondrial Ca^{2+} overload can be partially suppressed by the cytosolic Ca^{2+} chelator BAPTA-AM, suggesting that Ca^{2+} uptake into mitochondria occurs via diverse pathways and ER Ca^{2+} storage was the chief source. Furthermore, Pb-induced apoptosis was markedly inhibited by 2-APB and BAPTA-AM, respectively. Additionally, elevated IP_3 levels with up-regulated IP_3R-1 and IP_3R-2 (mRNA and protein) levels were revealed in Pb-exposed rPT cells. In summary, IP_3R-mediated ER Ca^{2+} release promoted the elevations of $[Ca^{2+}]_c$ and $[Ca^{2+}]_{mit}$ in Pb-exposed rPT cells, which played a chief role in apoptosis induced by impaired calcium homeostasis.

1. Introduction

Lead (Pb) is an important environmental and occupational pollutant that can cause serious damage to human health. Routes of exposure to Pb have been principally from contact with it in paints, fertilizers, cosmetics, automobiles, and batteries (Nevin, 2007). Pb is a multi-organ toxicant, which exerts potent toxic effects on different tissues including the liver, heart, kidney, brain and the hematopoietic system (Oyagbemi et al., 2014). As a known nephrotoxic metal, Pb-induced nephrotoxicity may occur at very low levels. Epidemiologic studies showed that a higher prevalence of chronic kidney disease at blood Pb levels of 10 μg/dl (0.48 μM) to 80 μg/dl (3.85 μM), while high blood Pb levels (>80 μg/dl) are rare nowadays (Loghman-Adham, 1997; Muntner et al., 2007; Kadir et al., 2008). Moreover, Pb accumulates primarily in the proximal tubule of the nephron, leading to renal tubular damage (Gonick, 2008). Consequently, primary cultures of rPT cells were established to investigate low-level Pb-induced nephrotoxicity in the present study.

Generally, mechanism of Pb nephrotoxicity is ascribed to the action of oxidative stress and apoptosis (Wang et al., 2009; Liu et al., 2012; Zhang et al., 2013). Our research group found that oxidative stress-mediated apoptotic death played a key role in Pb-induced nephrotoxicity in vitro. Moreover, it is important to

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester; BCA, bicinchoninic acid; CFM, calcium-free medium; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; rPT, rat proximal tubular; TPEN, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl) ethylenediamine; PI, propidium iodide; PBS, phosphate buffered saline; PIP, phosphatidylinositol phosphate; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_{mit}$, mitochondrial Ca^{2+} concentration; $[Ca^{2+}]_{ER}$, endoplasmic reticulum Ca^{2+} levels; Q-PCR, quantitative real-time PCR.

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note that Ca^{2+} plays an important role in apoptosis regulation and disturbance of intracellular Ca^{2+} homeostasis can induce apoptosis in response to a variety of pathological conditions (Zhivotovsky and Orrenius, 2011). The proapoptotic effects of Ca^{2+} are mediated by a diverse range of Ca^{2+}-sensitive factors that are compartmentalized in various intracellular organelles including the cytoplasm, ER, and mitochondria (Hajnóczky et al., 2003). ER is the most important intracellular Ca^{2+} stores, while mitochondria act as temporary sinks to maintain the intracellular Ca^{2+} levels (Hajnóczky et al., 2003). Prolonged changes in Ca^{2+} distribution including elevations in $[Ca^{2+}]_c$, $[Ca^{2+}]_{mit}$, and decreased $[Ca^{2+}]_{ER}$ can trigger a variety of signaling cascades that lead to cell death (Pinton et al., 2008). Moreover, some specific receptors are critical to maintain the Ca^{2+} homeostasis, e.g., mobilization of Ca^{2+} from ER to cytosol is regulated by the IP_3R (Zhivotovsky and Orrenius, 2011). Our previous study has demonstrated that Pb elevated the $[Ca^{2+}]_c$ in rPT cells, but the source of calcium (influx from extracellular medium and/or release from intracellular stores) was not well defined. This study will offer further evidences to clarify this question and investigate the effect of altered subcellular Ca^{2+} distribution on apoptosis in Pb-exposed rPT cells.

2. Materials and methods

2.1. Reagents

All chemicals were of highest grade purity available. 1,2-Bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) and 2-aminoethoxydiphenyl borate (2-APB) were from Tocris Bioscience (Bristol, UK). Pluronic F-127 and Fluo-4-AM were obtained from Dojindo Laboratories (Tokyo, Japan). Dihydro-rhod-2-AM, Mag-Fluo-4-AM, ER-tracker Red Mito-tracker Green and *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) were purchased from molecular probes (Eugene, OR, USA). Annexin V-FITC apoptosis detection kit was from Pharmingen (Becton Dickinson Company, USA). Three 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) 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 transferrin. BCA protein assay kit and enhanced chemiluminescence (ECL) kit were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). DMEM-F12 (1:1) medium, sodium borohydride ($NaBH_4$), lead acetate ($PbAc_2$) and all other chemicals were purchased from Sigma–Aldrich, USA.

2.2. Cell isolation, culture and treatment

Isolation, identification and culture of Sprague-Dawley rat proximal tubular (rPT) cells were as previously described (Wang et al., 2009). Based on the doses of Pb in our previous study, cell cultures were incubated in the presence of 0, 0.25, 0.5, and 1 μM Pb. BAPTA-AM (cytosolic Ca^{2+} chelator) and 2-APB (an inhibitor of IP_3R) 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 less than 0.1% and 0.1% DMSO has no effect on Ca^{2+} signaling (Takenouchi et al., 2005). Meanwhile, 0.1% DMSO, 2-APB and BAPTA-AM have no significant toxic effects on cells, as confirmed in this study (data not shown).

2.3. Quantification of apoptosis by flow cytometry

The apoptotic cell distribution was analyzed by annexin V/PI double staining method, which has been extensively described in our previous study (Wang et al., 2009). Cells were pretreated with 10 μM BAPTA-AM for 30 min, followed by treatment with various Pb doses (0.25, 0.5 and 1 μM) for another 12 h. In addition, 10 μM 2-APB was co-incubated with Pb for 12 h to assess its effect on apoptosis. After the treatment, the harvested cells were washed twice with PBS and incubated with annexin V/PI for the flow cytometric analysis.

2.4. Analysis of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$)

Firstly, changes of $[Ca^{2+}]_c$ were analyzed by confocal microscopy. Cells on coverslips exposed to 12-h Pb (0.25, 0.5 and 1 μM) treatment under regular DMEM- F12 medium (containing Ca^{2+}) were incubated with 0.5 mM of TPEN (lead chelator) at 37 °C for 10 min to discount Pb interference in the fluorescence signal, because Ca^{2+} and Pb^{2+} interact similarly with the Fluo-4-AM dye, and then loaded with 2.5 μM Fluo-4-AM for 30 min in dark at 37 °C. After washing with D-Hank's solution, fixed cells were imaged on the Zeiss LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany). Fluo-4 was excited at 488 nm using an argon laser line and emitted fluorescence was collected through a 530 nm band-pass filter. $[Ca^{2+}]_c$ was calculated by LSM imaging software (Zeiss, Oberkochen, Germany). Secondly, changes of $[Ca^{2+}]_c$ were assessed by flow cytometry. The first subculture of cells was refreshed with CFM (Ca^{2+}-free DMEM-F12 as described above) when it reached around 80% confluence. After 20 min adaptation, cells were exposed to 0.5 μM Pb 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 of 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 0.5 μM Pb-treated rPT cells cultured in regular DMEM-F12 medium (containing Ca^{2+}) for the same time intervals (0 h, 1 h, 2 h).

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

The dihydro-rhod-2-AM is a membrane-permeable form of Ca^{2+}-sensitive dye selectively accumulated in mitochondria, which enables it to be used to measure mitochondrial Ca^{2+} levels. 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 (Fukumori et al., 2010). Then Pb-exposed cells on coverslips 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. To confirm the mitochondrial localization of the rhod-2 probe, cells were also labeled with 1 μM Mito-tracker Green for 20 min, and then visualized by the confocal microscope (as described above). Laser lines of 488 nm and 543 nm was used to excite Mito-tracker Green and dihydro-rhod-2-AM, respectively, and $[Ca^{2+}]_{mit}$ was calculated by LSM imaging software (Zeiss, Oberkochen, Germany).

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

Given that the approximate ER Ca^{2+} levels in most types of cells is in the range of 100–1000 μM, the optimal dissociation constant (Kd) for measuring $[Ca^{2+}]_{ER}$ is between 22 and 250 μM (Park et al.,