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CaMKII mediates cadmium induced apoptosis in rat primary osteoblasts through MAPK activation and endoplasmic reticulum stress

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ABSTRACT

Ca^{2+} is an important ion in various intracellular metabolic pathways. Endoplasmic reticulum (ER) is a major intracellular calcium store and ER calcium homeostasis plays a key part in the regulation of apoptosis. We have previously shown that Cadmium (Cd) induces apoptosis in osteoblasts (OBs), accompany by increased cytoplasmic calcium. As the role of calcium in OBs apoptosis induced by Cd has not been clarified we investigated the effects of Cd exposure in rat OBs on intracellular Ca^{2+}, CaMKII phosphorylation, and the pathways implicated in inducing apoptosis. The results showed that cadmium(Cd) induced elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in OBs by the release of Ca^{2+} from ER and the inflow of Ca^{2+} from the extracellular matrix. Cd induced $[Ca^{2+}]_i$ elevation and phosphorylation of CaMKII which might be involved in activation of MAPKs and participated in Cd-induced mitochondrial apoptosis through the alteration of the ratio of Bax/Bcl-2 expression. Meanwhile, CaMKII phosphorylation activated unfolded protein response (UPR) during cadmium treatment and could enable the ER apoptosis pathway through the activation of caspase-12. These results indicated that CaMKII plays an important role in Cd induced ER apoptosis and MAPK activation. Our data provide new insights into the mechanisms underlying apoptosis in OBs following Cd exposure. This provides a theoretical basis for future investigations into the clinical therapeutic application of CaMKII inhibitors in osteoporosis induced by Cd exposure.

1. Introduction

Cadmium (Cd) is a toxic environmental contaminant which exerts adverse effects on most organ systems due to its protracted biological half-life or approximately 20–30 years in humans and its low rate of excretion from the body. In 1955 Cd was firstly defined as the cause of Itai-Itai disease in a Japanese population consuming Cd contaminated rice(Nishijo et al., 2017). In recent decades, more investigations have indicated that environmental cadmium exposure in the general population is associated with osteoporosis and bone fractures (Scallan et al., 2011). Poor bone health is an important outcome resulting from Cd pollution and osteoblasts (OBs) is the main targets of Cd toxicity.

At a cellular level, Cd could interact with various cellular events, including proliferation, differentiation, and cell death (Waisberg et al., 2003). Many studies have found that Cd can induce apoptosis in several different organs and tissues, including the kidney (Tattoli et al., 2012), hepatocytes (Okoro et al., 2015), neurons (Yuan et al., 2013), and thymocytes (Pathak et al., 2013). Our previous study demonstrated that Cd could also induce apoptosis in OBs (Liu et al., 2014). However, the mechanisms through which cadmium induces apoptosis are still to be clarified.

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) can precisely regulate many essential cellular events. The storage, release, and uptake of Ca^{2+} in all non-muscle cells is regulated by the endoplasmic reticulum (ER) (Bai et al., 2008). The concentration of $[Ca^{2+}]_i$ is very low in normal physiological conditions, and the concentration of Ca^{2+} in the extracellular matrix is ten thousand times, and ER is one thousand times, greater that of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ is precisely regulated by calcium pumps (Ca^{2+}-ATPase), ryanodine receptors (RyR), and the inositol-1,4,5-triphosphate receptor (IP_3R). Calcium pumps in mammals are mainly distributed in cytoplasmic and nuclear membrane, three isomers of RyR are mainly distributed in skeletal muscle (RyR1), cardiac muscle (RyR2), and brain

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(RyR3) but RyR are not distributed in bone (Bai et al., 2008). Therefore, ER homeostasis is closely related to IP_3R, the Ca^{2+} release channel, in OBs. As a fendamental molecule in calcium signaling, calmodulin-dependent II protein kinase II (CaMKII) plays an important role in proliferation and differentiation of OBs. Studies have shown that CaMKII could participate in apoptosis induced by Cd through various pathways, including acceleration of ER dysfunction via phosphorylation of IP_3R (Bai et al., 2008), and activation of MAPKs and mTOR pathways (Owen et al., 2014). In recent years, it has been found that CaMKII plays an essential role in the regulation of insulin resistance, cardiovascular disease (Bracken et al., 2016) and pulmonary fibrosis (Bravo et al., 2013) by regulating multiple apoptosis pathways. However, there have been few studies investigating the role of CaMKII in bone injury induced by Cd, while the upstream and downstream pathways and the target of CaMKII have not been fully elucidated.

Apoptosis is a process of programmed cell death accompanied by the activation, expression, and regulation of a variety of enzymes and genes. There are three apoptosis pathways, including the exogenous apoptosis pathway (death receptor pathway), endogenous apoptosis pathway (mitochondrial pathway), and the endoplasmic reticulum stress pathway. The first two are classical apoptotic pathways while the endoplasmic reticulum stress pathway has only recently been fully characterized. At present, most studies on the endoplasmic reticulum stress induced apoptosis pathway have focused on cell lines (Ha et al., 2016; Hague et al., 2000), the mechanism of endoplasmic reticulum stress mediated apoptosis induced by Cd in primary cells has not been reported. ER is the site of protein folding and aggregation, while also being the principal site of $[Ca^{2+}]_i$ storage. Protein misfolding, Ca^{2+} depletion, or oxidative stress in ER cavity can disrupt ER function resulting in ER stress response (Hu et al., 2016).

There are three main signal transduction factors on the ER membrane which sense endoplasmic reticulum stress including protein kinase R-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1). Endoplasmic reticulum stress can result in death signals through three ways. ATF4 and ATF6 can induce apoptosis by CCAAT/enhancer binding protein homologous protein (CHOP), the interaction between tumor necrosis factor receptor associated factor 2 (TRAF2). IRE1 can induce apoptosis by activation of caspase-12 on the membrane of ER. Finally, the IRE-TRAF2 interaction can recruit and activate apoptosis signal-regulating 1 kinase (ASK1) and its downstream c-Jun N-terminal kinase (JNK), thus participating in a variety of pro-apoptosis pathways, including induction of apoptosis by phosphorylation of Bcl-2 (Bravo et al., 2013). The ER membrane is rich in Bcl-2 family proteins, including anti-apoptotic proteins Bcl-2 and Bcl-xL, and pro-apoptosis proteins Bax and Bak, which play important role in the regulation of Ca^{2+} release from ER and apoptosis (Chotpantarat et al., 2014). Firstly, high expression of Bcl-2 inhibits Ca^{2+} release from the ER to the mitochondria, thus maintaining ER calcium homeostasis. In contrast, high expression of Bax and Bak protein can promote Ca^{2+} release from the ER to mitochondria resulting in mitochondrial calcium overload, which releases cytochrome C (cyt. C), leading to the induction of apoptosis (Khuzestani and Souri, 2013). Secondly, overexpression of Bcl-2, Bcl-xL, and knockout of both Bax and Bak can significantly reduce the phosphorylation of IP_3R and the Ca^{2+} output of ER (Chotpantarat et al., 2014). Therefore, the ratio of Bax/Bcl-2 determines the flow of Ca^{2+} and phosphorylation of IP_3R in ER, which is involved in the regulation of ER stress mediated apoptosis (Chotpantarat et al., 2014; Greenberg et al., 2014) and ER stress plays a central role in the regulation of the process of apoptosis.

ER calcium homeostasis is mainly mediated by the calcium channel and calmodulin. Calcium imbalance in ER would induce apoptosis mediated by $[Ca^{2+}]_i$ overload. Our previous study found that low concentration of Cd resulted in OBs toxicity, whereas $[Ca^{2+}]_i$ overload is one of the prominent apoptosis signals. $[Ca^{2+}]_i$ overload activated the mitochondrial apoptosis pathway by calcium-dependent calmodulin (Liu et al., 2014). We also found that Cd would activate the MAPKs pathway (Zhao et al., 2015). These results indicated that there is a connection between Cd-induced apoptosis and ER stress induce by $[Ca^{2+}]_i$ overload in OBs. In this study, we aimed to investigate the role of $[Ca^{2+}]_i$ in ER stress mediated apoptosis induced by Cd using primary OBs. We hypothesized that both $[Ca^{2+}]_i$ and CaMKII play a major role in mediating the effects of Cd toxicity in OBs.

2. Materials and methods

2.1. Reagents

Cadmium acetate ($CdAc_2$), Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2-aminoethoxydiphenyl borane (2-APB) (IP3 receptor antagonist), Thapsigargin (TG) (non-competitive inhibitor of the ER Ca^{2+} ATPase), and Hoechst 33258 were purchased from Sigma-Aldrich (St. Louis, MO, USA). KN-93 (CaMKII inhibitor) was purchased from Cayman Chemical (Ann Arbor, MI, USA). The Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Trypsin was obtained from Amresco (Solon, OH, USA). Antibodies against Erk1/2, phospho-Erk1/2 (Thr202/Tyr204), JNK, phospho-JNK (Thr183/Tyr185), p38, phospho-p38 (Thr180/Tyr182), BiP, ATF-4, CHOP, cleaved-caspase-3, Bax, Bcl-2, and β-actin were purchased from Cell Signaling Technology (Boston, MA, USA). Antibody against phospho-CaMKII (Thr286) was purchased from Abcam (Cambridge, MA, USA). Antibody against cleaved-caspase-12 was purchased from Biovision (Milpitas, CA, USA). Anti-beclin 1, horseradish peroxidase, and (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FITC-conjugated anti-rabbit IgG was purchased from Bioworld (Minneapolis, MN, USA). Fluo-4/AM and Enhanced chemiluminescence (ECL) solution was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Cell isolation and cell culture conditions

Sprague–Dawley rats used in this study were purchased from the Laboratory Animal Center at Yangzhou University, Yangzhou, China. This study was approved by the Animal Care and Use Committee of Yangzhou University and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Cranial OBs were obtained from Sprague-Dawley rat fetuses aged between 18 to 19 days old. The calvarias were incubated with 0.25% (w/v) trypsin at 37 °C for 10 min, then cut into slices and incubated with 0.1% collagenase at 37 °C for 40 min. The isolated primary OBs were cultured with DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 for 10–12 d (Liu et al., 2014). The phenotype was determined using an alkaline phosphatase (ALP) staining kit according to the manufacturer's protocol (Beyotime Institute of Biotechnology, Jiangsu, China).

2.3. Measurement of cell viability

Cell activity was monitored using the RTCA system (Roche, Mannheim, Germany), adhered cell degree was detected to reflect cell activity. The background level was initially determined by loading 100 μl/well of culture medium (DMEM with 10% FBS) into a 16-well E-plate. Changes in OBs activity were monitored by seeding approximately 10,000 cells/well in the E-plate and culturing for 14 h in order for the cells to adhere and reach their proliferative phase. The impedance was measured every minute for the first hour and then every 15 min from 2 to 14 h (Liu et al., 2014).

In our previous studies (Zhao et al., 2015), we used MMT assay to