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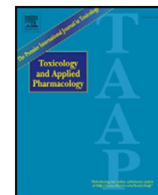

Cadmium induces autophagy through ROS-dependent activation of the LKB1–AMPK signaling in skin epidermal cells

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ABSTRACT

Cadmium is a toxic heavy metal which is environmentally and occupationally relevant. The mechanisms underlying cadmium-induced autophagy are not yet completely understood. The present study shows that cadmium induces autophagy, as demonstrated by the increase of LC3-II formation and the GFP-LC3 puncta cells. The induction of autophagosomes was directly visualized by electron microscopy in cadmium-exposed skin epidermal cells. Blockage of LKB1 or AMPK by siRNA transfection suppressed cadmium-induced autophagy. Cadmium-induced autophagy was inhibited in dominant-negative AMPK-transfected cells, whereas it was accelerated in cells transfected with the constitutively active form of AMPK. mTOR signaling, a negative regulator of autophagy, was downregulated in cadmium-exposed cells. In addition, cadmium generated reactive oxygen species (ROS) at relatively low levels, and caused poly(ADP-ribose) polymerase-1 (PARP) activation and ATP depletion. Inhibition of PARP by pharmacological inhibitors or its siRNA transfection suppressed ATP reduction and autophagy in cadmium-exposed cells. Furthermore, cadmium-induced autophagy signaling was attenuated by either exogenous addition of catalase and superoxide dismutase, or by overexpression of these enzymes. Consequently, these results suggest that cadmium-mediated ROS generation causes PARP activation and energy depletion, and eventually induces autophagy through the activation of LKB1–AMPK signaling and the down-regulation of mTOR in skin epidermal cells.

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Introduction

Cell death is generally classified into three categories: apoptosis, autophagy, and necrosis (Kitanaka and Kuchino, 1999). Apoptosis and autophagy are controlled tightly by a regulatory mechanism and these types of cell death play a central role in tissue homeostasis, development, and disease. The best known mode of cell death is apoptosis (Shimada et al., 1998; Waalkes et al., 2000), characterized by cellular shrinkage, nuclear condensation, and DNA fragmentation (Kroemer and Reed, 2000; Li et al., 1997; Susin et al., 1999). Biochemical changes such as the activation of caspases and/or endonucleases are also important characteristics of apoptosis (Arends et al., 1990; Patel et al., 1996). Autophagy is a cellular defense process in which cytosolic components, organelles, and invading bacteria are transported by autophagosomes to lysosomes for degradation (Dice, 2007; Levine and Klionsky, 2004; Mizushima, 2007; Muller et al., 2000). Thus, autophagy is evidenced by the early appearance of large inclusions in the cytoplasm derived from autophagic vacuoles or autolysosomes. Such autophagy could be caused by starvation, cytoplasmic renewal, elimination of intracellular components and pathogens, innate and acquired immune responses, and programmed cell death (Hara et al., 2006; Komatsu et al., 2006; Nakagawa et al., 2004; Paludan et al., 2005; Pattingre et al., 2005).

Beclin 1 and microtubule-associated protein 1 light chain 3 (LC3) are the critical components in autophagy. Beclin 1 is the mammalian orthologue of the yeast Atg6/Vps 30, and is involved in the regulation of autophagy (Liang et al., 1999; Qu et al., 2003; Tassa et al., 2003; Yue et al., 2003; Zeng et al., 2006). LC3 is the mammalian homologue of yeast Atg8 and localizes to autophagosomal membranes after post-translational modifications. LC3 exists in two molecular forms; LC3-I (18 kDa) is cytosolic form, whereas LC3-II (16 kDa) binds to autophagosomes (Kabeya et al., 2000; Mizushima et al., 2001). The

Abbreviations: ROS, reactive oxygen species; PARP, poly (ADP-ribose) polymerase; LC3, microtuble-associated protein 1 light chain 3; AMPK, AMP-activated protein kinase; PJS, Peutz–Jeghers syndrome; EMEM, Eagle's minimal essential medium; FBS, Fetal bovine serum; DPQ, 3,4-Dihydro-5[4-(1-peperindinyl)butoxy]-1(2H)-isoquinoline; 3-MA, 3-Methyladenine; CA-AMPK, constitutively active forms of AMPKα; DN-AMPK, Dominant negative forms of AMPKα; SOD, Superoxide dismutase; CAT, Catalase; si-RNA, Small interference RNA; SSB, Single-strand break; 8-OHdG, 8-hydrodeoxyguanosine; PI, Propidium iodide; ESR, Electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; MDC, Monodansylcadaverine; ACC, Acetyl CoA carboxylase; mTOR, Mammalian target of rapamycin; MMP, Mitochondrial membrane potential.

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amount of LC3-II directly correlates with the number of autophagosomes (Kabeya et al., 2000).

The energy-sensing LKB1–AMP-activated protein kinase (AMPK) pathway regulates cell survival under energy deprivation which increases AMP: ATP ratio (Lizcano et al., 2004; Shaw et al., 2004). AMPK is a heterotrimeric protein complex consisting of AMPK-α, AMPK-β, and AMPK-γ subunits. AMPK is downstream of LKB1 in a signaling pathway that regulates energy homeostasis (Hardie, 2004; Shaw et al., 2004; Woods et al., 2003). LKB1 was identified as the gene mutated in human Peutz–Jeghers syndrome (PJS) (Hemminki et al., 1998; Jenne et al., 1998) and necessary for the activation of AMPK (Hawley et al., 2003; Shaw et al., 2004). Changes in cellular AMP/ATP ratios promote allosteric interaction between AMP and AMPK-γ subunit, which promotes phosphorylation of AMPK-α subunit at T172 and activation of AMPK signaling (Andersson et al., 2004; Hardie, 2004; Sanders et al., 2007). Moreover, AMPK has been implicated in many aspects of cell proliferation, apoptosis, and autophagy (Liang et al., 2007; Luo et al., 2005; Motoshima et al., 2006).

Cadmium is a toxic heavy metal and human carcinogen. Food chain, cigarette smoke, and cadmium mining industry are the main sources of cadmium exposure to humans (Jarup, 2003). Cadmium induces either apoptosis or carcinogenesis depending on the conditions such as concentrations and times exposed. It was recently reported that cadmium induced autophagy in mesangial cells (Wang et al., 2008; Wang et al., 2009). Further, cadmium-induced increase of intracellular ROS is involved in cell death caused by this metal (Son et al., 2010b). These findings suggest that in addition to the induction of apoptosis, cadmium leads to autophagic cell death. However, the cellular mechanisms by which cadmium causes autophagy have not been extensively explored. Little information is available about the relationship between intracellular ROS generation and autophagy. We used mouse JB6 epidermal cell lines to study the autophagy response to cadmium. JB6 cells are widely used and well studied in carcinogenesis induced by tumor promoters and metals as well as oxidative stress (Dhar et al., 2002). Therefore, the present study using these cells will contribute to further understanding of cadmium mediated toxicity and carcinogenicity. The concentrations of cadmium which were used in our experiments were 1 to 10 μM. These concentrations are 10 to 100 times higher than the blood levels (15 μg/l) of industrial workers who are occupationally exposed to cadmium (Glahn et al., 2008). However, cadmium has a long biological half-life (15–20 years) and accumulates in various organs such as the liver, kidneys, lung, bone, and eyes (Henson and Chedrese, 2004; Jin et al., 1998). Thus, the concentrations used in the present study are highly relevant to occupational exposure.

The present study examined whether cadmium actually causes autophagic cell death using the mouse epidermal cell line, JB6. We also determined the possible roles of intracellular ROS generated by cadmium on the process of autophagy. In addition, we investigated the molecular mechanisms involved in cadmium-induced autophagy. Here we demonstrate the critical roles of ROS-mediated signaling and the involvement of LKB1–AMPK signaling pathways in cadmium-induced autophagy.

Materials and methods

Chemicals and laboratory wares. Unless specified otherwise, all chemicals and laboratory wares were purchased from Sigma Chemical Co. (St. Louis, MO) and Falcon Labware (Bectone-Dickinson, Franklin Lakes, NJ), respectively. Eagle's minimal essential medium (EMEM), fetal bovine serum (FBS), gentamicin, geneticin, and L-glutamine were purchased from Gibco Co. (Gibco BRL, NY). 3,4-Dihydro-5[4-(1-peperindinyl)butoxy]-1(2H)-isoquinoline (DPQ) and 3-Methyladenine (3-MA) were purchased from Calbiochem (San Diego, CA).

Cell culture and treatment. JB6 mouse epidermal cells (ATCC, CRL-2010) were cultured in EMEM supplemented with 5% FBS, 2 mM L-glutamine, and 50 μg/ml of gentamicin. GFP-LC3 JB6 cells generated by stable transfection of an expression vector encoding for the LC3-GFP fusion protein were maintained in EMEM with 5% FBS and 800 μg/ml of G418. The cells were grown at 37 °C in a 5% CO_2 atmosphere. One million cells per milliliter were resuspended in the growing medium and divided into 60 mm culture dishes or 96-well plates. When the cells reached to 80% confluence, the medium was replaced with a fresh EMEM containing various concentrations of $CdCl_2$ (0–10 μM) and chemicals.

Plasmids and transfection. GFP-LC3 plasmid was provided from Dr. Jia Luo (University of Kentucky). Constitutively active forms of AMPKα (CA-AMPK) and dominant negative forms of AMPKα (DN-AMPK) constructs were gifted from Dr. J. Suttles (University of Louisville). CAT-Myc-DDK-, SOD1-Myc-DDK-tagged plasmids were purchased from Origene (Rockville, MD) and SOD2-EGFP-tagged plasmid came from Addgene (Cambridge, MA). Transfections were performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. Briefly, JB6 cells were seeded in 6-well culture plates and transfected with 4 μg of plasmids at approximately 50% confluency. Transfected cells with GFP-LC3 were examined by fluorescence microscope. Overexpressed or knockdown cellular levels of the proteins specific for AMPKα, catalase (CAT), superoxide dismutase-1 (SOD1), and SOD2 were checked by immunoblotting, and the cells were maintained using G418 for stable cell lines.

Electron microscopy. To morphologically observe the induction of autophagy in cadmium-treated JB6 cells, we performed electron microscopy analysis as described elsewhere (Paglin et al., 2001). After being treated with cadmium (10 μM) for 24 h, cells were washed twice with PBS and fixed with ice-cold glutaraldehyde (3.5% in 0.1 M Sorenson's buffer, pH 7.4) for 30 min at 4 °C. Cells were postfixed in 1% osmium tetroxide (OsO_4) for 30 min at 4 °C and embedded in LX 122 before being cut and stained with uranyl acetate/lead citrate. Finally the cells were observed using a Hitachi H600 electron microscope (Hitachi Instrument, Tokyo, Japan).

GFP-LC3 localization. The GFP-LC3 puncta were quantified by counting the number of cells as described elsewhere (Alexander et al., 2010). Briefly, JB6 cells stably transfected with GFP-LC3 construct were divided on coverslips plated on 6-well plates (0.2×10^6/coverslip). Cells were exposed to cadmium (0–10 μM) with or without various activators or inhibitors for 24 h and fixed in ice-cold methanol. Total 50 GFP positive cells were counted under a fluorescence microscopy (Carl Zeiss, Germany), and cells with more than five puncta were considered autophagic GFP-LC3 puncta cells.

Western blot analysis. Cell lysates were made in a lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.25% Na-

Fig. 1. Cadmium induces autophagy in JB6 cells. (A) The cells were exposed to the increasing concentrations (0–10 μM) of cadmium for 24 h. (B) Cells were exposed to 10 μM cadmium for various times (0–24 h). Protein lysates were analyzed by a NuPAGE Bis-Tris electrophoresis system, and the levels of LC3-I and LC3-II were detected by Western blot analysis. (C) JB6 cells stably transfected with GFP-LC3 were treated with cadmium for various concentrations (0–10 μM) and visualized by microscopy for fluorescent puncta. The number of GFP-LC3 puncta positive cells were counted and presented at indicated concentrations (D) and times (E). (F) JB6 cells treated with or without cadmium for 24 h were analyzed on electron microscopy. Arrows indicate autophagosomes. In addition, before treated with cadmium (10 μM), the cell were pre-incubated with 3-MA (5 mM) for 1 h. After incubation 24 h, a fluorescence of GFP-LC3 puncta cells were visualized by microscopy (G) and the number of GFP-LC3 puncta positive cells were counted and presented on graph (H). β-actin was used as an internal control. The results are shown as the mean ± SE of three separate experiments. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ versus the control value or the cadmium treatment alone (ANOVA, Scheffe's test).

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