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MAPK/JNK1 activation protects cells against cadmium-induced autophagic cell death via differential regulation of catalase and heme oxygenase-1 in oral cancer cells

Keum-Young So [a], Sang-Hun Kim [a], Ki-Tae Jung [a], Hyun-Young Lee [a], Seon-Hee Oh [b,*]

[a] Department of Anesthesiology and Pain Medicine, Chosun University, 309 Pilmundaero, Dong-gu, Gwangju 61452, Republic of Korea
[b] School of Medicine, Chosun University, 309 Pilmundaero, Dong-gu, Gwangju 61452, Republic of Korea

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ABSTRACT

Antioxidant enzymes are related to oral diseases. We investigated the roles of heme oxygenase-1 (HO-1) and catalase in cadmium (Cd)-induced oxidative stress and the underlying molecular mechanism in oral cancer cells. Exposing YD8 cells to Cd reduced the expression levels of catalase and superoxide dismutase 1/2 and induced the expression of HO-1 as well as autophagy and apoptosis, which were reversed by *N*-acetyl-L-cysteine (NAC). Cd-exposed YD10B cells exhibited milder effects than YD8 cells, indicating that Cd sensitivity is associated with antioxidant enzymes and autophagy. Autophagy inhibition via pharmacologic and genetic modulations enhanced Cd-induced HO-1 expression, caspase-3 cleavage, and the production of reactive oxygen species (ROS). Ho-1 knockdown increased autophagy and apoptosis. Hemin treatment partially suppressed Cd-induced ROS production and apoptosis, but enhanced autophagy and CHOP expression, indicating that autophagy induction is associated with cellular stress. Catalase inhibition by pharmacological and genetic modulations increased Cd-induced ROS production, autophagy, and apoptosis, but suppressed HO-1, indicating that catalase is required for HO-1 induction. p38 inhibition upregulated Cd-induced phospho-JNK and catalase, but suppressed HO-1, autophagy, apoptosis. JNK suppression exhibited contrary results, enhancing the expression of phospho-p38. Co-suppression of p38 and JNK1 failed to upregulate catalase and procaspase-3, which were upregulated by JNK1 overexpression. Overall, the balance between the responses of p38 and JNK activation to Cd appears to have an important role in maintaining cellular homeostasis via the regulation of antioxidant enzymes and autophagy induction. In addition, the upregulation of catalase by JNK1 activation can play a critical role in cell protection against Cd-induced oxidative stress.

1. Introduction

Cadmium (Cd) originates from natural sources, as well as industrial and agricultural uses, and is a ubiquitous environmental contaminant. The main sources of Cd exposure to humans are inhalation of air, ingestion of contaminated food and water, and smoking (Waisberg et al., 2003). Cd causes nephrotoxicity, osteotoxicity, lung toxicity, and hepatotoxicity, and is implicated in the development of cancer (Waisberg et al., 2003; Rani et al., 2014). Therefore, Cd is classified as a type I carcinogen by the International Agency for Cancer Research (IARC, 1993).

Cells are continuously attacked by intra- and extracellular reactive oxygen species (ROS) produced in response to environmental stresses. Normally, ROS are detoxified by nonenzymatic or enzymatic antioxidants, including glutathione, superoxide dismutase (SOD), catalase, and peroxidase (Valko et al., 2007). However, ROS can accumulate due to an imbalance between ROS production and antioxidant levels, triggering signaling cascades for various cell death modes, such as apoptosis, autophagy, and necrosis (Templeton and Liu, 2010; Messner et al., 2016). Mounting evidence suggests that Cd toxicity is driven by ROS-mediated oxidative stress. Because Cd is unable to directly generate ROS, Cd-induced oxidative stress indirectly occurs via the disruption of various antioxidant enzymes (Waisberg et al., 2003; Nemmiche, 2017). Thus, to understand Cd cytotoxicity, it may be important to elucidate the mechanisms of antioxidant enzyme regulation. Nuclear factor-erythroid 2-related factor 2 (Nrf2α) dissociates from cytosolic repressor Kelch-like ECH-associated protein 1 (Keap1) and is

Abbreviations: HO-1, heme oxygenase-1; Cd, cadmium; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species; JNK, c-jun N-terminal kinase; SOD, superoxide dismutase; OSCC, oral squamous cell carcinoma; BaF1, bafilomycin A1; CQ, chloroquine; 3-AT, 3-amino-1,2,4-triazole; PI, propidium iodide; MTT, 3-(4,5-dimetylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; DCFH-DA, dichloro-dihydro-fluorescein diacetate; siRNA, short interfering RNA; LC3, microtubule-associated protein 1A/1B-light chain; PARP-1, poly (ADP-ribose) polymerase 1; IC_{50}, half maximal inhibitory concentration; keap1, Kelch-like ECH-associated protein 1; FACS, fluorescence-activated cell sorting.

* Corresponding author.
E-mail address: seonh@chosun.ac.kr (S.-H. Oh).

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translocated into the nucleus under oxidative stress, where it regulates the transcription of antioxidants, including HO-1, catalase, and SOD (Waisberg et al., 2003; Ma, 2013), and glutathione levels (Cullinan and Diehl, 2004). Alternative mechanisms of Nrf2α regulation by various protein kinases (PKC, Akt, GSK-3β, and MAPK) have been reported (Nemmiche, 2017). HO-1 catabolizes pro-oxidant free heme into ferrous ion (Fe^{2+}), carbon monoxide, and biliverdin, which is further processed into bilirubin (Maines, 1988). Although all three products are involved in cellular protection, Fe^{2+} as a pro-oxidant is debatable (Wang et al., 2016). Catalase is a tetramer composed of subunits containing a heme group and is involved in the conversion of H_2O_2 into nontoxic oxygen and water. Thus, catalase deficiency or inhibition enhances cytotoxicity via H_2O_2 (Gill et al., 1990). By contrast, antioxidant enzymes may be involved in cell death. For example, hemin- and adenovirus-mediated HO-1 induction induces caspase-3 activation (Liu et al., 2002; Lang et al., 2005). Furthermore, catalase is required for the persistent activation of the apoptotic JNK signal via Cd exposure in human lung cancer cells Chuang et al. (2003). However, the molecular mechanisms involved in the regulation of antioxidant enzymes and their roles in Cd-induced cytotoxicity are not well understood.

Regarding oral effects, Cd increases the risk of dental caries and affects salivary gland function (Abdollahi et al., 2003; Arora et al., 2008). An etiological study revealed that tobacco smoking is a main cause of oral cancer, and about 90% of oral cancer patients are tobacco users. Smoking can damage epithelial cells in the oral cavity and oropharynx, and DNA-damaging chemicals in tobacco have been linked to increased risk of oral cancer (Kolanjiappan et al., 2003). Various antioxidant enzymes are associated with oral diseases (Fan et al., 2011). HO-1 plays an important role in diverse oral diseases, including oral cancer via antiapoptotic effects (Min et al., 2006, 2008). SOD and catalase expression are significantly lower in tissue samples of oral squamous cell carcinoma (OSCC) patients than in normal tissues (Gokul et al., 2010). Previously, we found that OSCC cell lines YD8 and YD10B induced HO-1 expression in response to Cd, which was involved in cell protection via the regulation of autophagy and endoplasmic reticulum (ER) stress-induced apoptosis (So and Oh, 2016). However, the underlying molecular mechanisms of Cd-induced oral cytotoxicity remain unclear. Therefore, in this study, we have investigated the responses of two representative antioxidant enzymes, catalase and HO-1, to Cd-induced cytotoxicity in YD8 and YD10B cells showing different sensitivity to Cd, and the molecular mechanisms underlying the regulation of antioxidant enzymes during Cd-cytotoxocity.

2. Materials and methods

2.1. Cell culture and chemicals

Human oral squamous cell carcinomas (OSCC), that is, YD8 and YD10B cells, were maintained in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with heat-inactivated 10% fetal bovine serum, 50 μg/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a 5% CO_2–95% air humidified incubator. Bafilomycin A1, 3-amino-1,2,4-triazole (3-AT), chloroquine, propidium iodide (PI), and MTT [3-(4,5-dimetylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma (St. Louis, MO). DCFH-DA was obtained from Molecular Probes (Eugene, OR). z-VAD-fmk was purchased from Calbiochem (Darmstadt, Germany), and Hemin was from Enzo (Playmouth, PA, USA). Other chemicals used were of the purest grade available from Sigma (St. Louis, MO).

2.2. Cytotoxicity assays

Cell viabilities were determined using MTT assays. In brief, cells were suspended in complete media, at a concentration of 1×10^5 cells/ml, and samples (200 μl) of cell suspensions were seeded onto 48-well plates and cultured overnight. After treatment with chemicals, cells were incubated with MTT (0.5 mg/ml) for 4 h, the formazan crystals formed were dissolved in dimethyl sulfoxide, and absorbances were measured at 540 nm using an ELISA microplate reader (Perkin-Elmer).

2.3. Flow cytometric analysis

Cells were harvested and washed twice with cold phosphate buffered saline (PBS). To analyze the percentage of nuclei with hypodiploid content (sub-G1), nuclei were stained by using PI (1 mg/ml) and at least 20,000 events were analyzed by a FACScan and Kaluza Analysis 1.5a (Beckman Coulter).

2.4. ROS detection in cells by fluorescence microscopy

ROS formation in cells was detected by fluorescence microscopy. Cells were seeded in 35-mm dishes, performed experiments, and loaded with dichloro-dihydro-fluorescein diacetate (DCFH-DA, Molecular Probes) for 30 min in complete medium at 37 °C in a 95% air-5% CO_2 incubator. After incubation, the medium containing DCFH-DA was aspirated, cells were washed twice with complete medium, and examined under a Nikon Eclipse TE 300 microscope with excitation and emission set at 490 and 530 nm, respectively. Fluorescence of oxidized DCFDA in cells was captured with RS photometrics coolsnap-pro color digital imaging camera.

2.5. Transfection and gene silencing

Cells were transduced with control short interfering RNA (siRNA) and siRNAs for Atg5, JNK1, p38, HO-1, and pcDNA3.1 and JNK1 as previously described (So and Oh, 2016; Jung et al., 2015).

2.6. Immunoblot analysis

Immunoblot analysis was performed as described previously (Jung et al., 2015). Antibodies were as follows: anti-LC3B, cleaved caspase-3, p-JNK, JNK, p-p38, p38, Atg5 and cleaved caspase-3 were obtained from Cell Signaling Technology (Beverly, MA, USA). HO-1 and catalase were purchased from Enzo and calbiochem (San Diego, CA, USA), respectively. Anti-procaspase-3, p62, CHOP, and GAPDH were Santa Cruz Biotechnology (Santa Cruz, CA, USA). To verify equal loadings of proteins, the blots were stripped using 10% SDS, 25 nM glycine (pH 2.0) for 30 min and washed with phosphate buffer containing 0.3% Tween 20, and followed by reprobing with GAPDH. Densitometry of bands in Western blot was analyzed by ImageJ program (National Institutes of Health, Bethesda, USA). The relative amount of each protein was normalized to the ratio of GAPDH.

2.7. Statistical analysis

All experiments were repeated at least three times, and values are expressed as means ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test, or one-way ANOVA (Student-Newman-Keuls Method). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of antioxidant enzymes and induction of autophagy and apoptosis in Cd-exposed OSCCs and reversal by NAC

Many in vivo and in vitro studies have reported that Cd cytotoxicity induces various cell death modes via oxidative stress. To determine whether Cd-induced oxidative stress results from the disruption of antioxidant enzymes, we evaluated the expression of antioxidant enzymes, including catalase, SOD1, SOD2, and HO-1, and cell death–related proteins in YD8 and YD10B cells exposed to various