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Potential endocrine-disrupting effects of metals via interference with glucocorticoid and mineralocorticoid receptors☆

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ABSTRACT

As a result of human activities, the pollution of metals is becoming ubiquitous in the environment. Among various toxicological mechanisms of action, metals have been considered as endocrine-disrupting chemicals (EDCs) through interference with steroid receptors. However, information regarding the potential endocrine disruption of metals on glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) is especially scarce. In this study, a total of 16 metals were assessed for their GR/MR activities using luciferase reporter gene assay. None of the tested metals exhibited GR or MR agonistic activity, but a total of 7 and 5 candidate metals showed obvious GR and MR antagonistic properties, respectively. All 7 GR antagonistic metals [$BaCl_2$, $CoCl_2$, $CuCl_2$, $Pb(NO_3)_2$, LiCl, $SnCl_2$ and $ZnCl_2$] inhibited glucocorticoid-responsive gene GILZ expression in J774A.1 cells. Further investigations indicated that the 5 MR antagonistic metals [$CdCl_2$, $Pb(NO_3)_2$, LiCl, $MnCl_2$ and $SnCl_2$] antagonized aldosterone-inhibited hepatocellular carcinoma cell proliferation. Among these metals, $Pb(NO_3)_2$, LiCl, and $SnCl_2$ showed both anti-glucocorticoid and anti-mineralocorticoid activities. Comprehensive screening and evaluation of GR and MR antagonists and agonists among metals should be considered to better understand the ecological and health risks of metals.

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1. Introduction

As a result of natural and human activities, the occurrence of metals is becoming ubiquitous and highly mobile in the environmental components (Harmanescu et al., 2011). For instance, cadmium (Cd), Cobalt (Co), manganese (Mn), lead (Pb) were extensively detected in surface water and groundwater (Muhammad et al., 2011; Khan et al., 2016). Extremely high levels of metals were observed in soils of abandoned electronic waste sites in China, with mean concentrations of Pb and zinc (Zn) reaching 6082.9 mg/kg and 5995.6 mg/kg, respectively (Zhang et al., 2014). High Pb levels were detected in leek and ipomoea, with average concentrations reaching 0.53 and 0.39 ppm in contaminated areas in China, respectively (Zhuang et al., 2009; Zheng et al., 2007). Intake of metals through drinking water and food chain would pose health risks to human and wildlife due to their toxicity, persistence and bioaccumulative nature (Singh et al., 2010; Pekey et al., 2004; Muhammad et al., 2011). Some of the metals, such as copper (Cu) and Zn, are essentially required for normal functions of living organisms based on their roles as cofactors of a large number of enzymes, but these metals could also cause toxic effects when presented in excessive levels (Harmanescu et al., 2011). Some nonessential metals like Cd and Pb are potentially toxic even at low concentrations (Orisakwe et al., 2012). Metals were often observed to accumulate in vital organs and are implicated in numerous health disorders (Duruibe et al., 2007). For instance, high levels of Mn and Cu in drinking water were associated with Alzheimer's disease (Dieter et al., 2005). Exposure to Pb could induce behavioral disturbances and memory deterioration in children and also cause neurotoxic and carcinogenic effects in adults (Steenland and Boffetta, 2000; Jarup, 2003; Harmanescu et al., 2011).

Among various toxicological mechanisms of action, metals have been considered as endocrine-disrupting chemicals (EDCs) through

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interference with steroid receptors, such as estrogen receptor (ER) and androgen receptor (AR). For instance, bis(tri-*n*-butyltin), Cd, antimony (Sb), lithium (Li), barium (Ba), chromium (Cr), selenate (Se) and stannous (Sn) showed estrogenic activity in estrogen receptor-dependent transcriptional expression assay and E-Screen assay (Choe et al., 2003). Cu, Co, nickel (Ni), Pb, mercury (Hg), Sn, vanadate (V) and divalent Cr significantly induced the expression levels of estrogen-regulated progesterone receptor genes in human breast cancer cell line MCF-7 (Martin et al., 2003). However, another study reported that Cd, Cu and Zn had no effects on transactivation of the estrogen-responsive element, but these metals were able to potentiate the estradiol-induced response in a dose-dependent manner (Denier et al., 2009). Cd was also reported to activate AR transcriptional activity through interacting with its hormone-binding domain (Wu et al., 2014).

Our recent studies demonstrated that glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), members of steroid receptor subfamily, were potential targets for EDCs (Zhang et al., 2016a, 2016b; 2017, 2018a). GR mediates the effects of cortisol and other glucocorticoids on immune, metabolic, endocrine and nervous systems (Savory et al., 2001). GR knock-out mice had severely retarded lung maturation and died within perinatal period (Cole et al., 1995). The transgenic mice with forebrain GR blockade were reported to develop physiological and behavioral symptom, reminiscent of those observed in human major depressive disorder (Boyle et al., 2005). Many abnormalities were also observed in GR-deficient mice, including disrupted hepatic gluconeogenesis, dysregulation of the hypothalamus-pituitary-adrenal-axis and adrenal hyperplasia (Charmandari et al., 2004). MR binds to a class of mineralocorticoids, such as aldosterone, and plays instrumental roles in Na^+/K^+ homeostasis, cell proliferation, inflammation and fibrosis (Sekizawa et al., 2011; Pippal and Fuller, 2008). Lethal dehydration by renal sodium and water loss was reported due to the loss of MR function in both human and mice neonate (Berger et al., 1998; Fuller and Rogerson, 2002; Geller et al., 1998). Recent studies revealed that MR was able to suppress the development and progression of hepatocellular carcinoma, colorectal and lung cancer (Jeong et al., 2010; Nie et al., 2015; Tiberio et al., 2013). Several previous studies suggested that some metals might interfere with GR and MR. For example, a study reported that Cu^{2+} and Zn^{2+} inhibited the DNA binding activity of GR (Makino et al., 1996). Another study using *in vitro* binding assay indicated that metal ions treatment, such as Hg^{2+}, Zn^{2+}, Cu^{2+}, resulted in a significant loss of aldosterone binding capacity of MR (Galigniana and Piwien-Pilipuk, 1999). Data regarding the actions of metals on GR/MR are still scarce. It is valuable to identify potential agonists or antagonists for GR and MR among common metals for a better understanding of their ecological health risks.

In this study, a total of 16 metals were screened for their GR/MR activities using luciferase reporter gene assay, which is a highly sensitive *in vitro* bioassay. The influence of these metals on glucocorticoid signaling was subsequently evaluated by the expression of glucocorticoid-induced leucine zipper (GILZ) gene in mouse macrophage cell line J774A.1. The disruption of aldosterone-induced hepatocellular carcinoma growth inhibition was used to validate the effects of metals on MR. Our findings that many metals possessed antagonistic activities via GR or MR had significant implications for a comprehensive assessment of metals-induced health impacts.

2. Materials and methods

2.1. Chemicals

Cortisol (>98% pure), analytical standards of $BaCl_2$, $CdCl_2$, $Cr(NO_3)_3$, $CoCl_2$, $CuCl_2$, $Pb(NO_3)_2$, LiCl, $MnCl_2$, $K_2Cr_2O_7$, Na_2MoO_4, Na_2WO_4, $NiCl_2$, $SrCl_2$, $SnCl_2$, $Ti(SO_4)_2$, and $ZnCl_2$ were obtained from Sigma (St. Louis, MO). RU486 (>98% pure) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Aldosterone (>97% pure) and spironolactone (>99% pure) were obtained from J&K Scientific (Beijing, China) and Selleck Chemicals (Boston, USA), respectively.

2.2. Plasmid constructs

The expression plasmid pF25GFP-hGRα and the reporter plasmid pMMTV-luc containing mineralocorticoid response element (MRE) and glucocorticoid response element (GRE) were kindly provided by Dr. Evangelia Charmandari (Biomedical Research Foundation of the Academy of Athens, Greece) (Nicolaides et al., 2014). The expression plasmid pEGFP-C1-hMR was a gift from Dr. Claudia Großmann (Martin Luther University, Germany) (Grossmann et al., 2005; Ouvrard-Pascaud et al., 2004). The use of pRL-TK (Promega, Madison, USA) was described previously as an internal control (Zhang et al., 2016a).

2.3. Cell culture and cell proliferation assay

Chinese hamster ovary K1 (CHO-K1) cells, mouse macrophage cells J774A.1 and human hepatocellular carcinoma cell line (SMMC-7721) were maintained as previously described (Zhang et al., 2016a). For all exposure experiments, the cells were cultured in phenol red-free Dulbecco's modified eagle medium (DMEM) (Hyclone, Logan, UT) supplemented with charcoal/dextran-treated FBS (pH 6–8). The oxidation-reduction potential (Eh) remained stable during the exposure period. Cell proliferation was measured using CellTiter 96 AQueous One Solution Cell Proliferation (MTS assay) (Promega) according to the manufacturer's instruction after exposure to tested chemicals. The cell proliferation assays for CHO-K1 and J774A.1 were conducted after 24 h exposure. For SMMC-7721 cells, the exposure time of highest non-cytotoxic concentration evaluation in SMMC-7721 was extended to 48 h. When the cytotoxic effects were significant at high test concentration (10^{-5} M), a serial dilution test was performed for determining the highest non-cytotoxic concentrations.

2.4. Dual-luciferase reporter assays for hGR and hMR

The Dual-luciferase reporter gene assay for hGR and hMR was performed as previously described (Zhang et al., 2016a). Briefly, CHO-K1 cells were exposed to tested chemicals to measure the agonistic/antagonistic activity after transient transfection of pMMTV-luc, pRL-TK, together with pF25GFP-hGRα or pEGFP-C1-hMR for hGR and hMR assay, respectively. Dual-luciferase Reporter Assay Kit (Promega) was used to measure firefly luciferase and renilla luciferase activities after 24 h exposure. The relative transcriptional activity was presented as the ratio of firefly to renilla luciferase activity. Relative inhibition rate (RIR) was calculated as % decrease of cortisol or aldosterone response obtained at the highest tested concentrations of chemicals.

2.5. Real-time quantitative PCR

The isolation of RNA and Real-time Quantitative PCR were performed as previously described (Zhang et al., 2016a). Briefly, the J774A.1 cells were treated with the test chemicals at the highest non-cytotoxic concentration for 24 h and total RNA was isolated using TRIzol Reagent (Invitrogen Inc., Carlsbad, CA). Real-time quantitative PCR was performed using the SYBR Green PCR master mix (Toyobo) on Mx3000P real-time PCR system (Agilent Technologies, Palo Alto, CA). The primer sequences of mouse GILZ

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