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Cadmium stimulates myofibroblast differentiation and mouse lung fibrosis

Xin Hu, Jolyn Fernandes, Dean P. Jones, Young-Mi Go*

Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Emory University, Atlanta, GA 30322, United States

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ABSTRACT

Increasing evidence suggests that Cd at levels found in the human diet can cause oxidative stress and activate redox-sensitive transcription factors in inflammatory signaling. Following inflammation, tissue repair often involves activation of redox-sensitive transcription factors in fibroblasts. In lungs, epithelial barrier remodeling is required to restore gas exchange and barrier function, and aberrant myofibroblast differentiation leads to pulmonary fibrosis. Contributions of exogenous exposures, such as dietary Cd, to pulmonary fibrosis remain incompletely defined. In the current study, we tested whether Cd activates fibrotic signaling in human fetal lung fibroblasts (HFLF) at micromolar and submicromolar Cd concentrations that do not cause cell death. Exposure of HFLF to low-dose Cd ($\leq$1.0 μM) caused an increase in stress fibers and increased protein levels of myofibroblast differentiation markers, including α-smooth muscle actin (α-SMA) and extra-domain-A-containing fibronectin (ED-A-FN). Assay of transcription factor (TF) activity using a 45-TF array showed that Cd increased activity of 12 TF, including SMAD2/3/4 (mothers against decapentaplegic homolog) signaling differentiation and fibrosis. Results were confirmed by real-time PCR and supported by increased expression of target genes of SMAD2/3/4. Immunocytochemistry of lungs of mice exposed to low-dose Cd (0.3 and 1.0 mg/L in drinking water) showed increased α-SMA protein level with lung Cd accumulation similar to lung Cd in non-smoking humans. Together, the results show that relatively low Cd exposures stimulate pulmonary fibrotic signaling and myofibroblast differentiation by activating SMAD2/3/4-dependent signaling. The results indicate that dietary Cd intake could be an important variable contributing to pulmonary fibrosis in humans.

1. Introduction

Cadmium (Cd) is an environmental pollutant that causes multiple adverse health effects, including organ failure and cancer. Humans are exposed to Cd from diet at an intake level of approximately 30 μg per day (ATSDR, 2012), while tobacco smoking significantly adds to the exposure (2 μg per cigarette) (ATSDR, 2012; Mannino et al., 2004). Cd is not effectively excreted by humans and has 10–30y biological half-life (ATSDR, 2012). Thus chronic exposure of human to low doses of Cd mainly *via* food consumption results in Cd deposition in different tissues and can be a significant health hazard. Cd is classified as a carcinogen affecting multiple organ systems including lung, liver, kidney, and hematopoietic and other systems (ATSDR, 2012). Cd exposure is closely associated with lung diseases including lung cancer, chronic obstructive pulmonary disease (COPD), and emphysema (Hart, 2000). Previous studies also show that Cd exposure in low concentration stimulated proliferation in mouse lung cells and resulted in severe lung inflammation (Kundu et al., 2009). Despite extensive data on Cd toxicity at high doses, the roles of Cd at low dietary levels in pulmonary health remain to be fully elucidated.

Progressive and usually fatal fibrotic lung disease is characterized by fibroblast proliferation and extracellular matrix (ECM) remodeling, which results in irreversible distortion of the lung and reduction in gas exchange (Selman et al., 2001). The pathogenesis is attributed to abnormal wound healing in response to lung insult such as inflammation and environmental exposure (Taskar and Coultas, 2006). During wound healing, resident fibroblasts undergo proliferation and differentiation into myofiboblasts which display exaggerated ECM production and endow a contractile apparatus allowing them to close open wounds. The *de novo* synthesized contractile apparatus includes expression of α-smooth muscle actin (α-SMA) and formation of stress fibers that adhere to ECM proteins (Hinz et al., 2007). Incorporation of α-SMA into stress fibers increases the cellular contractile strength and the rigidity of ECM, which further promotes the expression of α-SMA and the tissue repair process. Therefore, abnormal expression of α-SMA can affect this feedback loop during the wound healing process and

Abbreviations: αSMA, α-smooth muscle-actin; Cd, cadmium; ECM, extracellular matrix; ED-A FN, extra-domain-A fibronection; HFLF, human fetal lung fibroblast; MT, metallothionein; MTF-1, metal response element-binding transcription factor-1; TF, transcription factor

* Corresponding author at: Department of Medicine, Pulmonary Division, Emory University, 225 Whitehead Biomedical Research Building, 615 Michael Street, Atlanta GA 30322, United States.

E-mail address: ygo@emory.edu (Y.-M. Go).

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contribute to pathological fibrosis.

Development of *in vitro* models to replicate *in vivo* exposures is challenging for many reasons, *e.g.*, time frame of exposure, binding of Cd to many ligands, artificial nature of cell culture conditions. We use 1 μM as our *in vitro* reference dose (Go et al., 2013a,b) because this is similar to the concentration found in human lung (Chandler et al., 2016), does not cause cell death, and activates proinflammatory signaling similarly to that observed with mouse models in which oral Cd is provided to raise lung Cd to values similar to human lung Cd content (Chandler et al., 2016). Our previous study of Cd in this range showed that Cd disrupts actin cytoskeleton regulation in lung fibroblast by stimulating actin polymerization (Go et al., 2013a). Results also show that interruption of actin dynamics by Cd disrupted subcellular compartmental redox homeostasis (Cuypers et al., 2010; Go et al., 2013a) and stimulated inflammatory signaling involving NF-κB activation in HeLa cells (Go et al., 2013b). Although Cd-stimulated inflammatory signaling and elevation of oxidative stress are well known (Cuypers et al., 2010; Go et al., 2013a), the effect of comparable exposures to Cd on regulation of lung physiology specifically on the molecular mechanistic responses of lung fibroblasts remains unexplored. Therefore, in the present study we studied lung fibrosis regulation in response to 0.5–2 μM Cd in human lung fibroblasts. The results show that Cd activates profibrotic signaling and promotes myofibroblast differentiation by increasing expression levels of differentiation marker proteins such as α-SMA *via* activation of SMAD2/3/4 transcription factor. An immunocytochemical analysis of α-SMA in lungs of mice exposed to low-dose Cd in drinking water provided evidence that this process also occurs *in vivo*.

2. Materials and methods

2.1. Cell culture and Cd treatment

Experiments were performed on normal human fetal lung fibroblasts (HFL1, passages ≤11) obtained from American Tissue Culture Collection (ATCC, Rockville, MD). HFL1 were cultured in F-12K medium (Kaighn's Modification of Ham's F-12 medium) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin, and maintained in a humidified incubator at 5% CO_2 at 37 °C. For low-dose Cd treatment (0, 0.5, 1.0 and 2.0 μM as $CdCl_2$, Sigma-Aldrich, St. Louis, MO), cells grown in cell culture plates (6-well, 96-well, cover slips) were exposed to Cd for 6 h and 24 h to examine mRNA and protein expression, respectively. Doses of 0.5 and 1.0 μM result in Cd concentrations comparable to human dietary intake while 2.0 μM represents a higher level from smoking. The condition labeled 0 μM has no Cd added and has been previously found to have negligible Cd (Go et al., 2013a,b). Human transforming growth factor-β (TGF-β, Sigma-Aldrich) was used as a positive control for myofibroblast differentiation (Thannickal et al., 2003).

2.2. Immunofluorescence imaging on mouse lung

C57BL/6 mice (male, 5–7 weeks old, n = 4–8) were exposed to Cd in drinking water (0, 0.3 or 1.0 mg/L $CdCl_2$) for 16 weeks, with the approval of Institutional Animal Care and Use Committee of Emory University. For these studies, the mouse food was nominally Cd-free, equivalent to intake of 0.04 mg/L in the drinking water (Chandler et al., 2016). Lung samples were harvested from the mice at the end of 16 weeks and the left lobes of lung tissues treated with 10% neutral formalin for fixation. The fixed lungs were processed and stained with anti-α-SMA and anti-vimentin purchased from Abcam (Cambridge, MA) for fluorescence microscopy following the procedures previously described (Rock et al., 2011). Immunofluorescence was visualized using an Olympus X-700 fluorescence microscope system.

2.3. Myofibroblast differentiation marker examination by fluorescence microscopy and western blotting

To examine α-SMA and F-actin by fluorescence microscopy, HFL1 cells grown on glass coverslips were treated with Cd or TGF-β for 24 h, washed with PBS, and followed by the same procedures as described in previous studies (Go et al., 2013a,b). Briefly, cells were incubated with anti-α-SMA (Abcam) followed by Cy3. Cells were then incubated with BODIPY FL Phallacidin for F-actin and Hoechst for nuclei staining (Thermo Fisher Scientific). Fluorescence was visualized using an Olympus X-700 fluorescence microscope, and fluorescence intensity was quantified with ImageJ software (NIH) (Go et al., 2013a,b). Quantification of α-SMA was further confirmed by measuring fluorescence of cells grown in 96-well plates and treated with Cd in an independent experiment using a fluorescence plate reader (SpectraMax M2, Molecule Devices). To examine expression levels of α-SMA and ED-A FN by Western blot analysis, cells after Cd or TGF-β treatment for 24 h were lysed, proteins were prepared, and protein expression levels from Western blots were quantified as previously described (Go et al., 2013b). Primary antibodies to detect α-SMA and ED-A FN were purchased from Abcam.

2.4. Transcription factor activity array

HFL1 cells were reverse transfected with luciferase constructs precoated to 96-well plates (Cignal 45-Pathway Reporter Array, QIAGEN, Valencia, CA) that profile the activity of transcription factors in 45 signal transduction pathways. Each reporter contains a mixture of an inducible transcription factor responsive *Firefly* luciferase construct and constitutively expressing *Renilla* luciferase (20:1). The Firefly construct contains tandem repeats of transcription factor binding sequence (listed in Supplementary Table S1). After 24 h of transfection following the procedures provided by the manufacturer, the cells were treated with Cd for 6 h. *Firefly* and *Renilla* luciferase activity were quantified using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). All luciferase data were normalized relative to the *Renilla* luciferase activity. Negative and positive controls were included in all experiments to assure transfection efficiency.

2.5. Determination of gene expression levels by quantitative reverse transcription (qRT) PCR

Total mRNA extracted from cells after Cd treatment for 6 h was used to generate cDNAs by reverse transcription (QIAGEN). Quantification by real-time PCR was performed in triplicate on an iCycler IQ Multicolor RT-PCR Detection System (Bio-Rad Laboratories, Hercules, CA) for 40 cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 30s) using iCycler software and normalized by 18 s RNA levels. The sequences of primers purchased from Integrated DNA Technologies (Coralville, IA) were as follows (*5′ to 3′*): MT1/2, forward: *GCACCTCCTGCAAGAAGAGCT*, reverse: *GCAGCCCTGGGCACACTT*; Collagen1α1, forward: *AGCCAGC AGATCGAGAACAT*, reverse: *TCTTGTCCTTGGGGTTCTTG*; αSMactin (ACTA2), forward: *GACCCTGAAGTACCCGATAGAAC*, reverse: *GGGCAA CACGAAGCTCATTG*; Fibronectin, forward: *TCGAGGAGGAAATT CCAATG*, reverse: *ACACACGTGCACCTCATCAT*; MMP2, forward: *ACATCAAGGGCATTCAGGAG*, reverse: *GCCTCGTATACCGCATCAAT*; Axin2, forward: *CCTGCCACCAAGACCTACAT*, reverse: *CTTCATTCAAGG TGGGGAGA*.

2.6. Statistics

Data from at least three independent replicates of each experimental condition were compared using one-way ANOVA followed by Tukey's *post-hoc* tests. A *p*-value less than 0.05 was considered significant. For 45 TF activity experiments, one-way ANOVA repeated measures were used to take plate-to-plate variation into account.