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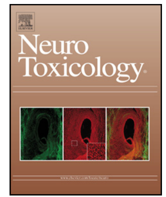

Full Length Article

Cadmium-induced IL-6 and IL-8 expression and release from astrocytes are mediated by MAPK and NF-κB pathways

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ABSTRACT

Chronic exposure to cadmium has been linked to brain cancers, learning disabilities and memory deficits. Previous studies of cadmium toxicity in the central nervous system report cadmium induces oxidative stress in neurons and astrocytes. In the peripheral system, cadmium promotes interleukin-6 (IL-6) and IL-8 production and release. Elevation of IL-6 expression is linked to the pathogenesis of neurodegenerative diseases and astrogliosis. IL-8 plays a role in angiogenesis of gliomas and neurodegenerative diseases. Herein, the effects of non-toxic concentrations of cadmium on the production of IL-6 and IL-8 and the underlying mechanisms were investigated. U-87 MG human astrocytoma cells and primary human astrocytes were exposed to cadmium chloride. At 24 h post-exposure to 1 and 10 μM, levels of intracellular cadmium in U-87 MG cells were 11.89 ± 3.59 and 53.08 ± 7.59 μg/g wet weight, respectively. These concentrations had minimal effects on cell morphology and viability. IL-6 and IL-8 mRNA levels and secretion increased in dose- and time-dependent manners post cadmium exposure. Acute exposure to cadmium increased phosphorylation of ERK1/2, p38 MAPK, and p65 NF-κB. Pretreatment with U0126–an inhibitor of MEK1 and MEK2 kinases–SB203580–a p38 MAPK inhibitor–and SC-514–an IKKβ inhibitor–suppressed cadmium-induced IL-8 expression and release. Upregulation of cadmium-induced IL-6 was inhibited by U0126 and SC-514, but not SB203580. On the other hand, SP600125–a JNK inhibitor–and celecoxib–a selective COX-2 inhibitor–had no effect on production of both cytokines. In conclusion, non-toxic concentrations of cadmium can stimulate IL-6 and IL-8 release through MAPK phosphorylation and NF-κB activation. Suppressing IL-6 and IL-8 production could be novel approaches to prevent cadmium-induced angiogenesis in gliomas and inflammation in the brain.

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1. Introduction

Cadmium is used extensively in various industries and produced as a by-product of metal smelting. It is also a contaminant in cigarette smoke. Cadmium is classified as a human carcinogen by the Department of Health and Human Services (DHHS) and the International Agency for Research on Cancer (IARC) (Jarup et al., 1998; Waisberg et al., 2003). After being absorbed into the bloodstream, cadmium can destroy the structural integrity of the blood-brain barrier (BBB), allowing cadmium to enter and accumulate in the brain (Wang and Du, 2013; Provias et al., 1994). Epidemiological studies identify cadmium as a risk factor for brain cancers and Alzheimer's disease (Min and Min, 2016; Wesseling et al., 2002). Cadmium exposure is associated with the lack of attention in children, adult with high consumption of contaminated foods, and workers in cadmium-contaminated areas (Ciesielski et al., 2012, 2013; Viaene et al., 2000). The average and maximum levels of cadmium detected in brain tissue of brain tumor patients were 2.02 ± 10.99 and 72.78 μg/g wet tissue, respectively (Al-Saleh and Shinwari, 2001).

Inflammation is one of the hallmarks of neurodegenerative diseases and cancers, and upregulation of many proinflammatory cytokines and chemokines including interleukin-6 (IL-6) and IL-8

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has been associated with these diseases. Cadmium increases IL-6 and/or IL-8 production in various types of human peripheral cells, including bronchial epithelial cells (Rennolds et al., 2012; Cormet-Boyaka et al., 2012), THP-1 monocytic cells (Freitas and Fernandes, 2011), and HepG2 hepatocyte cells (Souza et al., 2004). IL-6 is an inflammatory cytokine with neurotrophic and detrimental effects depending on its concentrations and expression of its receptors. IL-6 protects neurons from excitotoxicity; however, high concentrations of IL-6 induce neuronal death. IL-6 stimulates astrocyte proliferation and secretion of inflammatory mediators and growth factors (Spooren et al., 2011). IL-8 is a chemoattractant chemokine responsible for innate immunity by recruiting neutrophils and macrophages to inflammation sites (Brat et al., 2005). Over production of IL-8 in the brain promotes tumor growth and angiogenesis in gliomas (Brat et al., 2005). In humans, increased IL-6 and/or IL-8 levels in the central nervous system (CNS) were linked to pathogenesis of neurodegenerative diseases such as Alzheimer's disease (Liu et al., 2014), viral infection (Liu and Kumar, 2015; Nookala and Kumar, 2014; Vivithanaporn et al., 2010) and epilepsy (Pernhorst et al., 2013). In these disorders, astrocytes are one of the major sites of IL-6 and IL-8 production. Furthermore, IL-6 and IL-8 production in astrocytes is upregulated by MAPK and/or
NF-κB pathways (Nookala and Kumar, 2014; Shah and Kumar, 2010; Shah et al., 2012). In the peripheral system, cadmium-induced IL-6 and IL-8 is mediated by ERK1/2 and/or NF-κB pathways (Rennolds et al., 2012; Cormet-Boyaka et al., 2012; Freitas and Fernandes, 2011; Hyun et al., 2007; Rajendran et al., 2016).Previous studies report cadmium toxicity on astrocytes is mediated by glutathione depletion and oxidative stress (Im et al., 2006). Little is known about cadmium's effect on astrocytic inflammation. Herein, we determined whether cadmium is transported into astrocytes and subsequently induced inflammation. In addition, the mechanism of cadmium-induced inflammation was investigated. Cadmium transport into human astrocytes was observed, as was cadmium's stimulation of IL-6 and IL-8 expression and release via the ERK1/2, p38, and NF-κB pathways.

2. Materials and methods

2.1. Cell cultures and reagents

U-87 MG astrocytoma cells (U-87 MG) were obtained from American Type Culture Collection (ATCC) and cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% penicillin-streptomycin (Gibco, USA). Primary human astrocytes (PHA) were provided by Prof. Christopher Power (Department of Medicine, University of Alberta, Canada). PHA were prepared according to protocol 1420 approved by the University of Alberta Human Research Ethics Board (Biomedical) as reported previously (Vivithanaporn et al., 2010). PHA were grown in MEM containing 10% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin. All cultures were grown and maintained at 37 °C in a humidified chamber with 5% CO_2. Completed medium was renewed every 1-2 days. Both cell types were used from the fifth to the tenth passage.

Cadmium chloride ($CdCl_2$) was dissolved in sterile water at 1000 mM while manganese chloride ($MnCl_2$), nickel chloride ($NiCl_2$), copper(II) sulfate ($CuSO_4$) and mercury chloride ($HgCl_2$) were dissolved in sterile water at 100 mM. All stock solutions of heavy metals were stored at −20 °C until use. U0126 (Cell-signaling, USA), SB203580 (Tocris, United Kingdom), SP600125, SC-514 and celecoxib were dissolved in dimethyl sulfoxide (DMSO). All chemicals were from Sigma-Aldrich unless otherwise indicated. Cells were treated with inhibitors one hour prior to cadmium exposure. The final concentration of DMSO was 0.1%.

2.2. Measurement of intracellular cadmium

U-87 MG cells were plated in 100-mm dishes. At 70–80% confluence, cells were treated with 1 and 10 μM of $CdCl_2$ for 24 h in serum-free MEM. Level of cadmium uptake was measured as previously described (Soodvilai et al., 2011). Briefly, cells were washed twice with phosphate-buffer saline (PBS) containing 10 mM disodium EDTA and twice with PBS without EDTA. Cells were trypsinized with 0.1% trypsin and centrifuged at 3000 rpm for 10 min. Cell pellets were digested with 1 ml of 65% nitric acid and evaporated at 90 °C three times. Lysate was incubated with 2 ml of 65% nitric acid overnight and then diluted with 18 ml of distilled water. Cadmium content was measured using a flame furnace atomic absorption spectrophotometer (FFAAS) (PinAAcle 900T, Perkin Elmer). Amount of intracellular cadmium content was expressed as μg of cadmium/g protein of wet weight.

2.3. Observation of cell morphology

U-87 MG cells were plated at 5×10^5 cells in 60-mm dishes. At 70–80% confluence, cells were treated with 1 to 30 μM $CdCl_2$ for 6 and 24 h. Cell morphology was observed with an inverted light microscope (Eclipse E200, Nikon).

2.4. MTT cell viability assay

U-87 MG and PHA cells were treated with $CdCl_2$ from 0.01 to 100 μM for 6 and 24 h in serum-free MEM. At 2 h before the indicated incubation time, MTT solution was added to each well to a final concentration of 0.5 mg/ml. The formazan crystals were then dissolved in DMSO and measured spectrophotometrically at 562 nm using a microplate reader (ELx808™, Biotek). The percentage of cell viability was calculated as percentage in comparison to untreated cells.

2.5. Quantitative real-time PCR

U-87 MG cells were treated with 0.1, 1 and 10 μM $CdCl_2$ for 3 and 6 h. Total RNA was extracted and purified by Total RNA Purification kit according to manufacturer's protocol (Jena Bioscience, Germany). Complementary DNA (cDNA) was synthesized using 1 μg of total RNA mixed with Primer Random (Roche Diagnostics, USA) and superscript III reverse transcriptase (Invitrogen, USA). The primer sequences for human IL-6 were: forward, 5′-ACCCCTGACCCAACCACAAAT-3′; and reverse, 5′-AGC TGCGCAGAATGAGATGAG-3′. The primer sequences for human IL-8 were: forward, 5′-CACCGGAAGGAACCATCTCAC-3′; and reverse, 5′-TGGTCCACTCTCAATCAC
TCTCAG-3′. The primer sequences for human GFAP were: forward, 5′-ACACGTCTGACCCTCTCCAC-3′; and reverse, 5′-TGCTCGTGCCTCAGTTTTAC-3′. The primer sequences for human GAPDH were: forward, 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′; and reverse, 5′-CGGAGTCAACGGATTTGGTCG-3′. Quantitative real-time PCR analysis was performed using SensiFAST SYBR LO-ROX (Bioline, Canada) on an Applied Biosystems Real-time PCR 7500 system (ABI 7500, Appied Biosystems). Threshold cycle (C_t) value of each target gene was normalized to the expression of housekeeping gene, GAPDH. The difference between C_t value of the target gene and GAPDH of cadmium-treated cells was subtracted from the difference between C_t value of the target gene and GAPDH of untreated cells and expressed as relative fold

change (RFC) according to the $2^{-\Delta\Delta Ct}$ method. The RFC value of untreated cells was defined as 1.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Confluent monolayer of U-87 MG and PHA cells in 12-well plates was exposed to $CdCl_2$, $MnCl_2$, $NiCl_2$, $CuSO_4$ or $HgCl_2$ for 6 and 24 h. Supernatant was collected and centrifuged at 5000 rpm for 5 min and then stored at −80 °C until tested. The amount of IL-6 and IL-8 were measured by human IL-6 and IL-8 ELISA kits according to the manufacturer's protocol (eBioscience, USA).

2.7. Western blotting

U-87 MG cells were grown in 60-mm dishes and treated with 1 and 10 μM $CdCl_2$ for 5, 15, and 30 min. Cells were lysed in lysis buffer [20 mM Tris, 1% NP-40, 50 mM NaCl and Protease Inhibitor Cocktail (PIC) Set III (1:1000, Calbiochem, La Jolla, CA)] for GFAP and MAPK pathways. For NF-κB pathways, cells were first lysed in cytoplasmic extract buffer [10 mM HEPES, 10 mM KCl, 0.5 mM DTT, 1% NP-40 and PIC]. Lysates were briefly vortexed every 3 min for 3 times, and cytoplasmic extract was collected after centrifugation at 13,200g for 2 min. Pellets were further lysed in nuclear extract buffer [20 mM HEPES, 500 mM KCl, 1.5 mM $MgCl_2$, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1% NP-40 and PIC] by vortexing every 3 min for a total of 30 min. Nuclear extract was collected after centrifugation at 13,200g for 15 min. Crude protein lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes, which were blotted for GFAP (BD Biosciences, Cat. No. 556330, 1:1000 dilution), phospho-p44/42 MAPK (ERK1/2) (Cell Signaling, Cat. No. 4370, 1:4000 dilution), p44/42 MAPK (Cell Signaling, Cat. No. 9102, 1:2000 dilution), phospho-p38 MAPK (Cell Signaling, Cat. No. 9215, 1:1000 dilution), p38 MAPK (Cell Signaling, Cat. No. 9212, 1:2000 dilution), phospho-p65 NF-κB (Cell Signaling, Cat. No. 3033, 1:1000 dilution), β-actin (Cell Signaling, Cat. No. 4967, 1:2000 dilution), and lamin A/C (Cell Signaling, Cat. No. 4777, 1:3000 dilution). Chemiluminescent signal was visualized using enhanced chemiluminescence substrate (Bio-Rad, Cat. No. 170–5060) on film. Band density was determined using ImageJ software. β-actin or lamin A/C were used as internal loading control to normalize expression of proteins of interest in cytoplasm and nucleus, respectively.

2.8. Statistical analysis

Each experiment was performed at least three times and data were expressed as mean ± SEM. Multiple-sample comparisons were carried out using one-way ANOVA followed by Tukey's pairwise comparison. In all comparisons, differences were considered significant when p value is less than 0.05 ($p < 0.05$) and 0.01 ($p < 0.01$). All statistical analyses were performed using Graphpad® Prism statistical analysis software (version 6.0).

3. Results

3.1. Cadmium is accumulated in cultured human astrocytes

First whether cadmium can be taken up by U-87 MG cells was examined using FFAAS. Intracellular cadmium concentrations showed a significant dose-dependent increase. At 24 h post-exposure to 1 and 10 μM $CdCl_2$, intracellular cadmium concentrations were 11.89 ± 3.59 and 53.08 ± 7.59 μg/g wet weight, respectively (Fig. 1).

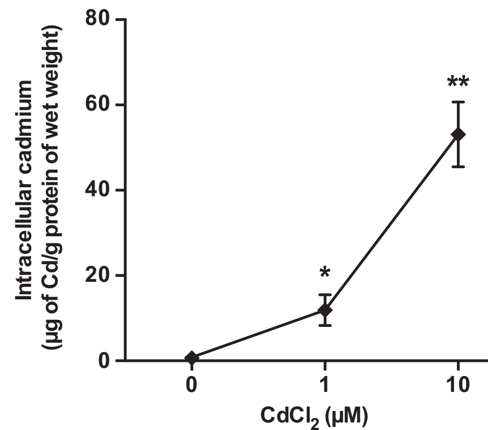

Fig. 1. Cadmium accumulation observed in cultured human astrocytes.
U-87 MG astrocytoma cells were treated with $CdCl_2$ 1 and 10 μM for 24 h and the intracellular level of cadmium was determined using FFAAS. Cadmium accumulated in a dose-dependent manner. Each data point represents mean ± SEM of intracellular cadmium expressed as μg of cadmium/g protein of wet weight from three independent experiments. One-way ANOVA was used for statistical analysis and statistical significance was donated as *($p < 0.05$) and **($p < 0.01$) in comparison between cadmium-treated cells and untreated cells.

3.2. Cadmium decreases cell viability of cultured human astrocytes

Previous studies showed cadmium at 20 μM or higher was toxic to primary rat astrocytes (Im et al., 2006; Yang et al., 2008). Morphological examination of U-87 MG cells at 6 and 24 h after exposed to $CdCl_2$ showed cadmium, up to 10 μM, did not induce any morphological change. Human astrocytes became round and lost their processes at 30 μM of $CdCl_2$ (Fig. 2). MTT assays revealed U-87 MG and PHA viability was reduced less than 15% at 6 h post-exposure to 10 μM $CdCl_2$, and viability of both cell types was still more than 70% after exposure for 24 h at this concentration. The median toxic concentrations of cadmium (TC_{50}) for U-87 MG cells at 6 and 24 h were 56.24 ± 1.16 μM and 20.65 ± 1.12 μM, respectively (Fig. 3A). Similarly, the TC_{50} values of $CdCl_2$ on PHA were >100 μM and 25.11 ± 1.12 μM at 6 and 24 h, respectively (Fig. 3B). To rule out the effect of cell death, 10 μM of $CdCl_2$ was used as the maximum concentration in subsequent experiments. Additionally, 1 and 10 μM of $CdCl_2$ did not alter mRNA and protein expression of glial fibrillary acidic protein (GFAP), an astrocyte activation marker (Supp. Fig. 1).

3.3. Cadmium induces IL-6 and IL-8 expression in cultured human astrocytes

Cadmium elevates IL-6 and IL-8 expression and secretion in bronchial epithelial and renal glomerulus endothelial cells (Cormet-Boyaka et al., 2012; Rajendran et al., 2016). In U-87 MG cells, 1 and 10 μM $CdCl_2$ induced expression IL-6 mRNA. At 10 μM of $CdCl_2$, IL-6 mRNA levels were 7.16 ± 0.86 fold and 4.67 ± 0.70 fold higher than untreated cells at 3 and 6 h post-exposure, respectively (Fig. 4A). In contrast, exposure to 1 μM $CdCl_2$ only showed a trend of IL-8 upregulation in U-87 MG cells. At 10 μM of $CdCl_2$, IL-8 mRNA levels were 6.20 ± 1.25 fold and 5.95 ± 1.73 fold higher than untreated cells at 3 and 6 h post-exposure, respectively (Fig. 4B). Increased IL-6 and IL-8 mRNA levels were associated with the release of both cytokines. Secretion of IL-6 and IL-8, as evidenced by their presence in culture media, increased with cadmium concentration and time (Fig. 4C and D). Release of IL-6 and IL-8 from U-87 MG cells exposed to 10 μM of $CdCl_2$ was detectable as early as 6 h post-exposure (Fig. 4C and D). At 24 h post-exposure, 1 μM $CdCl_2$ stimulated release of both IL-6 and IL-8; and even the

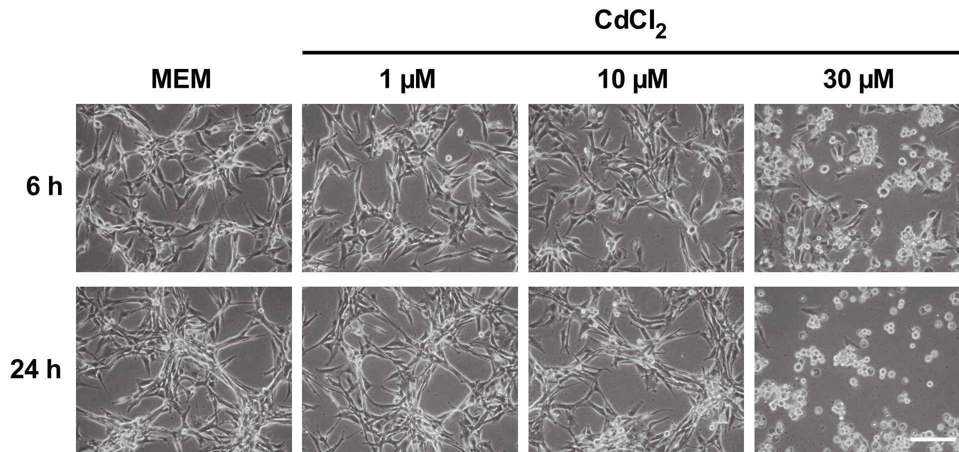

Fig. 2. Cadmium-induced morphological change of cultured human astrocytoma cells.
U-87 MG astrocytoma cells were treated with $CdCl_2$ at 1, 10, and 30 μM for 6 and 24 h. Morphological change occurred in cells treated with 30 μM $CdCl_2$ from 6 h. Pictures were representative data from at least three independent experiments. The scale bar was 20 μm.

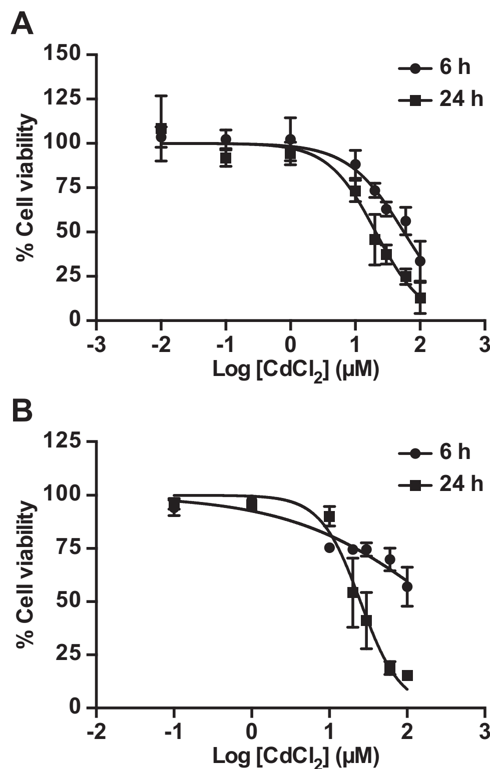

Fig. 3. Toxicity of acute cadmium exposure in cultured human astrocytes.
U-87 MG astrocytoma cells and primary human astrocytes (PHA) were treated with $CdCl_2$ at 0.01 to 100 μM for 6 and 24 h. Cell viability of U-87 MG astrocytoma cells (A) and PHA (B) were measured by MTT assays. Loss of cell viability after $CdCl_2$ exposure was found at 10 μM or higher, and cell viability was less than 15% when exposed to $CdCl_2$ at 100 μM for 24 h. Each dot and square represented the average of percentage of surviving cells (±SEM) from three independent experiments.

lowest concentration, 0.1 μM, elevated IL-8 in culture supernatant of U-87 MG cells. In contrast, $CdCl_2$ up to 10 μM did not significantly stimulate the release of IL-6 while $CdCl_2$ at 10 μM stimulated IL-8 release from PHA (Fig. 4E and F). Additionally, IL-8 levels in culture media from PHA were less than levels in U-87 MG cells after exposed to the same concentration of cadmium. These results indicated that non-toxic concentrations of cadmium induced both IL-6 and IL-8 expression and secretion from cultured human astrocytoma cells.

In addition to cadmium, other heavy metals, i.e., manganese and nickel, reportedly induce IL-6 or IL-8 (Freitas and Fernandes, 2011; Honda et al., 2015). Manganese chloride ($MnCl_2$), nickel chloride ($NiCl_2$), and copper(II) sulfate ($CuSO_4$) up to 10 μM were not toxic to cultured human astrocytes (data not shown). $MnCl_2$ at 10 μM increased IL-6 release at 24 h post-exposure while $NiCl_2$ and $CuSO_4$ at 1 and 10 μM did not significantly increase IL-6 and IL-8 production at 6 and 24 h post-exposure (Fig. 5A and B). Mercury chloride ($HgCl_2$) at 10 μM reduced cell viability more than 80% at 6 h post-exposure (data not shown); therefore, the effect of 10 μM $HgCl_2$ on IL-6 and IL-8 release was not tested. $HgCl_2$ at 1 μM did not alter IL-6 and IL-8 expression. These results suggested that cadmium induces IL-6 and IL-8 release from human astrocytoma cells at lower concentrations than other heavy metals.

3.4. Increased IL-6 and IL-8 production by cadmium is mediated by ERK, p38 and NF-κB pathways in cultured human astrocytes

MAPK pathways, including ERK and p38 MAPK, and NF-κB pathways play a role in IL-6 and IL-8 production in human astrocytes (Nookala and Kumar, 2014; Shah and Kumar, 2010; Shah et al., 2012). To determine whether cadmium-induced IL-6 and IL-8 was mediated by activation of ERK, p38 and NF-κB pathways; U-87 MG cells were treated with medium or $CdCl_2$ at 1 and 10 μM for 5, 15, and 30 min. At the end of incubation, expression of ERK and p38 MAPK were determined. Both concentrations of $CdCl_2$ increased levels of phosphorylated ERK1/2 at 15 and 30 min (Fig. 6A), while elevated phosphorylated p38 MAPK was found at 5 min and continued up to 30 min (Fig. 6B). Both concentrations of $CdCl_2$ increased levels of phosphorylated p65 NF-κB in cytoplasmic and nuclear extracts at 30 min (Fig. 6C). Next, U-87 MG cells were pretreated with vehicle (DMSO) and 20 μM U0126–a MEK1 and MEK2 inhibitor – or 25 μM SB203580–a p38 MAPK inhibitor–for 1 h. After that, 10 μM $CdCl_2$ was added for 15 min. U0126 and SB203580 suppressed cadmium-induced phosphorylation of ERK1/2 and p38, respectively (Supp. Fig. 2A and B). Similarly, pretreatment with 10 μM SC-514–an IKKβ inhibitor–suppressed cadmium-induced phosphorylation of p65 NF-κB in cytoplasmic extract at 30 min (Supp. Fig. 2C). These results showed that cadmium activates ERK1/2, p38 and NF-κB pathways.

To identify the pathways involved in IL-6 and IL-8 production, U-87 MG cells were pretreated with vehicle (DMSO), 20 μM U0126, 25 μM SB203580, 25 μM SP600125, 10 μM SC-514 or 25 μM

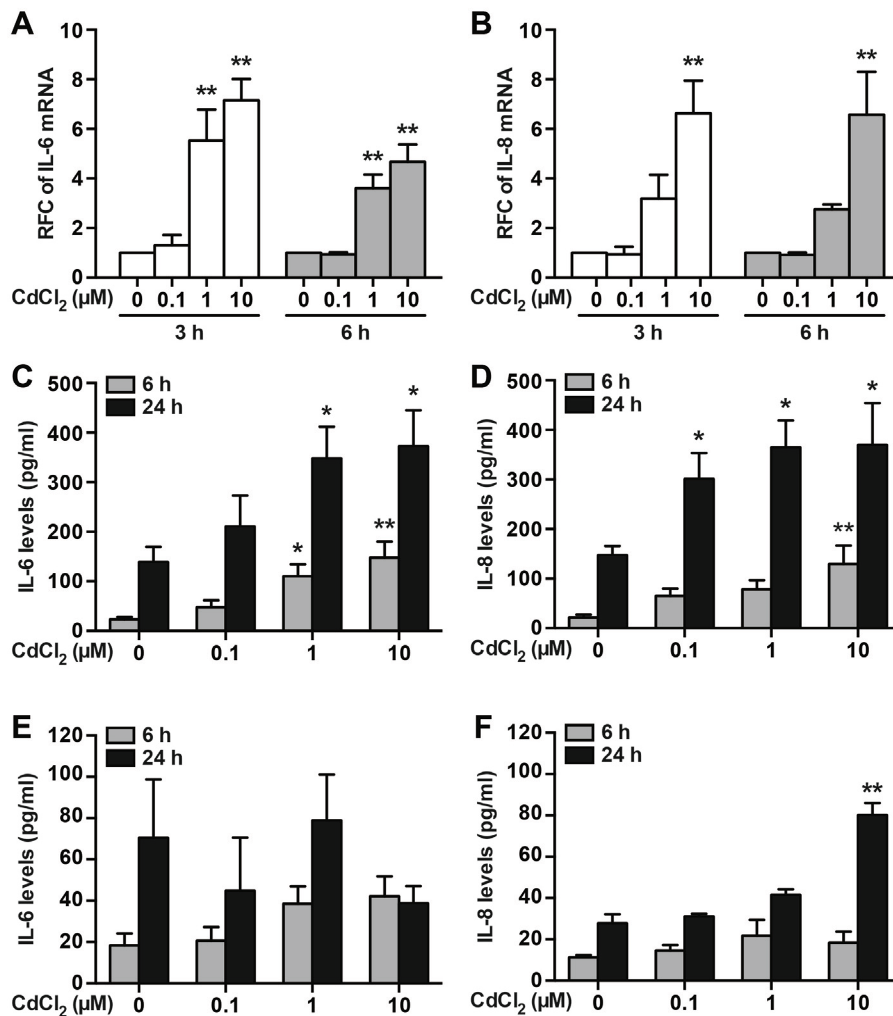

Fig. 4. Cadmium-induced IL-6 and IL-8 expression and secretion by cultured human astrocytes.
Levels of IL-6 and IL-8 mRNA transcripts were determined using real-time-PCR. Data were normalized to housekeeping gene GAPDH and presented as relative fold change (RFC) compared to untreated cells. $CdCl_2$ at 1 and 10 mM significantly elevated levels of IL-6 mRNA transcripts, while only 10 mM of $CdCl_2$ significantly increased levels of IL-8 mRNA transcripts in U-87 MG astrocytoma cells at 3 and 6 h post-exposure (A, B). At 6 and 24 h post-exposure to $CdCl_2$, levels of IL-6 and IL-8 presented in the medium culturing U-87 MG (C, D) and primary human astrocytes (E, F) were measured by human Il-6 and IL-8 ELISA kits and shown as pg/ml of cell culture supernatant. $CdCl_2$ increased both IL-6 and IL-8 release from U-87 MG; whereas only increased IL-8 was found in PHA cells. Each bar represented mean values (±SEM) of at least three independent experiments. Two-way ANOVA was used for statistical analysis and statistical significance was donated as * ($p < 0.05$) and ** ($p < 0.01$) in comparison between cadmium-treated cells and untreated cells at the same time point.

celecoxib for 1 h. These concentrations were selected based on previous studies of these pathways (Cormet-Boyaka et al., 2012; Shah et al., 2012; Zhu et al., 2015; Figueiredo-Pereira et al., 2002; He et al., 2015) and their effects on viability of U-87 MG cells. All concentrations used in the present study showed no cytotoxic effect on U-87 MG cells (Supp. Fig. 3). Treatment with MAPK inhibitors had no effect on basal levels of IL-6 and IL-8 in culture supernatant while SC-514 inhibited basal IL-6 release at 24 h (Supp. Fig. 4). Inhibition of ERK1/2 and NF-κB significantly decreased cadmium-induced expression of IL-6. U0126 and SC-514 suppressed IL-6 mRNA levels at 3 h post-exposure to 10 μM $CdCl_2$ by 49.98% and 71.28%, respectively (Fig. 7A). In addition to ERK1/2 and NF-κB, inhibition of p38 MAPK decreased the cadmium-induced expression of IL-8 mRNA at 3 h post-exposure by 56.32% (Fig. 7B). Consistently, pretreatment with U0126 and SC-514, but not SB203580, decreased IL-6 release at 6 and 24 h post-exposure; while all three inhibitors reduced IL-8 release at 6 and 24 h post-exposure (Fig. 7C and D). Combining U0126 and SC-514 suppressed cadmium-induced IL-6 and IL-8 to the same level as DMSO-treated cells; however, the reduction was not greater than the inhibitory effect observed from each inhibitor (Supp. Fig. 5A and B). Similarly, combining U0126 and SB203580 did not significantly reduce cadmium-induced IL-8 expression beyond the reduction observed when each inhibitor was applied separately (Supp. Fig. 5C). On the other hand, SP600125–a JNK inhibitor–did not prevent cadmium-induced IL-6 and IL-8 expression (Fig. 7A–D). Of note, celecoxib–a cyclooxygenase-2 (COX-2) inhibitor–stimulated IL-6 release at 6 h (Supp. Fig. 4); however, pretreatment with celecoxib did not potentiate cadmium-induced IL-6 and IL-8 expression (Fig. 7A–D). Together, these data showed the ERK1/2, p38 and NF-κB pathways play a key role in regulating expression and release of cadmium-induced IL-6 and IL-8 from human astrocytes.

4. Discussion

Cadmium induces inflammation in the peripheral system especially lungs of smokers; however, effects of cadmium on inflammation in the CNS are largely unknown. Herein, non-cytotoxic concentrations of cadmium accumulated in cultured human astrocytes and stimulated IL-6 and IL-8 expression and

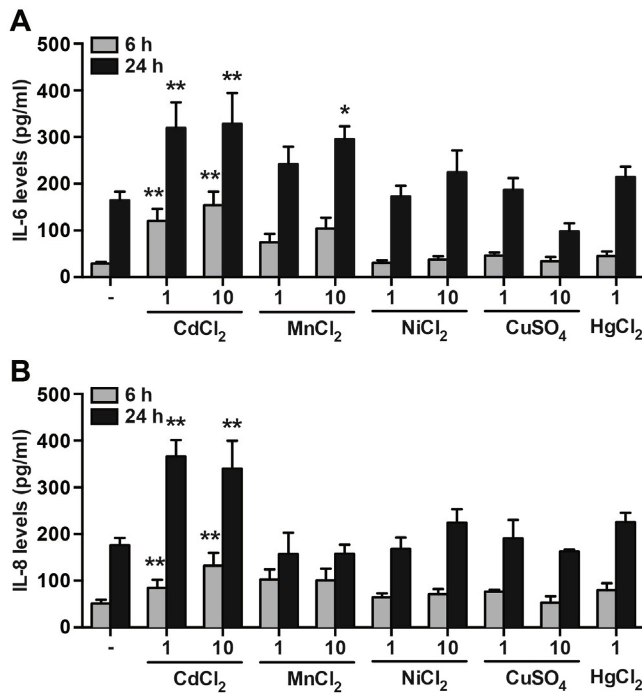

Fig. 5. Different induction of IL-6 and IL-8 secretion from cultured human astrocytes in response to various heavy metals.
U-87 MG astrocytoma cells were treated with 1 and 10 μM of $CdCl_2$, manganese chloride ($MnCl_2$), nickel chloride ($NiCl_2$) and copper (II) sulfate ($CuSO_4$) and 1 μM of mercury chloride ($HgCl_2$) for 6 and 24 h. Levels of IL-6 and IL-8 presenting in cultured media were measured by IL-6 and IL-8 ELISA kits. $CdCl_2$ and $MnCl_2$ at 10 μM induced IL-6 secretion (A) while only $CdCl_2$ induced IL-8 secretion from U-87 MG astrocytoma cells (B). Each bar represented mean values (±SEM) of at least three independent experiments. Two-way ANOVA was used for statistical analysis and statistical significance was donated as * ($p < 0.05$) and ** ($p < 0.01$) in comparison between heavy metal-treated cells and untreated cells at the same time point.

release via activation of the MAPK and NF-κB pathways. IL-8 levels increased with time and cadmium concentration in both U-87 MG and PHA cells; while elevation of IL-6 levels only occurred in U-87 MG cells. Interestingly, U-87 MG cells released higher levels of IL-8 than primary astrocytes and were activated by lower concentration of cadmium. The present study showed cadmium induces inflammation as well as delineated the mechanisms involved, providing rationale for future studies of cadmium-induced neuroinflammation and glioma in animal models.

Intracellular cadmium level in cultured astrocytes was within the range found in glioma tissues–Cadmium cannot penetrate intact BBB; therefore, levels of cadmium in healthy adult brains were low or undetectable (Wang and Du, 2013). In autopsied gray and white matter of healthy human subjects, average concentrations of cadmium were 0.06 and 0.04 μg/g dry weight, respectively. The maximum concentration in either area was 0.14 μg/g dry weight (Bush et al., 1995). Cadmium exposure caused BBB leakage in human and animals (Provias et al., 1994; Shukla et al., 1996). Additionally, cadmium elevated the level of ICAM-1, a marker of BBB injury (Jeong et al., 2004). Greater accumulation of cadmium in the brain was found in heavy metal-exposed individuals and in pathological conditions such as gliomas. Average concentrations of cadmium in hippocampus of mercury mine workers and unexposed individuals were 0.366 μg/g wet weight (0.032–0.700) and 0.109 μg/g wet weight (0.012–0.170), respectively (Falnoga et al., 2000). In human brain tumor, the average and maximum concentrations of cadmium measured by atomic absorption spectrometry were 2.02 and 72.78 μg/g wet weight, respectively (Al-Saleh and Shinwari, 2001). Average intracellular cadmium concentrations after treatment with cadmium chloride at 1 and 10 μM for 24 h were below the maximum reported value from glioma tissue, indicating the cadmium level found in brain tissues could lead to increased brain levels of IL-6 and IL-8.

Different cytotoxic levels of cadmium on neurons and astrocytes–Cadmium is toxic to neurons. Exposing primary rat cortical

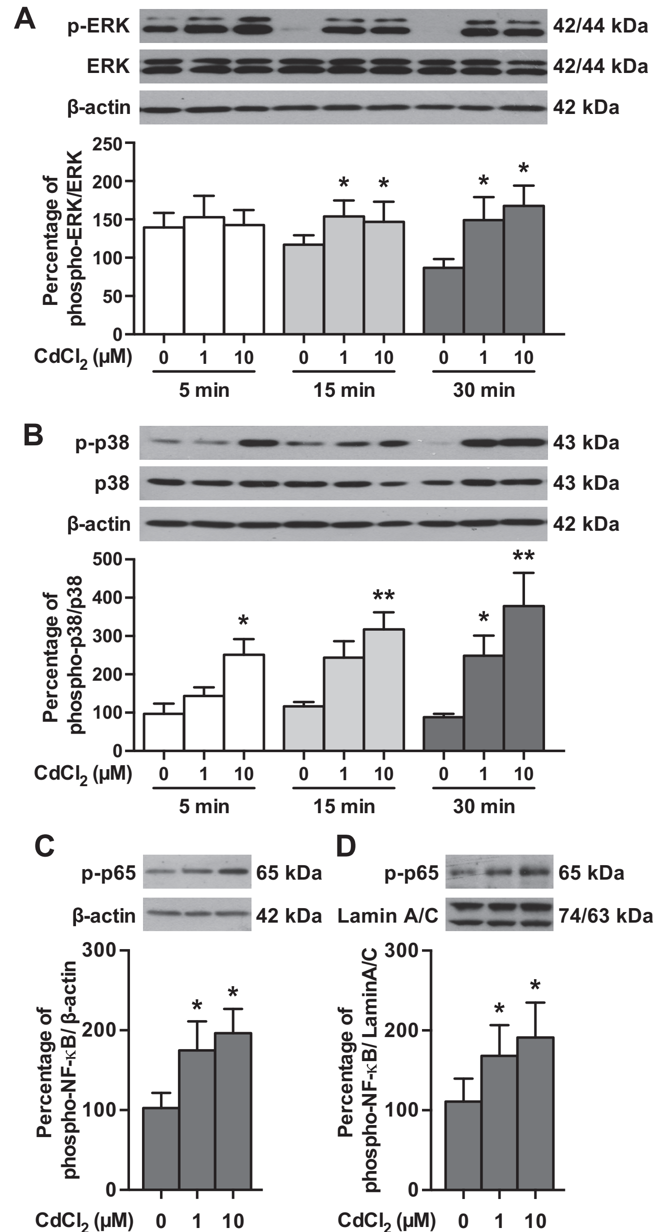

Fig. 6. Cadmium-induced activation of the ERK1/2, p38 MAPK and NF-κB pathways in cultured human astrocytes.
U-87 MG astrocytoma cells were incubated with 1 and 10 μM $CdCl_2$. Levels of phospho-ERK1/2 (p-ERK1/2), ERK, phospho-p38 MAPK (p-p38), p38 MAPK and phospho-p65 (p-p65) were detected by Western Blotting. $CdCl_2$ increased the level of phospho-ERK while the same amount of total-ERK or β-actin was detected at 15 and 30 min post-exposure (A). Increased levels of phosphorylated p38 MAPK were detected at 5 min and continued up to 30 min post-exposure to $CdCl_2$ (B). Elevated phospho-p65 levels were found at 30 min post-exposure to 1 and 10 μM $CdCl_2$ in cytoplasmic extract (C) and nuclear extract (D). b-actin and lamin A/C were used as loading controls for cytoplasmic and nuclear extracts, respectively. Data were representative of at least three independent experiments. Each bar represented mean values (±SEM). Two-way ANOVA was used for statistical analysis and statistical significance was donated as * ($p < 0.05$) and ** ($p < 0.01$) in the comparison between cadmium-treated cells and untreated cells at the same time point.

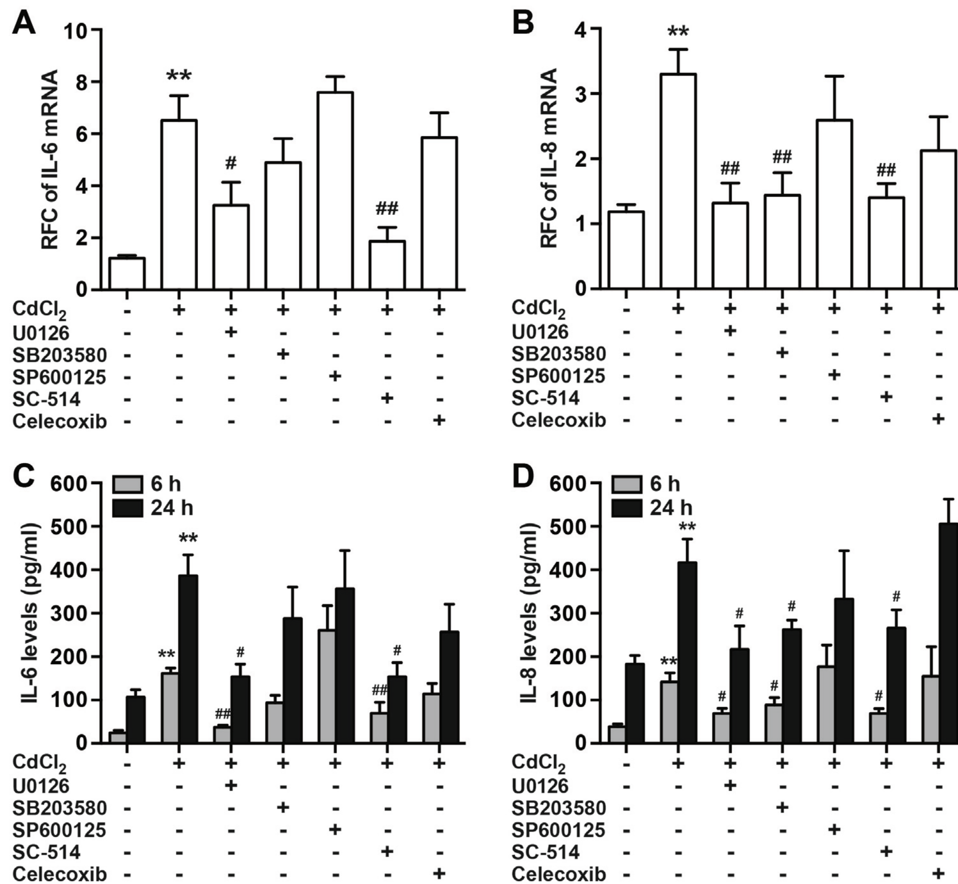

Fig. 7. Inhibition of MAPK and NF-κB pathways suppressed cadmium-induced IL-6 and IL-8 expression and secretion in cultured human astrocytes.
U-87 MG astrocytoma cells were treated with 10 μM $CdCl_2$ in the absence or presence of signaling inhibitors. Levels of IL-6 and IL-8 mRNA transcripts (A and B) and proteins (C and D) were determined using real-time-PCR and ELISA, respectively. 20 μM U0126–an ERK inhibitor–and 10 μM SC-514–an IKKβ inhibitor–decreased IL-6 and IL-8 expression and release (A, C). Additionally, 25 μM SB203580–a p38 inhibitor–inhibited the cadmium-induced IL-8 expression and release. 25 μM SP600125–a JNK inhibitor–and 25 μM celecoxib–a selective COX-2 inhibitor–did not inhibit cadmium-induced IL-6 and IL-8 production. Each bar represented mean values (±SEM) of at least three independent experiments. One-way ANOVA was used for statistical analysis and statistical significance was donated as * ($p < 0.05$) and ** ($p < 0.01$) in comparison between cadmium-treated cells and untreated cells and # ($p < 0.05$) and ## ($p < 0.01$) in comparison of the cadmium-treated cells with or without pretreatment with signaling inhibitors.

neurons to 1–5 μM cadmium for 24 h resulted in approximately 50% reduction in viability (Yang et al., 2008); while the present study showed that this concentration was not toxic to human astrocytes. Cadmium-induced neuronal toxicity resulted from increased reactive oxygen species formation via the MAPK and mTOR pathways (Chen et al., 2008, 2011). However, treatment with up to 30 μM of cadmium did not induce reactive oxygen species generation or increase malondialdehyde level in human astrocytoma cells (data not shown).

Exposure to cadmium was associated with neurocognitive deficits–One epidemiological study of 2183 U.S. children found a significant association between children with high cadmium levels in blood and urine and learning disabilities (Ciesielski et al., 2012). Another study of U.S. adults showed the link between cadmium exposure at the levels without adverse effects and decreased performance in tasks requiring attention and perception (Ciesielski et al., 2013). Occupational cadmium exposure has been linked to slowed visuomotor functioning in neurobehavioural test, peripheral neuropathy and inability to concentrate (Viaene et al., 2000). High blood cadmium levels (>0.6 μg/L) were recently identified as a risk factor for Alzheimer's disease with hazard ratio 3.83 and 95% confidence interval from 1.39 to 10.59 (Min and Min, 2016).

Cadmium exposure causes both peripheral and central inflammation–Cadmium in micromolar concentration increases expression of many proinflammatory markers including inflammatory cytokines, inducible nitric oxide synthase, cyclooxygenase and matrix metalloproteinase in *in vitro* and *in vivo* models of lung and macrophages (Olszowski et al., 2012). Rats that inhaled cadmium experienced an influx of polymorphonuclear leukocytes to airways and lung inflammation (Gavett and Oberdorster, 1994). The present study showed that cadmium at 1 and 10 μM stimulated IL-6 and IL-8 release from astrocytes. Previous studies in gastrointestinal epithelial cells (Hyun et al., 2007), bronchial epithelial cells (Rennolds et al., 2012; Cormet-Boyaka et al., 2012), hepatocytes (Souza et al., 2004; Dong et al., 1998) and PBMC (Horiguchi et al., 1993) reported cadmium promoted upregulation of IL-6 and/or IL-8 much like the present study; however, these studies used higher doses of $CdCl_2$ (>30 μM) compared with the present study, suggesting astrocytes are sensitive to cadmium-induced inflammatory responses.

Astrocytes, the most abundant glia cells, play a role in innate immune response by releasing various proinflammatory mediators. IL-6 triggers astrogliosis and microgliosis as well as the production of prostaglandin E_2 (Spooren et al., 2011; Chikuma et al., 2009). IL-8 is a major chemokine responsible for neutrophil and macrophage recruitment. Elevated brain IL-8 levels could attract neutrophils and macrophages, resulting in accumulation of these cells, and subsequently, stimulation of innate immune responses in the brain. Several studies reported increased IL-8 and/or IL-6 levels in astrocytes triggered by viral infections, i.e., Nef or

Tat proteins for HIV virus (Liu and Kumar, 2015; Nookala and Kumar, 2014) or Core proteins for hepatitis C virus (Vivithanaporn et al., 2010). Similarly, methamphetamine triggered IL-6 and IL-8 production in astrocytes (Shah et al., 2012). Increased IL-6 and IL-8 levels were found in serum, cerebrospinal fluid, and/or brain tissues of Alzheimer's disease and multiple sclerosis patients (Spooren et al., 2011; Kleine et al., 2003) Amyloid β-protein triggers IL-6 production in astrocytes, which in turn stimulates amyloid precursor protein expression in neurons. Additionally, upregulation of C-X-C chemokine receptor 2 (CXCR2) – an IL-8 receptor–has been found in brain tissue of glioma patients (Yang et al., 2015) as well as postmortem human brain tissue of Alzheimer's disease patients (Ryu et al., 2015). IL-8 stimulated the release of matrix metalloproteinase-2 and −9 from primary rat cortical neurons, leading to neuronal death (Thirumangalakudi et al., 2007). In the present study cadmium at concentrations below the cytotoxic threshold induced IL-6 and IL-8 release from astrocytes. Increased IL-6 and IL-8 levels from cadmium could lead to neuroinflammation, neuronal death, and subsequently, neurocognitive impairment.

Cadmium exposure to human astrocytes stimulates the release of cytokines related to invasion and angiogenesis–One epidemiological study of 413,877 Finnish women identified cadmium as a risk factor for brain cancers with a standardized incidence ratio at 1.26 and 95% confidence interval from 0.72 to 2.22 (Wesseling et al., 2002). IL-6 promotes astrocytic chemotaxis and glioblastoma cell invasion (Spooren et al., 2011). IL-6 increases expression of MMP-9 and fascin-1, which promoted cell invasion of U251 and T98G glioblastoma cells. Furthermore, IL-6 stimulates vascular endothelial cell migration, leading to new blood vessel formation (Liu et al., 2010). In addition to immune responses, IL-8 is an important angiogenic factor, enhancing endothelial cell proliferation and matrix metalloproteinase production (Li et al., 2003). Over production of IL-8 in the brain is associated with glioma formation and malignant progression (Brat et al., 2005). The present study showed cadmium at 1 μM stimulated IL-6 and IL-8 release in U-87 MG cells; while secretion of IL-8 from PHA was detectable after exposure to 10 μM cadmium, implying cadmium was more likely to trigger the inflammation cascade in astrocytoma cells than healthy astrocytes. Upregulation of IL-6 and IL-8 induced by low concentrations of cadmium in gliomas could play an important role in gliomagenesis, angiogenesis and metastasis.

Heavy metal induction of IL-6 and IL-8 release in astrocytes–In T98G human astrocytoma cells, $MnCl_2$ at 100–800 μM stimulated production of IL-6 and IL-8 together with increased oxidative stress and apoptotic markers in T98G human astrocytoma cells (Park and Park, 2010). In contrast, in primary rat cortical astrocytes, $MnCl_2$ at 100 μM alone did not stimulate IL-6 expression but potentiated IL-1β/IFN-γ-induced IL-6 expression (Chen and Liao, 2002). In the present study, in U-87 MG human astrocytoma cells, $MnCl_2$ at 10 μM increased IL-6 release at 24 h post-exposure while $CdCl_2$ at 10 and 1 μM promoted IL-6 release at 6 and 24 h post-exposure, respectively. In BEAS–2 B human bronchial epithelial cells, the highest concentration of manganese sulfate and nickel sulfate at 500 μM increased IL-6 and IL-8 levels; while other heavy metals – including vanadium, chromium, zinc, and lead–reduced IL-6 and IL-8 production at 24 h post-exposure (Honda et al., 2015). At 24 h post-exposure, 120 μM nickel nitrate did not induce cytokine production while 120 μM zinc chloride and 60 μM $CdCl_2$ promoted IL-8 production in THP-1 cells. Additionally, $CdCl_2$ increased the release of IL-6 and TNF-α (Freitas and Fernandes, 2011). In comparison, cadmium seems to have the most potent effect on induction of proinflammatory responses in peripheral and central systems.

Cadmium stimulates IL-6 and IL-8 expression by activating MAPK and NF-κB pathways–ERK1/2 and p38 are families of upstream MAPKs activating NF-κB and AP-1 as well as regulating expression of cytokines and chemokines. MAPK activation by cadmium is different between cell types (Matsuoka and Igisu, 2002). Previous studies in primary mice astrocytes demonstrated cytotoxic concentrations of cadmium at 10 μM activated MAPKs – ERK1/2, JNK, and p38 (He et al., 2015; Jiang et al., 2015; Jo and Koh, 2013). Cadmium-induced IL-6 and IL-8 expression is associated with activation of ERK1/2, p38 MAPK and/or NF-κB depending on cell types. The present study showed increased phosphorylation of ERK1/2 and p38 MAPK shortly after exposure to cadmium and inhibition of ERK1/2 and p38 MAPK signaling strongly suppressed IL-8 mRNA expression and release of this chemokine while increased IL-6 mRNA expression and release is mediated via ERK1/2, not p38. Additionally, cadmium induced phosphorylation of the p65 NF-κB pathway and inhibition of NF-κB pathway reduced IL-6 and IL-8 expression and release. In human bronchial epithelial cells, cadmium-induced IL-6 and IL-8 was mediated by two different pathways. Suppression of NF-κB signaling inhibited cadmium-induced IL-6, while ERK1/2 and p38 MAPK mediated cadmium-induced IL-8 (Rennolds et al., 2012; Cormet-Boyaka et al., 2012). In contrast, increased IL-8 mRNA and protein expression after exposure to toxic doses of cadmium in human intestinal epithelial cells and THP-1 monocytic was mediated by activation of the NF-κB pathway via I-κBα degradation (Rennolds et al., 2012; Freitas and Fernandes, 2011).

MAPK activation by cadmium is mediated through intracellular calcium elevation (Matsuoka and Igisu, 2002; Jiang et al., 2015). High concentrations of cadmium increased oxidative stress and cell death in primary rat astrocytes via the activation of calcium signaling (Yang et al., 2008). Elevation of intracellular calcium by cadmium stimulated the MAPK and PI3K/Akt signaling pathways, leading to cell death of primary mice astrocytes. BAPTA-AM–an intracellular Ca^{2+} chelator – inhibited phosphorylation of ERK1/2, p38, and JNK in mice astrocytes (Jiang et al., 2015). Similarly, BAPTA-AM decreased JNK phosphorylation in LLC-PK1 kidney proximal tubule cells (Matsuoka and Igisu, 2002). Determining whether elevated intracellular calcium concentration plays a role in cadmium-induced IL-6 and IL-8 could lead to another potential target to reduce cadmium-induced inflammation.

Upregulation of cadmium-induced IL-6 and IL-8 is not mediated by COX-2–Upregulation of COX-2 leads to production of prostaglandins including PGE_2. Cadmium increased COX-2 levels in C6 rat glioma and mouse neuronal cells (Figueiredo-Pereira et al., 2002; Park et al., 2012). PGE_2 increased IL-6 release in U373 MG human astrocytoma and primary rat astrocytes via activation of p38 MAPK and protein kinase C (Fiebich et al., 2001). In MCF-7 breast cancer cells, increased expression of COX-2 was correlated with IL-8 levels (Simeone et al., 2007). In the present study, inhibition of COX-2 activity by celecoxib–a specific COX-2 inhibitor–had no effect on cadmium-induced IL-6 and IL-8 mRNA expression and release, indicating COX-2 did not play a role in cadmium-induced cytokine production.

5. Conclusion

In addition to direct toxicity via oxidative stress, cadmium–at non-cytotoxic concentrations–promoted inflammatory responses in the brain by upregulating IL-6 and IL-8 expression and release from astrocytes via the MAPK and NF-κB pathways. Increased IL-6 and IL-8 levels may play a role in cognitive impairment, neurobehavioral disorders and metastasis of gliomas in cadmium-exposed individuals. Therefore, reducing IL-6 and IL-8 production

and effects could be potential targets for prevention of neuro-inflammation and neurotoxicity caused by pollutants, such as cadmium.

Conflict of interest

All authors declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2017.03.001.

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