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Cadmium induced ROS alters M1 and M3 receptors, leading to SN56 cholinergic neuronal loss, through AChE variants disruption

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ABSTRACT

Cadmium, an environmental neurotoxic compound, produces cognitive disorders, although the mechanism remains unknown. Previously, we described that cadmium induces a more pronounced cell death on cholinergic neurons from basal forebrain (BF). This effect, partially mediated by M1 receptor blockade, triggering it through AChE splices variants alteration, may explain cadmium effects on learning and memory processes. Cadmium has been also reported to induce oxidative stress generation leading to M2 and M4 muscarinic receptors alteration, in hippocampus and frontal cortex, which are necessary to maintain cell viability and cognitive regulation, so their alteration in BF could also mediate this effect. Moreover, it has been reported that antioxidant treatment could reverse cognitive disorders, muscarinic receptor and AChE variants alterations induced by cadmium. Thus, we hypothesized that cadmium induced cell death of BF cholinergic neurons is mediated by oxidative stress generation and this mechanism could produce this effect, in part, through AChE variants altered by muscarinic receptors disruption. To prove this, we evaluated in BF SN56 cholinergic neurons, whether cadmium induces oxidative stress and alters muscarinic receptors, and their involvement in the induction of cell death through alteration of AChE variants. Our results show that cadmium induces oxidative stress, which mediates partially the alteration of AChE variants and M2 to M4 muscarinic receptors expression and blockage of M1 receptor. In addition, cadmium induced oxidative stress generation by M1 and M3 receptors alteration through AChE variants disruption, leading to cell death. These results provide new understanding of the mechanisms contributing to cadmium harmful effects on cholinergic neurons.

1. Introduction

Cadmium is a persistent environmental neurotoxic pollutant (ATSDR, 2012). Exposure to this metal has been associated with numerous human pathologies as osteoporosis, kidney dysfunction, respiratory ailments, birth defects and neurodegenerative diseases (Jahan et al., 2014; Mendez-Armenta and Rios, 2007). Cadmium induces cognitive disorders similar to Alzheimer's disease (AD) symptoms (Wang and Du, 2013), signaling it as a possible etiological factor of AD (Jiang et al., 2007). However, the mechanisms through which cadmium induces cognitive dysfunctions remain unknown.

Previously, we described that cadmium induces a more pronounced, but not selective, cell death on primary cholinergic neurons from basal forebrain (Del Pino et al., 2014). Degeneration of septal cholinergic neurons from basal forebrain, as happens in AD, results in memory deficits (Scheiderer et al., 2006). Thus, cholinergic neuronal loss in this region may be related with cadmium impairment of memory function, among other actions (Andersson et al., 1997).

Cadmium induced basal forebrain cholinergic neuronal loss was partially mediated by selective blockade of M1R through alteration of AChE splices variants expression (Del Pino et al., 2016a), although other mechanisms should be involved. Muscarinic receptor subtypes

Abbreviations: Ach, acetylcholine; AChE, acetylcholine esterase; CHAT, acetylcholine transferase; GFAP, glial fibrillary acidic protein; DMSO, dimethylsulphoxide; EMEM, eaglés minimum essential medium; FBS, fetal bovine serum; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; LDH, lactate deshidrogenase; MAP2, microtubule associated protein-2; BSA, bovine serum albumin; iso-OMPA, tetraisopropylpyrophosphoramide

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M1-M5 are necessary to maintain cell viability and regulate learning and memory processes (Bainbridge et al., 2008; Bubser et al., 2014; Galloway et al., 2014; Zheng et al., 2012). Cadmium has been reported to downregulate M1, M2 and M4 muscarinic receptor gene expression in frontal cortex and hippocampus (Gupta et al., 2017), so cadmium could also alter other muscarinic receptors besides M1 in basal forebrain, which could induce the effect observed on cholinergic neurons.

In addition, cadmium induces oxidative stress through reactive oxygen species (ROS) formation, depletion of antioxidant defenses, and reduction of antioxidant enzymes (Gonçalves et al., 2010; Luchese et al., 2007; Nemmiche, 2017; Pari and Murugavel, 2007; Thévenod, 2009). Oxidative stress has been involved in the induction of cognitive disorders and cadmium-antioxidant co-treatment could reverse cognitive disorders induced by cadmium (Gonçalves et al., 2010; Gupta et al., 2017; Maodaa et al., 2016). Oxidative stress is also able to induce muscarinic receptor activity dysfunction, AChE splice variants gene expression alteration and basal forebrain cholinergic neurons cell death (Bond et al., 2006; Del Pino et al., 2016c; Giraldo et al., 2014; Gupta et al., 2017), which could also contribute to explain the effect observed.

According to this, we hypothesized that oxidative stress produced by cadmium exposure alters muscarinic receptors, which disrupts AChE variants expression, partially inducing the cell death observed in basal forebrain cholinergic neurons. The present work intends to study the mechanisms involved in basal forebrain cholinergic neuronal loss, due to the importance of this effect to explain toxicity of cadmium on memory function and neurodegenerative diseases symptoms like.

2. Materials and methods

2.1. Chemicals

The compounds cadmium chloride (99.99% trace metals basis), dimethyl sulfoxide (DMSO), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma (Madrid, Spain). [^{3}H] pirenzepine was obtained from Perkin Elmer (Madrid, Spain). Whatman GF/B glass fiber filter paper was obtained from Millipore (Madrid, Spain). All other chemicals were reagent grade of the highest laboratory purity available.

2.2. Culture of SN56 cells

SN56 cells (gift from Dr. Sandra Petersen, University of Massachusetts, USA), a cholinergic murine neuroblastoma cell line derived from basal forebrain septal neurons (Hammond et al., 1990), with stable expression of AChE and ChAT between 20 and 40th passage, were used as a model of cholinergic neurons from basal forebrain to evaluate whether oxidative stress, through muscarinic receptors alteration, mediates the cell death induced by cadmium on cholinergic neurons. The cells were maintained at 37 °C and 5% CO_2 in DMEM supplemented with 10% FBS, penicillin/streptomycin, 2 mM L-glutamine (Sigma, Madrid, Spain), and 1 mM sodium pyruvate. Medium was changed every 48 h (Hudgens et al., 2009). Cells were seeded in 6-well plates at a density of 10^6 cells/well. Cells were treated for 24 h with cadmium chloride (1 to 100 μM) with or without the antioxidant N-acetyl cysteine (NAC) (1 mM) to research the implication of oxidative stress, muscarinic receptors and AChE variants in the possible mechanisms related to cell death. A vehicle group containing 0.1% DMSO was employed in parallel for each experiment as a control.

In a previous work, we show that cadmium is able to block M1R, affect the AChE splice variants gene expression and induce cell death from 1 to 100 μM concentrations, which has been described to be relevant for chronic and highly acute human exposure (Del Pino et al., 2016b). According to this, we have chosen 1 to 100 μM concentrations to evaluate concentration-dependent effect on oxidative stress production and muscarinic receptors alteration induced by cadmium and the concentration of 10 μM to test the hypothesis stated in the present work.

2.3. Measurement of cell viability (MTT assay)

SN56 cells viability and mitochondrial dehydrogenase activity was measured by MTT after 48 h transfection. The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenase. Cells were incubated with 100 μl of yellow MTT solution (final concentration 0.5 mg/ml) for 4 h after 48 h transfection. After 4 h at 37 °C, the medium was removed and the formazan reaction product was dissolved in 250 μl DMSO. Absorbance was read at 562 nm (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Madrid, Spain) and results were expressed as mean percentage of viable cells relative to untreated controls. Control cells were taken as 100% viability.

2.4. Lactate dehydrogenase (LDH) assay

The extent of cell death was assessed by measuring the LDH released into the culture medium using Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions. Briefly, the culture medium was removed and pipetted into 96-well plates. The Master Mix reagent was added, and after 3 min colorimetric intensity was determined at 450 nm over every 5 min using a microplate spectrophotometer (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Madrid, Spain).

2.5. Protein determination

At the end of the treatments, SN56 cells were washed with pre-chilled phosphate-buffered saline (PBS), collected by scrapping, and lysed using RIPA buffer (Thermo Scientific, Madrid, Spain) with freshly added protease inhibitors cocktail (Thermo Fisher Scientific, Madrid, Spain). After centrifugation at 10,000 × *g* for 10 min at 4 °C, cell lysate supernatant was collected. Protein concentration was assayed using a BCA kit (Thermo Fisher Scientific, Madrid, Spain) and normalized.

2.6. Gene knockdown

SN56 cells were transfected with siRNAs in 6-well plates (1×10^6 cells/well) using HiPerfect Transfection reagent according to the manufacturer's instructions (Qiagen, Barcelona, Spain). Two sets of siRNA duplexes (Qiagen, Barcelona, Spain) homologous to mouse AChE, M1R, M2R, M3R, M4R and M5R sequences for each one were designed using the HiPerformance Design Algorithm (Norvatis AG) and were purchased from Qiagen (catalog numbers GS11423, GS12669, GS243764, GS12671, GS12672 and GS213788, respectively, for mouse). As a transfection control, an All Stars Negative Control siRNA (Qiagen, Barcelona, Spain) was used. 48 h after transfection, the efficiency of single or simultaneously siRNA-mediated AChE, M1R, M2R, M3R, M4R and M5R knockdown was determined by RT-PCR using primers specific for mouse AChE, M1R, M2R, M3R, M4R and M5R mRNA (Qiagen, Barcelona, Spain). The knockdown effects of AChE, M1R, M2R, M3R, M4R and M5R on cell injury were tested by MTT cell viability assay. After 24 h of incubation with the siRNAs, the cells were washed with PBS and incubated for a further 24 h in culture medium with or without cadmium.

2.7. Real-time PCR analysis

Total RNA was extracted using the Trizol Reagent method (Invitrogen, Madrid, Spain). The final RNA concentration was determined using a spectrophotometer Nanodrop 2000 (Thermo Fisher Scientific, Madrid, Spain) and the quality of total RNA samples was assessed using an Experion Lab Chip (Bio-Rad, Madrid, Spain) gel. First-