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Chloroacetic acid triggers apoptosis in neuronal cells via a reactive oxygen species-induced endoplasmic reticulum stress signaling pathway

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ABSTRACT

Chloroacetic acid (CA), a chlorinated analog of acetic acid and an environmental toxin that is more toxic than acetic, dichloroacetic, or trichloroacetic acids, is widely used in chemical industries. Furthermore, CA has been found to be the major disinfection by-products (DBPs) of drinking water. CA has been reported to be highly corrosive and to induce severe tissue injuries (including nervous system) that lead to death in mammals. However, the effects and underlying mechanisms of CA-induced neurotoxicity remain unknown. In the present study, we found that CA (0.5–2.0 mM) significantly increased LDH release, decreased the number of viable cells (cytotoxicity) and induced apoptotic events (including: increases in the numbers of apoptotic cells, the membrane externalization of phosphatidylserine (PS), and caspase-3/-7 activity) in Neuro-2a cells. CA (1.5 mM; the approximate to LD_{50}) also triggered ER stress, which was identified by monitoring several key molecules that are involved in the unfolded protein responses (including the increase in the expressions of p-PERK, p-IRE-1, p-eIF2α, ATF-4, ATF-6, CHOP, XBP-1, GRP 78, GRP 94, and caspase-12) and calpain activity. Transfection of GRP 78- and GRP 94-specific si-RNA effectively abrogated CA-induced cytotoxicity, caspase-3/-7 and caspase-12 activity, and GRP 78 and GRP 94 expression in Neuro-2a cells. Additionally, pretreatment with 2.5 mM *N*-acetylcysteine (NAC; a glutathione (GSH) precursor) dramatically suppressed the increase in lipid peroxidation, cytotoxicity, apoptotic events, calpain and caspase-12 activity, and ER stress-related molecules in CA-exposed cells. Taken together, these results suggest that the higher concentration of CA exerts its cytotoxic effects in neuronal cells by triggering apoptosis via a ROS-induced ER stress signaling pathway.

1. Introduction

Toxic agents in the environment (both those with natural origins and those that originate from manufacturing) are postulated to play important roles in the etiology and development of many diseases. Humans can be exposed to these unrestrained agents in the form of occupational pollutants or toxic waste during the production process or via the consumption of contaminated food or products. It has been indicated that exposure to these agents can trigger a multitude of deleterious reactions that induce tissue/organ injuries, and exacerbate the development of many diseases such as cancer, diabetes mellitus and neurodegenerative disorders [1–4].

Abbreviations: CA, chloroacetic acid; LDH, lactate dehydrogenase; ER, endoplasmic reticulum; GRP, glucose-regulated protein; CHOP, C/EBP homologous protein; XBP-1, X-box binding protein 1; ATF, activation transcription factor; PERK, protein kinase R-like ER kinase; IRE-1, inositol-requiring enzyme-1; eIF2α, eukaryotic translation initiation factor 2α; siRNA, small interference RNA; NAC, *N*-acetylcysteine; GSH, glutathione; ROS, reactive oxygen species.

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Chloroacetic acid (CA) is a chlorinated analog of acetic acid that is widely used in the applications of the chemical industry, which include its use as a postemergence contact herbicide, detergent, disinfectant, drug; it is also involved as an intermediate in the synthesis of a number of chemicals, including caffeine, vitamins, and chemical dyes [5–6]. CA is also one of the most commonly detected disinfection by-products (DBPs) during the drinking water chlorination disinfection process in the United States and other countries [7–8]. CA is rapidly and efficiently absorbed through the G-I tract and skin and is not only highly corrosive to tissues but can also cause systemic injury and death following accidental ingestion or dermal exposure [9–11]. Animal studies have revealed severe histopathological changes and tissue injuries (including mild to moderately enlarged livers with random bile duct proliferation, fibrosis, edema, and occasional inflammatory foci, foci of perivascular inflammation in the small pulmonary veins, alveolar injury, hemorrhagic and congested lungs, and increases in alanine aminotransferase, aspartate aminotransferase, creatinine, and blood urea nitrogen levels) after CA exposure (15–200 mg/kg/day) [12–16]. More importantly, Berardi et al. [17] and Bhat et al. [18] have indicated that CA has neurotoxic properties in mammals and damages the blood–brain barrier (BBB) function and can penetrate the brain, which leads to the loss of Purkinje cells in the cerebellum and the production of pyknotic nuclei in mice and rats. However, the toxicological effects and the possible mechanisms underlying CA-induced neurotoxicity remain mostly unclear.

Oxidative stress is a crucial factor for many undesirable biological reactions, and plays a role in functional cell and tissue injuries, as well as the development of many diseases. Physiological levels of reactive oxygen species (ROS) are important in maintaining several cellular functions, but overproduction of ROS that exceeds antioxidant capacity and destroys the prooxidant/antioxidant balance induces oxidative stress, thereby causing cell damage and death [19–20]. The formation of excessive ROS, which is induced by toxic insults, has been reported to cause DNA damage, to modify proteins and lipids functions, and to activate related signaling pathways under pathophysiological conditions that are indicative of the development of numerous diseases, including neurological disorders [1,21–22]. Furthermore, an increasing number of studies have indicated that toxic insults can induce endoplasmic reticulum (ER) stress, which elicits the excessive and long-term upregulation of the unfolded protein response (UPR; a mechanism that restores homeostasis in the ER), leading to apoptosis [23–24]. The ER is an important organelle and the cellular compartment that is responsible for the biosynthesis, proper folding, post-translational modification, and transport of nascent proteins to different destinations [25–26]. ER damage, which is caused by disturbances in the structure and function of the ER with the accumulation of misfolded proteins and changes in calcium homeostasis, results in overloading of the ER chaperones and their failure to fold and export newly biosynthesized proteins, leading to ER stress and cell apoptosis [26–27]. ER stress protein-mediated apoptosis has been demonstrated to be involved in the development of various diseases [28], and neuronal cells have a highly developed ER, which allows them to easily destroy the ER function following exposure to various agents, thereby leading to cell death and apoptosis and the development of neurodegenerative diseases [29–30]. Recently, an increasing number of studies have indicated that disruption of ER function by chemicals or stress stimuli, such as heavy metals, hypoxia, and the inhibition of protein glycosylation, significantly alters the expression of ER stress-related proteins (such as protein kinase R-like ER kinase (PERK), eukaryotic translation initiation factor 2α subunit (eIF2α), activation transcription factor (ATF)6, C/EBP homologous protein (CHOP), X-box binding protein 1 (XBP-1), glucose-regulated protein (GRP) 78, and GRP 94), which leads to apoptosis [24,31–33].

ER stress has been reported to play a key role in several human diseases, including neuronal diseases [29–30,34]. Recent studies have indicated that toxic insults that cause mammalian cell (such as pancreatic β-cells, renal cells, and myoblasts) death are involved in the induction of oxidative stress and ER stress-regulated apoptotic pathways [23–24,31]. However, the relationship between oxidative stress and the ER stress-regulated apoptotic signals involved in CA-induced neuronal cell death are not yet fully understood. In this study, we attempted to elucidate the crucial role of ER stress in CA-induced cytotoxicity in neuronal cells. We also investigated whether ROS can regulate the CA-induced ER stress signals-mediated apoptotic pathway in neuronal cells.

2. Materials and methods

2.1. Cell culture

Murine neuroblastoma cell line: Neuro-2a (CCL-131, American Type Culture Collection) was cultured in plastic tissue culture dish in a humidified chamber with a 5% CO_2–95% air mixture at 37 °C. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco BRL, Life Technologies, Inc., USA). The cells were seeded on 6-, 12-, or 24-well culture plates for each experiment and allowed to grow for 12–18 h prior to treatment with CA (the final pH of the culture media was approximately 7.4–7.6) for different time intervals.

2.2. Cell viability and cytotoxicity assay

Neuro-2a cells were washed with fresh media, cultured in 96-well plates (2×10^4 cells/well), and then incubated with CA (0.5–2 mM) for 24 h. After incubation, the medium was aspirated and fresh medium containing 30 μL of 2 mg/mL 3-(4,5-dimethyl thiazol-2-yl-)-2,5-diphenyl tetrazolium bromide (MTT) was added. After 4 h, the medium was removed and replaced with blue formazan crystals that were dissolved in dimethyl sulfoxide (100 μL; Sigma, St. Louis, MO, USA). The absorbance at 570 nm was measured using an enzyme linked immunosorbent assay (ELISA) microplate reader (Bio-Rad, model 550, Hercules, CA, USA).

Cytotoxicity was determined based on the amount of lactate dehydrogenase (LDH) that leaked out of the cytosol of damaged cells into the medium after CA exposure for 24 h. The cells were seeded as described for the MTT assay. After 24 h of treatment, 40 μL of the supernatant was added to a new 96-well plate to determine LDH release, and cell lysis buffer was also added to the positive control group to determine total LDH. The amount of LDH that had been released from the cells was quantified using the LDH Cytotoxicity Assay Kit (BioVision, Inc., USA) according to the manufacturer's instructions. The absorbance was measured using an ELISA microplate reader (Bio-Rad, model 550, Hercules, CA, USA) at 490–500 nm.

2.3. Analysis of apoptosis

2.3.1. Apoptotic cell detection

Apoptosis (the process of programmed cell death) is an important process that is used by most cells to negatively select for cells with deleterious damage. Dual staining of Annexin V-Cy3 (Ann Cy3) and 6-carboxy fluorescein diacetate (6-CFDA) fluorescence probes was used to detect apoptotic cells [4]. The cells were treated with or without CA for 24 h. After treatment, the cells were washed twice with PBS (pH 7.4), and incubated with Annexin V-Cy3 (Ann-Cy3) and 6-carboxy fluorescein diacetate (6-CFDA) simultaneously (Annexin V-Cy3™ Apoptosis Detection Kit). After being labeled at