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Acrylamide-induced mitochondria collapse and apoptosis in human astrocytoma cells

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ABSTRACT

Acrylamide (ACR) can be produced during food processing and has neurotoxic effects in humans. This study aims to determine ACR induced apoptotic responses in human astrocytoma U-1240 MG cells to realize the incurred toxic mechanisms. Under 1 and 2 mM ACR exposure, cell viability decreased as time increased. The increments in sub-G_1 phase were 87.5-fold, and pro-caspase 3 and PARP protein expressions decreased 35% and 54.5% respectively relative to the control after 2 mM ACR treatment. Molecular evidence of Bax/bcl-2 ratio and cytochrome c expression increased 8.86-fold and 6.81-fold as well as pro-caspase 9 decreased 67.8% relative to the control respectively under 2 mM ACR exposure. Trolox, an ROS scavenging agent, attenuated cell death and induced ROS production by 2 mM ACR. The ultrastructure alterations of mitochondria showed marked vesicular matrix compartmentalization and cytoplasmic vacuole formation after 2 mM ACR was treated for 48 h, whereas those treated for 72 h showed chromatin condensation, pyknosis, and swelling. These results indicate long-term exposure to ACR induced mitochondria collapse and finally led to apoptosis. Although 2 mM ACR is higher than average daily intake dosage, workers in chemical industries may be exposed to sufficient doses to entail health risks.

1. Introduction

Acrylamide (ACR), a water-soluble vinyl monomer, has many applications in chemical industries and laboratories (Tareke et al., 2000). ACR can be produced during food processing under high temperature via the Maillard reaction (Tareke et al., 2002), especially during processing of food containing asparagine and glucose (Friedman, 2003) such as coffee and potato chips (Eberhart et al., 2005; Soares et al., 2010). Many studies reveal that ACR has neurological and reproductive toxicities (Kutting et al., 2009; Parzefal, 2008) and has induced tumors in multiple organs in animals (Friedman et al., 1995; Johnson et al., 1986). Increasing incidences of endometrial, ovarian, and renal cancer (but not brain cancer) with increased dietary ACR intake have been reported in humans (Hogervorst et al., 2009).

Oxidative stress results from excess reactive oxygen species (ROS), which include hydroxyl radical, superoxide anion, and hydrogen peroxide. ROS can lead to the destruction of cellular macromolecules, including lipids, proteins, and DNA, and ultimately to cell death via apoptosis (Rodriguez et al., 1997; Halliwell, 2006). There are two major apoptotic pathways involved in the activation of caspases (Earnshaw et al., 1999; Strasser et al., 2000). The extrinsic pathway is triggered by Fas and the tumor necrosis factor (TNF) family through death receptors and leads to the activation of initiator caspase 8 followed by cleavage of downstream effector caspases. The intrinsic pathway is triggered by the release of cytochrome c from mitochondria and results in the activation of the initiator caspase 9, which then cleaves pro-caspase 3 for caspase 3 activation and cleaves poly(ADP-ribose) polymerase (PARP) for PARP inactivation (Nagata, 2000; Enari et al., 1998).

The bcl-2 protein family is a protein group mediating mitochondria functions and includes anti-apoptotic proteins (bcl-2 and bcl-XL) and pro-apoptotic proteins (Bax and Bad) (Hockenbery et al., 1993). During apoptosis, Bax recruited to mitochondrial membranes causes pore formation and cytochrome c release (Jürgensmeier et al., 1998), and then activates downstream

Abbreviations: ACR, acrylamide; BER, base excision repair; BS, bovine serum; DCHFDA, 2,7-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; MEM, minimum essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MMP, mitochondria membrane potential; PARP, poly(-ADP-ribose) polymerase; ROS, reactive oxygen species; TEM, transmission electron microscope.

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caspases. Bcl-2 can inhibit the pore-forming activity of Bax and stabilizes mitochondrial integrity (Tatton and Olanow, 1999).

ACR has been reported to induce apoptosis in various studies, including *in vitro* studies on human liver embryo L-02 (Xu et al., 2009) and human neuroblastoma SH-SY5Y (Sumizawa and Igisu, 2007) cell lines, and *in vivo* studies of the testes in Sprague-Dawley rats (Yang et al., 2005) and of CNS and PNS tissue in Wistar rats (Li et al., 2006). ACR also affects redox status and generates oxidative stress in hepatocytes (Cao et al., 2008), colon cells (Rodríguez-Ramiro et al. 2011), and nervous system cells (Park et al., 2010; Pernice et al., 2009), and then diminishes cell functions and leads to cell death ultimately.

Astrocytes are supportive, sensory cells in neural tissue (Sofroniew and Vinters, 2010) and protect neurons from nitric oxide toxicity (Chen et al., 2001). Previous studies showed that ACR has apoptotic effects on neurons (Sumizawa and Igisu, 2009) and ACR can pass through the blood–brain barrier (Sweeney et al., 2010). In our earlier studies, ACR caused DNA damage, inhibition in cellular proliferation, and cell cycle arrest in human astrocytoma cells (U-1240 MG), and the phosphorylation of p53 was also involved (Chen et al., 2009, 2010), while the mechanism was not well established. We propose that ACR increases ROS accumulation, and then induces apoptosis via mitochondrial related proteins. Therefore, the aim of this study is to ascertain the hypothesized mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

ACR, dimethyl sulfoxide (DMSO), 2,7-dichlorodihydrofluorescein diacetate (DCHFDA), ethanol, formaldehyde, JC-1, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide, Triton X-100, Trolox, and Tween 20 were from Sigma–Aldrich (St. Louis, MO); Tris (base) was from J.T. Baker (Phillipsburg, NJ); skim milk powder was from Anchor (Auckland, NZ); and RNase A was from Fermentas (Vilnius, Lithuania). All cell culture reagents and saline buffers were from Gibco (Rockville, MD). Antibodies against pro-caspase 9, Bax, bcl-2, and α-tubulin were from GeneTex (Irvine, CA); bcl-XL, Bad, and DNA ligase III (Lig III) were from Abcam (Cambridgeshire, UK); cytochrome c was from Invitrogen (Grand Island, NY); and pro-caspase 3 and PARP were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture and treatments

The human astrocytoma cell line U-1240 MG was obtained from the Ohio State University (Columbus, OH). U-1240 MG were cultured in minimum essential medium (MEM) supplemented with 10% (v/v) heat-inactivated bovine serum (BS), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2, 100% humidified atmosphere. ACR with various concentrations (i.e., 0, 0.1, 0.5, 1, and 2 mM) were prepared in the culture medium and added to the cells. The treated cells were cultured for various duration (0, 24, 48, and 72 h).

2.3. Cell viability analysis

Cell viability was evaluated by MTT assay (Denizot and Lang, 1986). Tetrazolium salts are metabolized by active mitochondrial dehydrogenases to form a blue formazan dye for the measurement of cell viability. Cells were plated at a density of 5×10^3 cells/well into 96-well plates, followed by treatment with different concentrations of ACR or co-treatment with Trolox (final concentration 5 mM) simultaneously. After washing the cells, culture medium containing 0.5 mg/mL of MTT was added to each well. The cells were incubated at 37 °C for 3 h, and the formazan crystals formed in viable cells were solubilized with 100 μL of dimethyl sulfoxide. The absorbance was measured at 590 nm with an ELISA Reader (TECAN, Switzerland).

2.4. Cell-cycle analysis

U-1240 MG cells were fixed in 70% cold ethanol at −20 °C overnight according to our previous protocol (Chen et al., 2010). The cells were treated with RNase A (0.8 μg/mL) in PBS with 0.5% Triton X-100 at 37 °C for 30 min and incubated in 0.5 mL of PBS with propidium iodide (20 μg/mL) for another 10 min at room temperature in the dark. The cell cycle was analyzed for DNA content with a BD LSR-Fortessa flow cytometer (BD, NJ) and Win-MDI (Version 2.9) software.

2.5. Determination of mitochondria membrane potential

Changes in mitochondrial membrane potential (MMP) were detected and measured using JC-1 staining through a flow cytometer (BD, NJ). The use of the JC-1 dye was modified from the protocol set out by Yao et al. (2007). In brief, after being treated, the cells were rinsed with PBS and then stained with 2.5 μg/mL of JC-1 dye for 20 min in the dark at room temperature. The cells were then washed with PBS once more prior to the MMP analysis. Photomultiplier settings were adjusted so as to detect JC-1 monomer fluorescence signals with the FITC detector (green fluorescence, centered at around 525 nm) and JC-1 aggregate fluorescence signals with the PE detector (red fluorescence, centered at around 590 nm).

2.6. Determination of ROS production

The production of ROS was monitored by ELISA Reader using DCHFDA (Jang and Surh, 2001). This dye is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield dichlorofluorescein (DCHF), which is trapped within cells. Hydrogen peroxide or low-molecular-weight hydroperoxides produced by cells oxidize DCHF to the highly fluorescent compound, 2,7-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by cells. After treated with ACR and ACR with Trolox (positive control) simultaneously, cells were washed twice with PBS to remove the extracellular compounds, and DCHFDA (final concentration 10 μM) was added for 30 min incubation. After washing twice with PBS, the production of ROS was detected by ELISA Reader at an excitation wavelength of 492 nm and an emission wavelength of 517 nm (Beckman, CA).

2.7. Western blot analysis

Western blot analysis was carried out based on our previous protocol (Chen et al., 2009). After treatment with ACR, total proteins were extracted with CelLytic™ Cell Lysis Reagent (Sigma, MO) supplemented with 100 μL/mL of protease inhibitor cocktail (Fermentas, Lithuania). Protein concentrations were determined by Pierce® BCA Protein Assay kit (Thermo, IL). For analysis, the protein (15 μg per slot) was subjected to dodecyl sulfate polyacrylamide gel (SDS–PAGE, 8% polyacrylamide gel) electrophoresis at 100 V for 1 h and subsequently transferred to a poly-vinylidene fluoride membrane (Millipore, MA) at 100 mA for 1 h. After blocking with skim milk for 1 h, the membrane was then incubated with primary antibody at 4 °C overnight and with a peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein levels were detected with a Western lightning kit (Millipore, MA), with α-tubulin as an internal control. Films were scanned with a Geliance 600 Imaging system (Perkin Elmer, MA), and the data were quantified with Multi Gauge V3.0 software (Fujifilm, Stamford, CT).

2.8. Electron microscopic observation

The electron microscopic observations were carried out according to Cheng et al. (2002) at Joint Center for Instruments and Researches, College of Bioresources and Agriculture in National Taiwan University. After treatment with ACR, the cells were washed with PBS and then prefixed in 2.5% (v/v) glutaraldehyde in 0.1 M/pH 7.3 PBS at 4 °C overnight, washed with PBS three more times, and then postfixed in 1% osmium tetroxide in the same buffer for 1 h at room temperature. The cells were dehydrated in a graded ethanol series and embedded in Spurr's medium. Ultra-thin sections were made using an ultramicrotome with a glass knife and then stained with 5% uranyl acetate for 20 min, followed by lead citrate for 4 min. The sections were examined and photographed under a transmission electron microscope (TEM, JEM1400, Joel, Tokyo, Japan) operated at 80 kV.

2.9. Statistical analysis

Data were presented as mean ± standard deviation. Each experiment was performed in triplicate at least three times on different days. The statistical significance was determined independently by the Student's *t* test for comparisons of two groups. The differences were considered statistically significant when *p* values were less than 0.05.

3. Results

3.1. ACR induced cell death and activated apoptosis regulatory molecules

The U-1240 MG cell death induced by the ACR was analyzed via MTT assay. As shown in Fig. 1, ACR caused a significant decrease in cell viability in both a time- and dose-dependent manner. The genomic DNA of cells under apoptosis were degraded into final DNA fragments that were 200 bp long, which were observed on