Toxicology in Vitro 39 (2017) 119–125

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Effect of acrylamide-induced neurotoxicity in a primary astrocytes/microglial co-culture model

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ARTICLE INFO

Article history:
Received 28 September 2016
Received in revised form 4 November 2016
Accepted 5 November 2016
Available online 9 November 2016

Keywords:
Acrylamide
Neurotoxicity
Oxidative stress
Co-cultural cells
Microglia
Astrocytes

ABSTRACT

Acrylamide (AA), is a common food contaminant generated by heat processing. Astrocytes and microglia are the two major glial cell types in the brain that play pivotal but different roles in maintaining optimal brain function. The objective of this study is to investigate the neurotoxicity of AA, using a primary astrocytes/microglia co-culture model. Co-cultural cells obtained from Balb/c mice were cultured and treated with 0–1.0 mM AA for 24–96 h. Cell viability, reactive oxygen species (ROS) generation, oxidative end produces formation and glutathione (GSH) levels were measured. The expression of nuclear-E2-related factor 2(Nrf2), and nuclear factor kappa-beta (NF-κB) and selected down-stream genes were measured. Results showed that AA treatment led toa dose-dependent toxicity. Oxidative stress was induced as indicated by an increase of ROS, a decrease of GSH levels, and an increase in the formation of 4-hydroxynonenal-adduct and 8-hydroxy-2-deoxyguanosine-adduct. Both Nrf2 and NF-κB pathway contributed to the initiation of oxidative stress but the timing of two factors was different. Nrf2 and its related downstream genes were activated earlier than that in NF-κB pathway. In conclusion, AA-induced neurotoxicity attribute to oxidative stress via Nrf2 and NF-κB pathway. Moreover, the co-culture cell model was proven to be a viable model to study AA neurotoxicity.

1. Introduction

Due to its formation during food heating processing, acrylamide (AA) poses a potential health hazard and has attracted attention of many health professionals and researchers alike (Tareke et al., 2002).AA has been classified as “probably carcinogenic to humans” (Group 2A) by the International Agency for Research on Cancer (IARC, 1993). It has been shown that acrylamide is primarily metabolized by cytochrome P450 2E1 (CYP2E1) to the epoxide, glycidamide, which is more reactive toward DNA and proteins than the parent compound (Besaratinia and Pfeifer, 2007). AA has also been shown to be neurotoxic and its exposure caused gait abnormal, skeletal muscle weakness, weight loss both in human and experiment animals (He et al., 1989; Kuhlmann and Guilarte, 2000; Muralidhara, 2013). There are preliminary evidences indicating that the mechanism of AA-induced neurotoxicity is associated with oxidative stress. For example, when AA is metabolized, there was an increase in reactive oxygen species (ROS) and the AA-induced biotransformation involved glutathione and glutathione-related enzymes (Pennisi et al., 2013). Oxidative damage may contribute to AA-caused neurotoxicity, but the pathways between the molecular event and cellular outcomes remain elusive. In previous studies, Nrf2 and/or NF-κB pathway have been shown to play important roles on oxidative damage caused by other environmental toxicants including acrylonitrile, methylmercury, African dust storms (Caito et al., 2013; Caito et al., 2014; Ni et al., 2011; Rodríguez-Cotto et al., 2015).

In central nervous system (CNS), there are different types of cells, i.e. astrocytes, microglia, neurons, oligodendrocytes which exhibited different kinds of functions (Anderson and Swanson, 2000). In order to maintain CNS development and function, these cells demonstrate a high degree of plasticity and collectively responsible for many biological processes together (Streit et al., 1988). Recently, disruptions in cell-cell communications among these cells have been considered to play a critical role in the development of neurodegeneration disease and initiation and progression of neurotoxicity induced by xenobiotics (Bezzi et al., 2001; Cerbai et al., 2012; Li et al., 2014). For example, a study on ageing showed that astrocytes and microglia could participate together in the clearance of cellular debris associated with programmed cell death and contribute to neuronal damage in pathological conditions in the hippocampus of aged rats (Cerbai et al., 2012). In a study of infection in neonatal Borna disease virus, activation of astrocytes and/or microglia in culture was observed and inflammation mediators were released causing further neuronal damage (Ovanesov et al., 2008). Chronic activation of the glia cells in the brain can cause neuronal injury even if the xenobiotic is not directly toxic to the neurons (Ovanesov et al., 2008). It

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http://dx.doi.org/10.1016/j.tiv.2016.11.007
0887-2333/

has been shown that exposure to high dose (over 2 mM) of AA can cause apoptosis in various glial cell types including BV2 microglia (Liu et al., 2015), rat primary astrocytes, U-87MG, U-1240MG and U-251MG (Lee et al., 2014). The results of these studies using mono-culture have limitations as they did not account for the effects of interactions between different glial cells. Co-culture systems using binary combinations of different cells have been established to better simulate the in vivo system and include the cell interactions in nervous microenvironment (Miki et al., 2012). This study aims to determine study the mechanisms of AA-induced neurotoxicity using a co-culture microglia and astrocytes system. We hypothesize that the effect of AA-induced neurotoxicity is related to the Nrf2 and/or NF-κB pathway.

2. Materials and methods

2.1. Materials

Balb/c mice were from Charles River (Montreal, QC). Acrylamide was obtained from Sigma (Oakville, ON). Minimum essential medium (MEM/EBSS with 2 mM L-glutamine, Earle' balanced salts), fetal bovine serum (FBS), Hanks Balanced Salt Solution (HBSS), Phosphate buffered saline (PBS), 0.25% Trypsin-EDTA, and penicillin/streptomycin (10,000 U/mL), 2′,7′-dichlorodihydrofluoresceindiacetate (H_2DCFDA) were acquired from GE life science (Logan, Utah). CD11b microbeads and magnetic columns were obtained from Miltenyi Biotech (Auburn, CA). IC-07 hybridoma cells were acquired from HPA culture collections (Salisbury, UK) and A2B5 hybridoma cells were acquired from American Type Culture Collection (ATCC) (Manassas, VA). The rabbit complement was from Cedarlane (Burlington, ON). Anti-mouse CD11b antibody was from BD Pharmigen (Mississauga, ON). Anti-GFAP antibody, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)were from Invitrogen (Burlington, ON). HNE adduct competitive ELISA kit was from Cell Biolabs (San Diego, CA). Nrf2-anti rabbit IgG antibody, NF-kB -anti rabbit IgG antibody, reduced glutathione kit, 8-hydroxy 2 deoxyguanosine (8-OHdG) ELISA Kit were from Abcam (Toronto, ON). Cytokines ELISArray was obtained from Qiagen (Redwood City, CA). All the reagents for qPCR were purchased from BioRad (Mississauga, ON).

2.2. Cell culture and exposure

Research ethics approval for animal use was obtained from the Animal Care and Use Committee of the University of Ottawa according to the guidelines of the Canadian Council on Animal Care. Primary cell culture was prepared as previously described (Bassett et al., 2012; Marek et al., 2008). The purity of cell was checked by immunofluorescence using cell specific markers. Microglia labeled by CD11b were >99% pure and free from astrocytes, while astrocytes labeled by GFAP were 90–95% pure and completely free from microglia. Primary glial cells were plated into either 6-well or 96-well flat-bottom culture plates at microglia/astrocytes ratio of one to one according to different assays. All of them were cultured in MEM/EBSS supplemented with 10% (v/v) FBS, 1% penicillin/streptomycin and grown in a humidified incubator containing 5% CO_2 and 95% air at 37 °C overnight, and then changed to dosing medium containing AA from 0 to 1.0 mM for 24–96 h exposure according to different experiments.

2.3. Cell viability assay

Cells viability was performed by MTT assay. Briefly, cells were incubated with 0.8 mg/mL MTT at 37 °C for 2 h. After this, the medium was removed and replaced with 200 μL of DMSO. After general pipetting, absorbance was measured at 595 nm on the Tecan microplate reader (Männedorf, Switzerland).

2.4. Measurement of ROS and oxidative stress end products

ROS were measured according to reagent's instruction. Briefly, the cells were incubated with 5 μM H_2DCFDA/well for 45 min, then washed twice with PBS and measured with the Tecan microplate reader at an excitation wave length of 485 nm and an emission wave length of 530 nm.

4-Hydroxy-2-nonenal-histidine covalent adducts (4-HNE) and 8-hydroxy-2 deoxyguanosine levels (8-OHdG) were measured using the OxiSelect ELISA kit provided by CellBio Labs (CellBio Labs, San Diego) and 8-OHdG ELISA Kit provided by Abcam (Toronto, ON) according to the manufacturer's instructions.

2.5. GSH/GSSG ratio

GSH and GSSG were determined by fluorescents Green kits (Abcam, Toronto, ON) according to the manufacturer's instructions.

2.6. Western Blot

Nrf2 and NF-kB were measured using western blot. The cell lysate was prepared in ice-cold RIPA buffer with protein inhibitor cocktail tablet (Sigma, Oakville, ON). Protein samples (15–40 μg) were loaded and separated by 10% pre-cast gel (Bio-Rad). Membranes were blocked for 1 h with 5% skim milk in Tris buffered Saline -Tween-20 (TBST), and incubated with primary antibodies: Nrf2 (1:3000), NFkB p65 (1:50,000), at 4 overnight. The membranes were washed by TBST, and incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies (Santa Cruz Biotechnology Inc., Dallas, Texas), goat anti-rabbit IgG-HRP (1:3000), 1 h at room temperature. The blots were stained by ClarityWestern ECL Substrate (Bio-Rad), and target bands were visualized by using the Fusion imaging system (Montreal Biotech. Inc., Montreal, QC). The target band was quantified using the image Jsoftware (NIH image, Maryland) and the density of each band was normalized against Actin.

2.7. Quantification PCR

Co-cultural cells pellet at 24 and 96 h after 0.1 and 1.0 mM of AA treatment were used for qPCR analysis. RNA was extracted using BioRad Mini kits (Mississauga, ON) according to manufacturer's instructions. The RNA quality was checked using Nanodrop (Thermo, Burlington, ON). First strand cDNA was synthesized using the BioRadIscriptc DNA kit (Mississauga, ON) according to manufacturer's instructions. mRNA expression was measured using BioRad Evagreen supermix by real time-quantitative PCR (RT-qPCR). Each 10 μL PCR contain 5 μL supermix, 4 μL 1:10 diluted cDNA, 0.8 μL 10 μM primers, 0.2 μL nuclease-free water. The following primers (IDT, Coralville, IA) were used: hemeOxygenase 1 (HO1), NAD(P)H quinone dehydrogenase 1 (NQO1), glutathione S-transferase *mu* (GST *mu*), interleukin (IL)1β,IL-6,tumor necrosis factor-α (TNF-α), Granulocyte colony-stimulating factor (G-CSF), β-Actin (Sequences list see Supplementary material Table 1). The amplification protocol comprised an initial 3 min denaturation at 95 °C, followed by 40 cycles of denaturation for 10s at 95 °C and annealing/extension for 30 s at 60 °C/57 °C on a CFX96 (BioRad, Mississauga, ON). Melting curve analysis was performed to ensure that only one PCR product was obtained. For estimation amplification efficiency, a standard curve was generated using serial dilutions of cDNA samples. The relative amount of target mRNA was calculated by the comparative cycle threshold (C_t) method ($2^{-\Delta\Delta Ct}$) by normalizing target mRNA C_t values to those for actin.

2.8. Cytokines ELISArray

ELISArray was used to detect the changes of 12 cytokines including IL1α, IL1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, Interferon (IFN-γ), TNF-