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Acrylamide inhibits cellular differentiation of human neuroblastoma and glioblastoma cells

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ABSTRACT

This study explores human neuroblastoma (SH-SY5Y) and human glioblastoma (U-1240 MG) cellular differentiation changes under exposure to acrylamide (ACR). Differentiation of SH-SY5Y and U-1240 MG cells were induced by retinoic acid (RA) and butyric acid (BA), respectively. Morphological observations and MTT assay showed that the induced cellular differentiation and cell proliferation were inhibited by ACR in a time- and dose-dependent manner. ACR co-treatment with RA attenuated SH-SY5Y expressions of neurofilament protein-L (NF-L), microtubule-associated protein 1b (MAP1b; 1.2 to 0.7, $p < 0.001$), MAP2c (2.2 to 0.8, $p < 0.05$), and Janus kinase1 (JAK1; 1.9 to 0.6, $p < 0.001$), while ACR co-treatment with BA attenuated U-1240 MG expressions of glial fibrillary acidic protein (GFAP), MAP1b (1.2 to 0.6, $p < 0.001$), MAP2c (1.5 to 0.7, $p < 0.01$), and JAK1 (2.1 to 0.5, $p < 0.001$), respectively. ACR also decreased the phosphorylation of extracellular-signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) in U-1240 MG cells, while caffeine reversed this suppression of ERK and JNK phosphorylation caused by ACR treatment. These results showed that RA-induced neurogenesis of SH-SY5Y and BA-induced astrogliogenesis of U-1240 MG cells were attenuated by ACR and were associated with down-regulation of MAPs expression and JAK-STAT signaling.

1. Introduction

Acrylamide (ACR), a neurotoxin causing ataxia, skeletal muscle weakness and numbness of the hands and legs in animals and humans (Exon, 2006), has been applied in many industries and laboratories, and can be found in many food and tobacco products after heating (Kutting et al., 2009; Rice, 2005; SNFA, 2002; Sunayama et al., 2010; FAO/WHO, 2002). The mean intake of ACR for humans is 10–40 μg/day (Wilson et al., 2012), which is higher than the lifelong intake of 0.08 μg/kg body weight/day known to result in an additional cancer risk of 1 out of 10,000 people (NSFA, 2002). Therefore, toxicological concerns regarding ACR intake go beyond occupational settings into daily long-term and low-dose human exposure.

ACR causes central-peripheral neuropathy (LoPachin et al., 2002, 2004). Axons are the primary site at which ACR causes axonopathy after impairment of the neurotransmitter by increasing the number of vesicles in the synapses, swelling the distal nerve terminal axon, and filling it with neurofilaments (LoPachin et al., 2002). The disturbance of kinesin-related motor protein has been proposed to occur in ACR-induced axonal toxicity (Exon, 2006; LoPachin, 2005). Microtubule-associated proteins (MAPs) are important for neurite outgrowth during neurogenesis and extension development of glia cells (Couchie et al., 1985; Gonzalez-Billault et al., 2004). Dysfunction of MAP1b and MAP2c causes microtubules to be structurally unstable and leads to neurodegenerative diseases (Gonzalez-Billault et al., 2004). ACR exposure has been reported to affect the distribution of MAP1 and MAP2 proteins in different brain areas of rats (Chauhan et al., 1993), and the relative binding affinity of ACR with MAP proteins has been reported to be greater than that of ACR with tubulin (Lapadula et al., 1989). Moreover, signal pathways of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) and the mitogen-activated protein kinase (MAPK) are critical for various cellular events involved with cellular differentiation (both neurogenesis and astrogliogenesis), inflammation, apoptosis, proliferation, and cell survival (Douglas, 2012; Kyriakis and Avruch, 2012; Rawlings et al., 2004). Although ACR affects the distribution of rat brain MAPs, the effects of MAP expressions and JAK-STAT signal pathways, which are related to differentiation in neurogenesis and astrogliogenesis, have not been clarified.

Human neuroblastoma cells (SH-SY5Y) can differentiate into neurons in the presence of retinoic acid (RA) (Hartley et al., 1997). In addition, glioblastoma cells can differentiate into neuronal lineage cells in the presence of butyric acid (BA) (Sidiropoulou et al., 2009). Different concentrations (0, 2.5, 5 and 10 μM) of RA and BA were

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used to stimulate SH-SY5Y and U-1240 MG cells for 72 h, which resulted in cellular differentiation in a dose-dependent manner. Significantly different morphologies were observed in 10 μM RA-stimulated SH-SY5Y and 10 μM BA-stimulated U-1240 MG cells (data not shown). This study showed that RA-induced neurogenesis of SH-SY5Y cells and butyric acid (BA)-induced astrogliogenesis of U-1240 MG cells were attenuated by ACR and that MAPs expression and JAK-STAT signaling were down-regulated.

2. Materials and methods

2.1. Chemicals and reagents

ACR, ammonium persulfate (APS), BA, ethanol, glycine, methanol, NaCl, phosphatase inhibitor, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), protease inhibitor, and RA were from Sigma-Aldrich (St Louis, MO). Dimethyl sulfoxide (DMSO), KCl, tetramethylethylenediamine (TEMED), and Triton X-100 were from J.T. Baker (Phillipsburg, NJ). Protein markers and protein dyes were from Fermentas (Vilnius, Lithuania), and SDS, tris-base, and acrylamide/bis (37.5:1) were from AMRESCO (Solon, OH). Non-fat milk was from Anchor (Auckland, NZ), caffeine was from Calbiochem (Gibbstown, NJ), and polyvinylidene fluoride (PVDF), enhanced chemiluminescence (ECL) substrate, and Na_2HPO_4 were from Merck KGaA (Darmstadt, Germany). Anti-p38 antibody was from Abcam (Abcam, Cambridgeshire, UK), and anti-GAPDH, MAP1b, MAP2, neurofilament-L (NF-L), glial fibrillary acidic protein (GFAP), JAK1 antibody, and rabbit IgG antibody (HRP) were from GeneTex (Irvine, CA). Anti-extracellular-signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) antibody were from Cell Signaling Technology (Beverly, MA). All of the cell culture reagents and saline buffers were purchased from Gibco (Rockville, MD).

2.2. Cell culture

Human neuroblastoma cell line SH-SY5Y was purchased from the American Type Culture Collection (Manassas, VA, USA) and was grown in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient F12 medium (DMEM/F12) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Human astrocytoma cell line U-1240 MG was obtained from The Ohio State University (Columbus, OH). U-1240 MG cells were grown in minimum essential medium (MEM) supplemented with 10% (v/v) calf serum (CS), 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were maintained at 37 °C in a 5% CO_2, 100% humidified atmosphere.

2.3. Cell differentiation and morphological observation

For differentiation studies, the SH-SY5Y cells were transferred into differentiation medium (DMEM/F12 (1:1) medium supplemented with 1% (v/v) FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin) with different concentrations of RA (0, 2.5, 5, and 10 μM) for 72 h. The U-1240 MG cells were transferred into differentiation medium (MEM medium supplemented with 1% (v/v) CS, 100 units/mL penicillin, and 100 μg/mL streptomycin) with different concentrations of BA (0, 2.5, 5, and 10 μM) for 72 h. All cells were maintained at 37 °C in a 5% CO_2, 100% humidified atmosphere. Differentiation of SH-SY5Y and U-1240 MG cells with or without ACR treatment was observed using an inverted microscope (IX71; Olympus, Tokyo, Japan).

2.4. Cell treatments

ACR stock solution (1 M) was prepared with double-distilled water (ddH_2O) and sterilized through 0.22 μm filters. Appropriate ACR concentrations were prepared with culture medium of differentiation in the petri dish. Cells were then treated with ACR and cultured at 37 °C with 5% CO_2 for 72 h according to our previous study (Chen et al., 2009). For the determination the blocking effects of signal upstream of ACR-impairing the phosphorylation of MAPKs, U-1240 MG cells were pretreated with 0, 0.25, 0.5, and 1 mM caffeine for 30 min and then with 2 mM ACR for another 48 h.

2.5. Cell proliferation analysis

Cell viability was analyzed with an MTT assay according to our previous protocols (Chen et al., 2010). In brief, cells were placed in 96 well microtiter plate at a density of 1000 cells per well in a final volume of 100 μL of culture medium for 1 day prior to the experiment. Cells after ACR treatment were immediately incubated with 10 μL MTT solution (0.5 mg/mL) for 3 h at 37 °C, and the medium was then removed and 100 μL DMSO was added for 5 min at room temperature. Cell viability was quantified by ELISA reader (Spectra MAXM5 photometry, Molecular Devices, Sunnyvale, CA) at OD_{595} nm.

2.6. Western blot analysis

Protein expressions were measured with Western blot analysis, which was modified from our previous protocol (Chen et al., 2009, 2010). Cells were harvested in lysis buffer (Sigma, St Louis, MO) and left on ice for 20 min and then were centrifuged (8000 × *g* for 30 min at 4 °C). The proteins in the supernatant were measured by Thermo BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). The supernatant was diluted to 15–30 μg of protein/mL with loading buffer (Fermentas, Vilnius, Lithuania) and ddH_2O to 20 μL. Samples were heated to 92 °C for 5 min to denature the proteins. Proteins were separated by electrophoresis on a 10% (v/v) polyacrylamide gel at 60 V for 30 min and 100 V for 1 h, transferred onto a polyvinylidene fluoride membrane at 100 mA for 1 h at 4 °C, and then immunoblotted with anti-actin, GAPDH, MAP1b, MAP2, NF-L, GFAP, JAK1, ERK, JNK, and p38 antibody (with 1:10,000, 1:10,000, 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:2000, and 1:1000 dilution, respectively) purified from rabbit serum at 4 °C overnight. Immunoreactive bands were detected using the horseradish peroxidase-conjugated anti-rabbit IgG and enhanced by Geliance 600 Imaging System (J&H Technology Co., Ltd). Bandwidths were quantified by MultiGauge V1.1 (Fujifilm, Stamford, CT).

2.7. Statistical analysis

Data were presented as means ± standard deviation. Each experiment was performed in triplicate at least three times independently. The statistical significance of differences among groups was performed with one-way analysis of variance (ANOVA), followed by Duncan's test using SSPS (ver 17.0; Chicago, IL). The differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Acrylamide inhibits RA-induced neurite outgrowth and cell proliferation of SH-SY5Y cells

As shown in Figure 1A, after 10 μM RA stimulation, SH-SY5Y cells developed a round cell body and an extended axon-like neurite. Under co-exposure to the RA and different concentrations (0, 0.1, 0.5, 1, and 2 mM) of ACR, 0.5 mM ACR caused the SH-SY5Y cells to exhibit shorter neurite morphology, while 1 and 2 mM ACR resulted in cells with no neurite morphological extensions (Fig. 1A). Moreover, Western blot analysis found that expression levels of NF-L, a neurofilament subunit expressed in mature neurons, were increased after RA treatment and decreased under ACR co-treatment in SH-ST5Y cells (Fig. 1B). MTT assay showed that different concentrations (0, 2.5, 5, and 10 μM) of RA treatment significantly induced SH-SY5Y cell proliferation at 24, 48, and 72 h when compared with the control (0 h) (Fig. 2A). Cell proliferation of SH-SY5Y cells at 72 h did not show statistically significant differences as a result of different concentrations (0, 2.5, 5, and 10 μM) of RA treatment (Fig. 2A). However, induction of SH-SY5Y cell proliferation by 10 μM RA at 72 h was significantly decreased in 0.5, 1, and 2 mM ACR co-treatment when compared with the control (0 mM) (Fig. 2B). These results indicated that neurite outgrowth of SH-SY5Y cells was inhibited by ACR and that proliferation of SH-SY5Y cells was attenuated by ACR treatment in a time- and dose-dependent manner.

3.2. Acrylamide inhibits BA-induced extension development and cell proliferation of U-1240 MG cells

As shown in Figure 3A, the round cell bodies of U-1240 MG cells were differentiated into an axon-like extended neurite after 10 μM BA stimulation for 72 h. ACR exposure interfered with BA-induced U-1240 MG cell development such that the cells developed shorter extensions after exposure to 0.5 and 1 mM ACR, while no cell extensions were developed after exposure to 2 mM ACR. GFAP, a marker for glia cell differentiation, was analyzed by Western blot for U-1240 MG cells, which were co-treated with BA and different concentrations of ACR (Fig. 3B). Relative levels of GFAP in U-1240 MG cells increased after 10 μM BA treatment but decreased significantly in BA and ACR co-treatment groups. As shown in Figure 4A, different concentrations (0, 2.5, 5, and 10 μM) of BA treatment induced U-1240 MG cell proliferation significantly at 24, 48, and 72 h when compared with the control (0 h). There were no statistically significant differences in U-1240 MG cell proliferation resulting from