NeuroToxicology 63 (2017) 120–125

Contents lists available at ScienceDirect

NeuroToxicology

Full Length Article

Resistance of mouse primary microglia and astrocytes to acrylonitrile-induced oxidative stress

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ARTICLE INFO

Article history:
Received 29 August 2017
Received in revised form 26 September 2017
Accepted 28 September 2017
Available online 28 September 2017

Keywords:
Astrocyte
Microglia
Acrylonitrile
Nrf2

ABSTRACT

Acrylonitrile (ACN) is a widely used chemical in the production of plastics, resin, nitrile, acrylic fibers, synthetic rubber, and acrylamide. ACN is considered a Group 2B possible carcinogen in humans and is known to cause gliomas in rats. These gliomas are predominantly composed of microglia and not astrocytes. Interestingly, ACN treatment does not cause gliomas in mice, suggesting that mouse astrocytes and microglia may be resistant to ACN. We investigated the effects of ACN treatment on primary mouse microglia and astrocytes to investigate their sensitivity to the chemical. Cell viability, ACN uptake, glutathione (GSH) levels and the expression of NF-E2-related factor 2 (Nrf2) were evaluated in primary mouse microglia and astrocytes following ACN treatment. Our results indicate that mouse glial cells are resistant to ACN-induced oxidative stress. Both cell types accumulated ACN; however, there was a minor effect of ACN on cell viability in astrocytes and microglia. Nrf2 and GSH levels were unchanged in ACN-treated as compared to the untreated cells. These observations suggest that primary mouse glial cells are resistant to ACN.

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1. Introduction

Occupational exposure to acrylonitrile (ACN) occurs during the synthesis of acrylic fibers, synthetic rubber, resins, nitriles, and acrylamide (Acrylonitrile, 1999; Byrd et al., 1990). While there have been historical reports of high level acute ACN exposures resulting in death, the majority of exposures modern workers are exposed to are low dose and chronic in nature. ACN is primarily a neurotoxicant, acutely causing labored or irregular breathing, dizziness, impaired judgment, hallucinations, cyanosis, nausea, collapse, loss of consciousness, convulsions (Buchter and Peter, 1984; Vogel and Kirkendall, 1984) and chronicly causing liability of autonomic function such as arterial pressure, labile pulse, diffuse dermographia, increased sweating and change in orthostatic reflex (Buchter and Peter, 1984). Mice and rats exposed to ACN show similar symptoms as humans, however it is also a carcinogen (Bigner et al., 1986; Kirman et al., 2005). ACN is a multi-site carcinogen in rodents, however brain tumors have only been observed in rats (Ghanayem et al., 2002). This suggests that there is differential metabolism of ACN across species or that the carcinogenicity of ACN varies by species. Indeed, epidemiology data has not shown a causal association between ACN exposure and cancer in humans (Cole et al., 2008; Kirman et al., 2005), which has prompted a classification as a Group 2B possible carcinogen by the International Agency for Research on Cancer (Acrylonitrile, 1999).

Gliomas comprise the majority of tumors found in the brain of rats exposed to ACN (Bigner et al., 1986). Glial cells are a numerous and vital cell type in the brain, encompassing about 90% of the total cells in the brain and 50% of brain cellular volume (Allen and Barres, 2009; Parpura et al., 2012). Astrocytes are the predominant glial cells and responsible for various cellular functions including regulation of ion concentrations in the extracellular fluid, development of tight junctions in the blood-brain barrier (BBB), differentiation of myelinated axons, neurotransmitter uptake and release, metabolic support, nervous system repair, vasomodulation, and the promotion of myelination by oligodendrocytes (Allen and Barres, 2009; Parpura et al., 2012). Microglia are important glial cells that have a role in inflammation and extracellular signaling in addition to responding to neuronal injury by releasing both neurotoxic and neurotrophic factors (Aschner et al., 1999). Immunohistochemistry studies have shown that ACN-induced gliomas in rats are primarily composed of microglia, not astrocytes (Kolenda-Roberts et al., 2013). The effects of ACN on astrocytes have been well documented in *in vitro* rat models. ACN exposure in

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https://doi.org/10.1016/j.neuro.2017.09.013
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primary and transformed astrocytes leads to gliosis, while also inhibits gap junction intercellular communication. Glutathione (GSH) and ATP are both depleted by ACN, which can lead to increased reactive oxygen species (ROS) and increased oxidative DNA damage (Enongene et al., 2000; Esmat et al., 2007; Kamendulis et al., 1999a,b; Whysner et al., 1998). Studies of ACN on microglia are fewer. Previous work from our lab has shown that rat microglia are more sensitive to ACN treatment than astrocytes (Caito et al., 2013). Microglia accumulated less ACN, showed increased lipid peroxidation markers, increased GSH content and activation of the antioxidant transcription factor NF-E2- related factor 2 (Nrf2), suggesting that microglia may be more sensitive to ACN (Caito et al., 2013).

Reports of differential responses to ACN in mice and rats show that mice are more resistant to ACN than rats. Indeed, more types of cancers were observed in rats exposed to ACN (Bigner et al., 1986; Kirman et al., 2005). Mice show faster metabolism of ACN than rats (Jacob and Ahmed, 2004). Metabolism of ACN produces an epoxide metabolite that conjugates to glutathione (GSH), the most prevalent intracellular thiol, allowing for its elimination (Kedderis et al., 1996). Comparisons between rat and mouse GSH tissue levels following ACN exposure showed that rat GSH levels in brain, liver, lung, and kidney were more altered than mouse (Cote et al., 1984). Therefore, we were interested in understanding how mouse primary astrocytes and microglia respond to ACN, hypothesizing that mouse primary astrocytes and microglia may be more resistant to ACN compared with the same cells in the rat. We found ACN entered both cell types, but did not significantly affect viability, antioxidant response transcription factor Nrf2, or GSH levels. Taken together these results highlight the differential responses of rodent species to ACN.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, all biochemical reagents used in this study were purchased from Sigma Chemicals (St. Louis, MO, USA).

2.2. Primary cell culture and treatments

Primary microglia and astrocytes were isolated from cortical tissue of postnatal day-1 neonatal C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA). After dissociation, centrifugation and resuspension, the mixed glial cell culture for astrocyte isolation was maintained in 75 cm^2 tissue culture treated flasks fed with minimum essential medium (MEM: Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated horse serum (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin. The mixed glial cell culture for microglia isolation was maintained in 75 cm^2 tissue culture treated flasks fed with Dulbecco's Modified Eagle Medium (DMEM: Thermo Fisher) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were maintained in a humidified incubator at 37 °C of 5% CO_2. The media for astrocytes were changed twice a week. The media for microglia were changed once a week. After two weeks of culture, when cells reached ~95% confluence, microglia and astrocytes were separated by gentle shaking for 1 h at room temperature using an orbital shaker. Astrocytes from this separation process were plated in poly-L-lysine coated plates (Corning). Microglia were isolated as previously described (Saura et al., 2003). Microglia derived from this process were plated at an equal density to astrocytes in poly-L-lysine coated plates (Corning). One day after the cell attachment, cells were exposed to 0, 0.1, 0.5, 0.75, or 1 mM ACN (Sigma Aldrich) for different durations. The treatment concentrations and durations were identical to the treatment conditions we have previously reported in rat primary glial cells (Caito et al., 2013).

2.3. Cytotoxicity assays

The *in vitro* cytotoxic effects of ACN were evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the lactate dehydrogenase (LDH) toxicity assay. The MTT assay was used as a measure of viable cells, as the reduction of MTT occurs in active living cells' mitochondria, while the LDH assay was used as a measure of dead cells due to LDH leakage upon death and membrane compromise. The absorbance for MTT assay was measured at 570 nm and LDH assay at 490 nm. The LDH assay (TOX7, Sigma) was followed as manufacturer's instructions using the cell's culture media following the ACN treatment. The background absorbance (650 nm) was subtracted out for both assays.

2.4. Radiolabeled ACN uptake assay

Equal density of astrocytes and microglia were plated in poly-L-lysine coated 24-well plates. Three days after cell attachment, cells were exposed to 0, 0.1, 0.5, 0.75 or 1 mM ACN with 0.25 μCi/ml of ^{14}C ACN (American Radiolabeled Chemicals, ARC 1745-1 mCi/37 MBq, St. Louis, MO) for 1, 5, 10 and 30 min. The uptake assay was then performed following a published protocol (Sidoryk-Wegrzynowicz et al., 2011). The cells were washed two times with cold freshly prepared sodium-HEPES buffer with a pH of 7.4 to stop the treatment. Cells were then lysed with 1 M NaOH for 30 min at room temperature. A portion of cell lysate was transferred to scintillation vials and mixed with 4 ml ScintiSafe 30% Cocktail (Fisher Scientific, Pittsburgh, PA) and radioactivity was measured by a scintillation counter (the model of our scintillation counter). Protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) with bovine serum albumin as the standard.

2.5. Measurement of reduced glutathione and oxidized glutathione

After ACN treatment, cells were washed with 1× Phosphate Buffered Saline solution and then harvested with 5% metaphosphoric acid according to the counted number of cells. The reduced GSH and GSSG concentrations were measured by a GSH/GSSG detection kit (Enzo Life Sciences, Farmingdale, NY). GSH and GSSG (oxidized GSH) concentrations were normalized according to the number of cells initially harvested.

2.6. Western blot analysis

Following ACN treatment, cells were scraped and collected in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail on ice. Western blot was performed with the primary antibody of rabbit-anti Nrf2 (1:40,000) (sc-722, Santa Cruz Biotechnology, Santa Cruz, CA) and secondary antibody of goat anti-rabbit IgG (1:10,000) (31460, Thermo Scientific, Rockford, IL) to detect Nrf2 protein. β-actin was detected by applying primary antibody of mouse anti β-actin (1:100,000) (A5316, Sigma) and secondary antibody of goat anti-mouse IgG (1:5000). The density of the Nrf2-specific bands was normalized to β-actin and quantified with ImageJ.

2.7. Statistical analysis

All results were expressed as means ± standard errors with a minimum of three independent experiments. One-way analysis of variance (ANOVA) and two-way ANOVA followed by Bonferroni's

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