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Preconditioning of endoplasmic reticulum stress protects against acrylonitrile-induced cytotoxicity in primary rat astrocytes: The role of autophagy

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ABSTRACT

This study explored the protective effects of endoplasmic reticulum (ER) stress preconditioning induced by 2-deoxy-D-glucose (2-DG) or oxidized dithiothreitol (DTTox) on acrylonitrile (AN)-induced cytotocity in primary rat astrocytes. Cells were pretreated with 2-DG or DTTox for different times at various concentration. Next, astrocytes were treated with 2.5 mM AN for an additional 12 h. Cell viability and cytotoxicity were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) leakage, respectively. Reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi$m) were determined. Expression of glucose-regulated protein 78 (GRP78), phosphorylated-eukaryotic translation initiation factor 2α (p-eIF2α), microtubule-associated protein light chain 3 (LC3), P62, and Beclin1 were used to assess autophagy. In addition, 3-methyadenine (3-MA), an autophagy-specific inhibitor, was used to assess the role of autophagy in ER stress preconditioning-induced protection against AN cytotoxicity. The results showed that AN alone significantly decreased astrocytic viability and enhanced cytotoxicity. Compared to the AN-alone group, preconditioning with 2-DG or DTTox significantly increased cell viability and reduced cytotoxicity to indistinguishable levels. Decreased ROS generation and increased $\Delta\Psi$m were also inherent to ER stress preconditioning with these compounds. Furthermore, autophagy was activated by both 2-DG and DTTox. Blockage of autophagy attenuated the protection afforded by 2-DG or DTTox preconditioning in AN-treated astrocytes. These results establish that ER stress preconditioning affords cellular protection against AN, and that activation of autophagy mediates the cytoprotection. Modulation of ER stress and resultant activation of autophagy may be a novel target for to ameliorate AN toxicity.

1. Introduction

Acrylonitrile (AN) is an important chemical intermediate that is widely used in the production of acrylic fiber, plastics, resins and other chemicals. Contemporary occupational exposures to AN have sharply increased (International Agency for Research on Cancer, 1999; National Toxicology Program, 2011). The major target of AN is the central nervous system, predominantly cortical pyramidal cells (Nerland et al., 1989). It has been proposed that long-term exposure to AN may result in astrogliomas in rats (Pu et al., 2015; Woutersen, 1998; Wang et al., 2015). However, the underlying mechanisms of AN toxicity remain obscure.

Preconditioning of endoplasmic reticulum (ER) stress facilitates cellular adaptation and protection from the effects of ischemia, hypoxia and toxic chemicals (Hara et al., 2011; Peyrou and Cribb, 2007; Prachasilchai et al., 2008; Zou et al., 2014; Chandrika et al., 2015; Zhang et al., 2015). The accumulation of unfolded or misfolded proteins in the ER generates a stress condition that engages the unfolded protein response (UPR). The UPR is an adaptive response that aims to restore ER proteostasis by increasing the capacity of folding and degrading misfolded proteins. Recent research has shown that modulating the activity

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of different UPR signaling arms may reduce stress levels (Mollereau, 2015; Rivas et al., 2015). The three membrane-associated ER proteins that act as stress sensors and set in motion the UPR are: protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1(IRE1), and activating transcription factor 6 (ATF6). Although the molecular mechanisms of cytoprotection during the ER hormetic response have yet to be fully elucidated, it seems clear that increased folding capacity and attenuated protein translation resulting from UPR activation allow the cell to eliminate pathogenic proteins (Cominacini et al., 2015; Mollereau et al., 2014; Mollereau, 2015).

Among the known ER stress inducers, 2-deoxy-D-glucose (2-DG), the glucose analog and glycolytic inhibitor, not only blocks glycolysis resulting in reduced cellular ATP but also interferes with *N*-linked glycosylation, thus leading to ER stress and an unfolded protein response (UPR) (Pahl, 1999; Xi et al., 2011). Oxidized dithiothreitol (DTTox), another ER stress-inducing agent, can increase GRP78 protein expression in a concentration-dependent manner (Peyrou and Cribb, 2007). GRP78, a typical ER stress protein marker, promotes the recovery of optimal protein conformation and maintains cellular homeostasis (Pahl, 1999).

In addition, ER stress may activate autophagy, a bulk cellular degradation process that may result in either a cytotoxic or cytoprotective effect (Higa and Chevet, 2012). Autophagy is a cellular defense mechanism that occurs by degradation and recycling of cytoplasmic constituents. ER stress is linked to autophagy (Fouillet et al., 2012; Petrovski et al., 2011; Sheng et al., 2012). Toxic proteins that accumulate in the ER are removed by autophagy, suggesting that ER stress-induced autophagy may play a role in cytoprotection (Matus et al., 2012; Xi et al., 2011; Zou et al., 2014). In contrast, excess autophagy contributes to cell death (Sano and Reed, 2013). Thus, ER stress-mediated autophagy regulates cell survival or cell death. Autophagy acts via an ER-associated degradation system, playing a fundamental role in preventing toxic accumulation of disease-associated mutant proteins in the ER (Petrovski et al., 2011). In addition, ER itself represents the major autophagosomal cargo during ER stress, suggesting the pro-survival effect of autophagy may be due to increased removal of unfolded proteins (Mariño and López-Otín, 2004).

We investigated in astrocytes the potential protection of ER stress pre-conditioning induced by 2-DG or DTTox against AN-induced cytotoxicity, and the role of activation of autophagy in this process.

2. Material and methods

2.1. Animals, drugs and reagents

Postnatal 1–2 days Sprague-Dawley (SD) rats were provided by the Laboratory Animal Center of Jiangsu University. AN (Purity: 99.5%) was obtained from Shanghai Petrochemical Company. Dimethylsulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl diphenyltetrazolium bromide (MTT) were purchased from Gibco Company (San Francisco, CA, USA); newborn calf serum was obtained from Hangzhou Evergreen Company (Hangzhou, Zhejiang, China). 96-well plates and 6-well plates were purchased from the Corning Incorporated (Corning, NY, USA). Protein-extraction kits were provided by Kangcheng Reagent Company (Shanghai, China). The LDH kit was obtained from Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). JC-1 was purchased from Molecular Probes (Eugene, OR, USA). DTTox, 2-DG, 3-MA and acridine orange (AO) were all purchased from Sigma Company (St. Louis, MO, USA). The polyclonal antibodies to GRP78, p-eIF2α, LC3, P62 and Beclin1 were obtained from Cell Signaling Technology (Beverly, MA, USA). β-Actin and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were of analytical grade.

2.2. Primary astrocyte isolation and culturing

Primary neonatal rat cerebral cortical cell cultures were generated as previously detailed (Yin et al., 2011; Yuntao et al., 2014). Whole brains were removed under sterile conditions, minced with a sterile razor and trypsinized for 15 min in a 37 °C humidified incubator. Cells were suspended in fresh Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum and centrifuged at 1500*g* for 5 min. The cell suspensions were plated and cultured in DMEM media supplemented with 10% newborn calf serum, 100 units/L penicillin and 0.1 mg/L streptomycin in a 100% humidified 5% CO_2/95% air incubator at 37 °C. The culture medium was changed after 24 h and, subsequently, once every 2 days. Approximately two weeks after plating, astrocytes purity reached an estimated 95% concentration based on glial fibrillary acidic protein (GFAP) staining (dada not shown). Cells were seeded into 96-well plates at a density of 1.0×10^4 cells/well or 6-well plates at a density of 1.0×10^5 cells/well. The next day, cells at 80% confluency were used for experimentation as previously described.

2.3. Design

2-DG and DTTox were prepared in phosphate buffer saline (PBS) after solubilization in DMSO (10 μL/L), at a final concentration <0.01%. Astrocytes were pretreated with 2-DG (1, 5 or 10 mM) for 12 h or 24 h, or DTTox (1, 5 or 10 mM) for 1 h or 3 h, followed by exposure to AN (2.5 mM) for an additional 12 h. The control group was treated with only DMEM media in the absence of AN, 2-DG and DTTox. The AN-alone group was also set up (AN 2.5 mM for 12 h). Each experiment was carried out in 96- or 6-well plates and repeated at a minimum 3 times with at least three independently derived astrocytes cultures.

2.4. Evaluation of cell viability and cytotoxicity

2.4.1. Cell viability assay

Cell viability was measured with the MTT assay kit according to the manufacturer's instructions. Briefly, astrocytes, in a 96-well plate, were treated as previously described. MTT (0.5 mg/L; 10 μL) was added to each well for 4 h, followed by DMSO (150 μL) for an additional 15 min. The colorimetric intensity was analyzed with a Bio-Rad 680 microplate reader at a wavelength of 490 nm. Relative cell viability values for the experimental group were calculated by setting control group as 100%.

2.4.2. Cytotoxicity assay

Cytotoxicity was measured with the LDH assay (Decker and Lohmann-Matthes, 1988). Astrocytes were pretreated in a 96-well plate with 2-DG (5 mM) for 12 h or DTTox (5 mM) for 3 h, respectively. The supernatant was assayed for LDH release into the media using the LDH assay kit. The absorbance of all samples was measured at 450 nm using a microplate reader. The relative LDH release rate was calculated as units (U) per milliliter (ml) media in the experimental group/U per ml media in the control group × 100%.

2.5. Determination of intracellular ROS

After treatment with 2-DG (1, 5 or 10 mM) for 12 h or DTTox (1, 5 or 10 mM) for 3 h, the cells were incubated for 30 min with 10 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA) at 37 °C. In the