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Role of the Nrf2-ARE pathway in acrylamide neurotoxicity

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ABSTRACT

Acrylamide (ACR) intoxication is associated with selective nerve terminal damage in the central and peripheral nervous systems. As a soft electrophile, ACR could form adducts with nucleophilic sulfhydryl groups on cysteine residues of kelch-like erythroid cell-derived protein with CNS homology-associated protein 1 (Keap1) leading to dissociation of the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 activation of the antioxidant-responsive element (ARE) and subsequent upregulated gene expression of phase II detoxification enzymes and anitoxidant proteins might provide protection in neuronal regions with transcriptional capabilities (e.g., cell body). In contrast, non-transcriptional cell regions (axons, nerve terminals) might be vulnerable to electrophile-induced damage. To test this possibility, immunoblot analysis was used to measure protein products of Nrf2-activated ARE genes in nerve terminals and in cytosolic/nuclear factions of neuronal cell bodies isolated from rats intoxicated at two different ACR dose-rates; i.e., 50 mg/kg/d × 10 days, 21 mg/kg/d × 38 days. To detect possible differences in cell-specific induction, the cytoprotective response to ACR intoxication was determined in hepatic cells. Results show that control brain and hepatic cell fractions exhibited distinct subcellular distributions of Nrf2, Keap1 and several ARE protein products. ACR intoxication, however, did not alter the levels of these proteins in synaptosomal, brain cytoplasm or liver cell fractions. These data indicate that ACR was an insufficient electrophilic signal for ARE induction in all subcellular fractions tested. Because a cytoprotective response was not induced in any fraction, nerve terminal vulnerability to ACR cannot be ascribed to the absence of transcription-based defense mechanisms in this neuronal region.

1. Introduction

Exposure of humans and laboratory animals to acrylamide (ACR) produces ataxia, skeletal muscle weakness and changes in cognition. In laboratory animal models, this neurotoxicity is associated with highly selective damage to distal axon and nerve terminal regions (reviewed in LoPachin et al., 2002a, 2003; LoPachin and Gavin, 2008). Although the exact mechanism is unknown, this selective damage might be due to the differential abilities of cell bodies and nerve terminals to mount inducible cytoprotective processes (LoPachin and Gavin, 2008). Specifically, ACR is an electrophile that forms Michael-type adducts with nucleophilic residues on proteins (Barber and LoPachin, 2004; Barber et al., 2007; LoPachin et al., 2004, 2006, 2007a,b; reviewed in LoPachin et al., 2008). Cells are protected from electrophilic attack by xenobiotic chemicals (e.g., acrolein) and reactive oxygen species (ROS) through induction of the antioxidant-responsive element (ARE) and other transcriptionally based mechanisms. The ARE is a *cis*-acting regulatory element found in the promotor regions of genes encoding many phase II biotransformation enzymes (e.g., hemeoxygenase-1, γ-glutamyl cysteine ligase) and antioxidant proteins (e.g., glutathione reductase; Friling et al., 1990; Lee and Johnson, 2004; Zhu et al., 2008). Binding of the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), to the ARE activates gene transcription. During basal conditions, Nrf2 is associated with cytosolic kelch-like erythroid cell-derived protein with CNS homology-associated protein 1 (Keap1), which targets the transcription factor for ubiquitination and proteosomal degradation. Electrophile adduction of specific cysteine residues (Cys^{257}, Cys^{273}, Cys^{288} and Cys^{297}) on Keap1 promotes dissociation of the Nrf2-Keap1 complex, which leads to nuclear translocation of Nrf2 and subsequent transcriptional activation of ARE-driven genes and their cytoprotective protein products (Dinkova-Kostova et al., 2002; Hong et al., 2005; Wakabayashi et al., 2004; Zhang and Hannink, 2003; Zhang et al., 2004). In contrast to other cell types, nerve cells are unique since the cell body and nucleus are anatomically separated from their nerve terminals by an axon of variable

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length. Nerve terminals are anuclear regions that lack transcriptional machinery and, consequently, the distances separating these distal regions from the perikaryon could limit the availability of cell body-derived cytoprotective proteins. The absence of inducible defense mechanisms could render nerve terminals vulnerable to electrophilic attack.

The present study was designed to test the hypothesis that distal nerve regions, in contrast to the perikaryon, are selectively vulnerable to ACR attack due to their inability to mount a transcriptionally based ARE response. ARE induction is a possible consequence of ACR intoxication since the α,β-unsaturated carbonyl structure of this neurotoxicant is common among electrophilic chemicals that induce the ARE (Talalay et al., 1988). Glycidamide, the epoxide metabolite of ACR, is also an electrophile and could, therefore, activate the ARE. Furthermore, ACR might indirectly stimulate the antioxidant response via glutathione depletion leading to oxidative stress and ROS production (Catalgol et al., 2009; Yousef and El-Demerdash, 2006). In the present study we measured the levels of selected ARE-derived cytoprotective proteins in nerve terminals and in cytosolic/nuclear factions of neuronal cell bodies isolated from rats intoxicated at two different ACR dose-rates. To detect possible differences in cell-specific induction, the ARE response to ACR intoxication was determined in hepatic cells. Results indicate that, despite the induction of significant neurotoxicity, ACR exposure did not alter the content of phase II enzymes/antioxidants proteins in any cell fraction examined. These data do not support our original hypothesis and, instead, suggest previously unrecognized limitations in the electrophile responsiveness of the Nrf2-ARE pathway.

2. Materials and methods

2.1. Chemicals and materials

All chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI). EDTA-free Protease Inhibitor Cocktail was purchased from Roche Applied Sciences (Indianapolis, IN). Antibodies were purchased from following sources: Novus–Nrf2, the modifier subunit of glutamate-cysteine ligase (GCLM), heme oxygenase-1 (HO-1) and cystine/glutamic acid transporter (xCT); Santa Cruz–nuclear transcription factor Y subunit α (NF-Ya), NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione reductase (GR); Abcam–glutathione transferase M1 (GST-M1); R&D System–Keap1; Sigma – glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Goat anti-mouse or anti-rabbit IgG conjugated to alkaline phosphatase and Western Blue® (alkaline phosphatase substrate) were purchased from Promega Life Sciences (Madison, WI). Pre-made gels were purchased from Invitrogen Co. (Carlsbad, CA). Pierce bicinchoninic acid (BCA) protein assay kits and radio-immunoprecipitation assay (RIPA) buffer were purchased from Thermo Fisher Scientific Inc. (Rockford, IL). Amicon Ultra-4 centrifuge filter units (3 kDa molecular weight cutoff) were purchased from Millipore Co. (Billerica, MA).

2.2. Animals and ACR intoxication

All aspects of this study were approved by the Montefiore Medical Center Animal Care Committee. Adult male Sprague-Dawley rats (300–325 g; Taconic Farms, Germantown, NY) were housed individually with drinking water and chow available *ad libitum*. Rats ($n = 4$–6 per group) were exposed to ACR at dose-rates of either 50 mg/kg/d × 10 days (i.p.) or 21 mg/kg/d × 38 days (p.o.). Both dosing schedules produced moderate-severe levels of neurotoxicity as assessed via bi-weekly measurements of body weights and gait scores. Previous neurological studies have shown that body weight changes and gait scoring were sensitive indices of developing chemical-induced neurotoxicity (LoPachin et al., 2002b). To measure the development of gait abnormalities, rats were placed in a plexiglass box and were observed for 3 min by a trained, blinded observer who was not involved in animal care or ACR exposure. Following observations, a gait score was assigned from 1 to 4 where: 1 = a normal gait; 2 = a slightly abnormal gait (slight ataxia, hopping gait and foot splay); 3 = moderately abnormal gait (obvious ataxia and foot splay with limb abduction during ambulation); 4 = severely abnormal gait (inability to support body weight and foot splay).

2.3. Differential centrifugation methods

The tissue fractionation procedures described in the following subsections are well-characterized and have been used extensively to determine the respective subcellular distribution of various soluble proteins and factors (e.g., see Gullo et al., 1987; Mishra et al., 2002). Furthermore, our general approach is based on previous studies (e.g., see Watai et al., 2007) using subcellular fractionation and immunoblot analysis to define the responses of the Nrf2-Keap1 system to oxidative/electrophilic stress. Brain and liver fractions were prepared 24 h after termination of ACR exposure.

2.3.1. Hepatic cell fractionation

Hepatic cell nuclear/endoplasmic reticulum and cytosolic fractions were prepared by the method of Dyer and Herzog (1995). Briefly, liver (1 g) was minced and then homogenized in liver lysis buffer (pH 7.9) containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2% β-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) using a Dounce Tissue Grinder. The homogenate was filtered through 70-μm nylon mesh and the filtrate was centrifuged at 11,000 × *g* for 20 min. The supernatant was retained as the liver cell cytosolic fraction. To prepare nuclei, the corresponding pellet was washed in lysis buffer and was resuspended with extraction buffer (0.66 volume) containing 20 mM HEPES, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA and 25% glycerol (pH 7.9). The pellet suspension was shaken gently for 30 min (4 °C) and then centrifuged at 11,000 × *g* (20 min). The supernatant was de-salted using Amicon Ultra-4 centrifuge filter units. The retained filtrate was resuspended in RIPA buffer and was designated the liver nuclear/ER (LNE) fraction.

2.3.2. Brain cell fractionation

Brain cell nuclear/ER and cytosolic fractions were prepared by a modification of Giufrida et al. (1975) method. Briefly, whole brains were minced and then homogenized in a brain lysis buffer (pH 6.4) containing 0.32 M sucrose, 1.0 mM KH_2PO_4, 3.0 mM $MgCl_2$, 1.0% Brij-35, 0.2% β-mercaptoethanol and 0.5 mM PMSF using a Dounce Tissue Grinder. The homogenate was then centrifuged at 11,000 × *g* for 20 min and the supernatant was retained as the brain cell (neuronal plus glial) cytosolic fraction. To prepare nuclei, the pellet was washed in brain lysis buffer (excluding Brij-35) and resuspended in extraction buffer (see Section 2.3.1). The pellet suspension was shaken gently for 30 min (4 °C) and then centrifuged at 11,000 × *g* (20 min). The supernatant was de-salted using Amicon Ultra-4 centrifuge filter units. The retained filtrate was resuspended in RIPA buffer and was designated the brain nuclear/ER (BNE) fraction.

2.3.3. Preparation of synaptosomes

Synaptosomes were prepared by the Percoll gradient method of Dunkley et al. as modified by LoPachin et al. (2004). In brief, whole brains were rapidly removed and minced in cold (4 °C, pH 7.4) gradient buffer containing 0.32 M sucrose, 1 mM EDTA and 0.25 mM dithiothreitol (SED). Tissue was gently homogenized in SED buffer (10 passes in a Teflon-glass homogenizer; 700 RPM) and the resulting homogenate was centrifuged at 1000 g (10 min, 4 °C). The pellet (P1) was washed once and supernatants (S1 and S2) were combined. Protein content of the pooled supernatant was determined by the Pierce BCA protein assay and was adjusted with SED to 5 mg/ml and then layered on top of a freshly prepared 4-step discontinuous Percoll gradient (3%, 10%, 15% and 23% Percoll in SED, pH 7.4). Gradients were centrifuged at 32,000 g for 6 min and synaptosomes were collected at the last interface (15%/23%) and homogenized in RIPA buffer.

2.4. Gel electrophoresis and semi-quantitative immunoblot analysis

Tris–glycine continuous gradient gels (8–16%) were used to separate proteins. Protein contents were measured by the Pierce BCA assay and proteins were loaded on gels at 25 μg per lane. Following electrophoretic separation, proteins were transferred to polyvinyldene fluoride (PVDF) membranes overnight (20 mA current). After transfer, membranes were blocked with 5.0% dried milk in TBS/0.1% TWEEN 20 for 1 h and then rinsed. Membranes prepared from fractions were incubated for 1hr with a selected antibody diluted in 5% dried milk/TBS. Following primary antibody incubation, membranes were washed in TBS and incubated (1hr) with alkaline phosphatase (AP)-conjugated secondary antibody (goat anti-mouse or anti-rabbit IgG). Membranes were washed again and immunoreactive bands were visualized with alkaline phosphatase substrate (Western Blue®). Immunoreactive protein bands were scanned with a densitometer, digitized and analyzed as pixels per total area of the immunoreactive band using the freehand selection tool of the NIH Imaging Program (LoPachin et al., 2004). Densitometric data from the different subcellular fractions were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To determine statistical differences in the fractional distribution of ARE and associated proteins, normalized data (mean ± SEM; $n \geq 3$) from control liver and brain cell fractions were compared using Student's *t* test ($p < 0.05$). ANOVA followed by Dunnett's *post hoc* test ($p < 0.05$) was used for multiple range comparisons among treated and control group mean data.

2.5. Calculation of HSAB parameters

The Lowest Unoccupied Molecular Orbital (LUMO) energy (E_{LUMO}) and Highest Occupied Molecular Orbital (HOMO) energy (E_{HOMO}), were calculated using Spartan08 (version 1.1.1) software (Wavefunction Inc., Irvine CA). For each chemical, ground state equilibrium geometries were calculated with Density Functional BSLYP 6-31G* in water starting from 6-31G* geometries. Global (whole molecule) hardness (η) was calculated as $\eta = (E_{LUMO} - E_{HOMO})/2$ and softness (σ) was calculated