Amino Acid Analog Toxicity in Primary Rat Neuronal and Astrocyte Cultures: Implications for Protein Misfolding and TDP-43 Regulation

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Amino acid analogs promote translational errors that result in aberrant protein synthesis and have been used to understand the effects of protein misfolding in a variety of physiological and pathological settings. TDP-43 is a protein that is linked to protein aggregation and toxicity in a variety of neurodegenerative diseases. This study exposed primary rat neurons and astrocyte cultures to established amino acid analogs (canavanine and azetidine-2-carboxylic acid) and showed that both cell types undergo a dose-dependent increase in toxicity, with neurons exhibiting a greater degree of toxicity compared with astrocytes. Neurons and astrocytes exhibited similar increases in ubiquitinated and oxidized protein following analog treatment. Analog treatment increased heat shock protein (Hsp) levels in both neurons and astrocytes. In neurons, and to a lesser extent astrocytes, the levels of TDP-43 increased in response to analog treatment. Taken together, these data indicate that neurons exhibit preferential toxicity and alterations in TDP-43 in response to increased protein misfolding compared with astrocytes. © 2011 Wiley-Liss, Inc.

Key words: aging; Alzheimer’s disease; cell death; neurodegeneration; neurotoxicity; protein aggregation; ubiquitin

Protein misfolding and protein aggregation are characteristic features of many neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD), frontotemporal lobar degeneration (FTLD), and amyotrophic lateral sclerosis (ALS). The amino acid analogs canavanine (Can) and azetidine-2-carboxylic acid (AZC) mimic the natural amino acids arginine and proline and are incorporated into nascent polypeptides promoting irreversible abnormal protein confirmation (Fowden and Richmond, 1963; Fowden et al., 1967; Grant et al., 1975; Prouty et al., 1975; Rosenthal et al., 1989; Zagari et al., 1990; Rodgers and Shiozawa, 2008; Bessonov et al., 2010) and alterations in global protein synthesis (Kretz-Remy et al., 1998; Qian et al., 2010). Can and AZC can therefore be used to mimic the increased levels of abnormal proteins observed in aging cells, and potentially model increased protein misfolding observed in a variety of neurodegenerative conditions.

To prevent proteotoxicity from increased protein misfolding, cells rely on the function of numerous heat shock proteins (Hsps), including Hsp70 and Hsp40 (Li and Laszlo, 1985; Ananthan et al., 1986; Watowich and Morimoto, 1988; Hightower, 1991; Trotter et al., 2002; Barrett et al., 2004). Both Can and AZC have been shown to induce a variety of Hsps (Thomas and Mathews, 1984; Li and Laszlo, 1985; Watowich and Morimoto, 1988; Kozutsumi et al., 1998; Trotter et al., 2002; Qian et al., 2010), consistent with both analogs promoting proteotoxic stress. Currently it is not known whether neurons and astrocytes differ with regard to their sensitivity to toxicity or Hsp induction in response to amino acid analogs such as Can and AZC.

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Several abnormal proteins have been shown to accumulate in neurodegenerative diseases such as AD, PD, and FTLD (Koo et al., 1999; Agorogiannis et al., 2004; Ross and Poirier, 2004; Meridith 2005; Zhu et al., 2005; Reddy, 2006; Uversky 2008), suggesting the genesis of proteotoxic stress. Recent studies have suggested an important role for TAR DNA-binding protein of 43-kDa (TDP-43) in modulating proteotoxicity associated with increased protein misfolding (Neumann et al., 2006; Chen-Plotkin et al., 2010). TDP-43 is abundantly expressed in neurons and glia and has been identified as a major component of ubiquitinated neuronal cytoplasmic inclusions (NCI) and neuronal intranuclear inclusions. Full-length TDP-43, as well as cleavage products of ~25 kDa and 35 kDa, are observed in ALS and FTLD (Dalal et al., 2004; Halawani and Latterich, 2006; Kabashi et al., 2008; Zhang et al., 2009; Chanson et al., 2010; Ritson et al., 2010; Barmada et al., 2010). Currently, it is not known whether analogs such as Can and AZC modulate TDP-43 homeostasis in primary neuron and astrocyte cultures.

The present study demonstrates that treatment of primary rat neurons and astrocytes results in a dose-dependent increase in cell death, with neurons being more vulnerable to the toxicity of Can and AZC. The preferential increase in neuronal toxicity did not appear to be linked to differences in ubiquitinated proteins, oxidized proteins, or Hsp induction. Amino acid analogs induced increased levels of TDP-43 and its cleavage products. Taken together, these data have implications for understanding how increased levels of aberrant proteins during aging and neurodegenerative disease contribute to neuronal death and dysfunction in the brain.

**MATERIALS AND METHODS**

**Materials**

The antibodies to β-actin (SC-47778) and ubiquitin (SC-8017) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies to TDP-43 (3448S) were purchased from Cell Signaling Technology (Cambridge, MA). The antibodies to Hsp70 (SPA-8101D) and Hsp40 (SPA-400D) were purchased from Enzo Life Sciences (Plymouth Meeting, PA). The oxyblot kit was purchased from Millipore (Billerica, MA). All the chemicals including Hoechst 33342 (bis-benzamide trihydrochloride) stain, Triton X-100, protease inhibitor mix, EDTA, DNase I, L-azetidine-2-carboxylic acid), and L-canavanine were purchased from Sigma-Aldrich (St. Louis, MO). All electrophoresis and immunoblot reagents were purchased from Bio-Rad (Hercules, CA). All cell culture supplies were obtained from Gibco Life Sciences (Gaithersburg, MD). The BCA reagent was purchased from Thermo Scientific (Pittsburgh, PA).

**Establishment and Maintenance of Primary Neuron and Astrocyte Cultures: Treatment With Analogos**

Neuronal cultures were established as described previously from our laboratory (Ding et al., 2006; Dasuri et al., 2010b; Ebenezer et al., 2010). Briefly, primary rat cortical neuronal cells were cultured from E18 Sprague-Dawley rats and maintained in 5% CO2 at 37°C in MEM or Neurobasal medium containing 5% fetal bovine serum (heat inactivated), N2 supplement, B27 supplement, and 1% antibiotic. Cells were used in experiments between days 6 and 9 (neurons) and between 12 and 15 (astrocytes) postplating. Rat astrocyte cultures were established from E18 Sprague-Dawley rats as described previously from our laboratory (Ding et al., 2006; Dasuri et al., 2010b; Ebenezer et al., 2010). Astrocytes were maintained in 5% CO2 at 37°C in MEM containing 5% fetal bovine serum (heat inactivated), N2 supplement, and 1% antibacterial solution. All animals were utilized in accordance with IACUC-approved protocols at the Pennington Biomedical Research Center. For the analysis of protein levels following analog treatment, the primary cultures of neurons and astrocytes were treated with various concentrations of analogs (AZC or Can), and the pelleted cells were frozen at −80°C until further use.

**Analysis of Cell Viability**

Cell survival was determined by quantification of apoptotic and necrotic nuclei using Hoechst 344 staining as described previously (Keller et al., 1998; Ding et al., 2006; Dasuri et al., 2010b, Ebenezer et al., 2010). Briefly, Neuronal and astrocyte cells were treated with increasing concentrations of analogs (AZC or Can) for 3 hr or with 5 mM of analog for the indicated intervals. The treated cells were stained with the fluorescent DNA-binding dye Hoechst 344 at a concentration of 1 μg/μl, and the percentage of viable cells was determined by counting the number of dead cells (condensed and fragmented nuclei) using a fluorescence microscope equipped with a ×32 objective. Additional confirmation of cell viability was determined using MTT reduction as a measure of cell viability as reported previously (Kruman et al., 1997; Ding et al., 2006; Dasuri et al., 2010b; Ebenezer et al., 2010).

**Protein Estimation**

Protein concentration of the cell lysates made in cell lysis buffer was estimated using BCA (Thermo Scientific) reagent as described by the manufacturer.

**Western Blotting**

The protein samples were analyzed by SDS-PAGE and immunoblotted with specified antibodies as described previously (Dasuri et al., 2010a).

**Analysis of Protein Oxidation Levels**

Protein carbonyl levels were analyzed using Oxyblot kit (Millipore) as described by the manufacturer. Briefly, 10 μg protein lysate was derivatized with 2,4-dinitrophenylhydrazone (DNPH), and then the derivatized products were detected by Western blot analysis as described by the manufacturer.

**Triton X-100 Fractionation of Cell Lysates and Analysis**

Triton X-100 fractionation was done as described previously (Dasuri et al., 2010b). Briefly, cells were suspended in Journal of Neuroscience Research
Levels of TDP-43 in Neurons and Astrocytes

To determine whether amino acid analog treatment alters the levels of TDP-43, we conducted studies looking at the total levels of TDP-43 in neurons and astrocytes. In this analysis, we observed that the total levels of TDP-43 were robustly increased in neurons (Fig. 5). In astrocytes, increases were transient or occurred to a lesser degree (Fig. 5) than was observed in neuronal cultures. Analysis revealed that the levels of the known ~35 kDa cleavage product of TDP-43 were selectively observed in neurons treated with the highest concentrations of amino acid analogs and were not observed in astrocyte cultures (Fig. 5). See Supporting Information Figure 2 for quantification of TDP-43.

DISCUSSION

Amino acid analogs AZC and Can are used to induce protein misfolding (Goldberg and Dice, 1974; Trotter et al., 2002) It is known that AZC causes changes in the confirmation of the protein backbone leading to functional alterations in multiple proteins (Trotter et al., 2001, 2002; Hoshikawa et al., 2003; Bessonov et al., 2010). Our study shows for the first time that AZC and Can both cause a loss of cell viability in primary CNS cultures. These postmitotic neurons in the current study exhibited dramatically elevated sensitivity to the toxicity associated with increased protein misfolding compared with mitotic astrocyte cultures. Neurons are known to be more vulnerable than astrocytes to a variety of stressors relevant to aging, including oxidative stressors and proteasome inhibitors (Keller et al., 1998, 1999; Schmuck et al., 2002; Watts et al., 2005; Ding et al., 2006; Dasuri et al., 2010b). This increased vulnerability may be due in part to the increased propensity of neurons to undergo increases in protein hydrophobicity following proteasome inhibitor treatment (Dasuri et al., 2010a) compared with astrocyte cultures. The key to understanding the basis for this toxicity likely resides in defining whether toxicity is mediated by gross abnormalities in the proteome or conversely is mediated by selective or key perturbations within the proteome, during periods of increased protein misfolding.

In the current study, we observed that the levels of ubiquitinated and oxidized proteins are increased in both neurons and astrocytes following amino acid analog treatment. The increases in both ubiquitinated and oxidized proteins were similar in both neurons and astrocytes, suggesting that the increased neuron death in neurons compared with astrocyte cultures likely is not mediated by gross increases in either ubiquitinated or oxidized proteins. Presumably, the misfolding of proteins by AZC and Can treatment results in their rapid ubiquitination, with the increase in ubiquitinated proteins in

Statistical Analysis

Statistical analyses were conducted in Prism 3.0 software (GraphPad Software, San Diego, CA). Student’s two-tailed t-test was used to determine whether observed differences were statistically significant (P < 0.05).

RESULTS

Neuronal Cells Exhibit Increased Sensitivity to Amino Acid Analog Toxicity

Canavanine and AZC are amino acid analogs that are incorporated into newly synthesized proteins, resulting in protein misfolding and the accumulation of abnormal proteins (Kelley and Schlesinger, 1978; Hightower 1980; Ananthan et al., 1986). To study the relative susceptibilities of primary rat neuronal and astrocyte cultures to the toxicity of misfolded and abnormal proteins, we conducted studies analyzing the acute toxicity of Can and AZC. We have observed that AZC and Can promoted a dose-dependent increase in cell death in both neurons and astrocytes (Figs. 1, 2). The toxicities of AZC and Can were nearly identical, with both agents observed to induce more pronounced cell death in neurons (Figs. 1, 2) at every dose analyzed. Treatment with analogs induced a time-dependent increase in cell death, with neurons observed to undergo more severe loss of viability than astrocytes (Figs. 1, 2) at every dose analyzed.

Neuronal and Astrocyte Cells Exhibit a Higher Level of Ubiquitinated and Oxidized Proteins Following Amino Acid Analog Treatment

To overcome the accumulation of abnormal or misfolded proteins, cells rely on the function of multiple proteolytic pathways, including ubiquitin-dependent pathways (Hershko and Ciechanover, 1992; Hochstrasser 1992; Navon and Ciechanover, 2009). In our analysis, we observed that Can and AZC increased the levels of ubiquitinated protein in both neurons and astrocytes (Fig. 3). Although much more variable, amino acid analog treatment also increased the levels of oxidized proteins in both neurons and astrocytes (Fig. 3).

Amino Acid Analog Treatment Increases the Levels of Hsps in Neurons and Astrocytes

Next, we sought to elucidate whether Can and AZC treatment altered the levels of Hsps in neurons and astrocytes. In our studies we observed that both neurons and astrocytes exhibited increases in Hsp70 and Hsp40 levels following Can and AZC treatment (Fig. 4). See Supporting Information Figure 1 for quantification of blots from Figure 4.

Effect of Amino Acid Analog Treatment on the Levels of TDP-43 in Neurons and Astrocytes

Levels of TDP-43 in Neurons and Astrocytes

To determine whether amino acid analog treatment alters the levels of TDP-43, we conducted studies looking at the total levels of TDP-43 in neurons and astrocytes. In this analysis, we observed that the total levels of TDP-43 were robustly increased in neurons (Fig. 5). In astrocytes, increases were transient or occurred to a lesser degree (Fig. 5) than was observed in neuronal cultures. Analysis revealed that the levels of the known ~35 kDa cleavage product of TDP-43 were selectively observed in neurons treated with the highest concentrations of amino acid analogs and were not observed in astrocyte cultures (Fig. 5). See Supporting Information Figure 2 for quantification of TDP-43.

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Statistical Analysis

Statistical analyses were conducted in Prism 3.0 software (GraphPad Software, San Diego, CA). Student’s two-tailed t-test was used to determine whether observed differences were statistically significant (P < 0.05).
the present study consistent with the proteolytic pathways responsible for ubiquitinated protein degradation being overwhelmed or inhibited following amino acid analog treatment. In our search of the literature, this study appears to be the first to demonstrate that amino acid analogs are capable of increasing the levels of oxidized protein in any cell type. Neither AZC or Can would be expected to be capable of producing reactive oxygen species, suggesting that the increased levels of oxidized protein in the current study are due to the misfolded proteins themselves being more vulnerable to the endogenous oxidative stressors in both neurons and astrocytes in culture. Alternatively, the increased protein misfolding induced by both AZC and Can may indirectly increase oxidative stress within both neurons and astrocytes. For example, misfolding (and presumably loss of function) of antioxidant enzymes and key cell signaling components results in amino acid analogs promoting increases in oxidized proteins by shifting the intracellular environment to a prooxidant environment. Interestingly, previous studies have implied a role for increased reactive oxygen species as a mechanism for amino acid analog-induced increases in nuclear factor-κB activation (Kretz-Remy et al., 1998).

Our results suggest that increased protein misfolding in the brain during aging and in a variety of neurodegenerative conditions promotes neurocognitive abnormalities through direct effects on neurons as well as indirect mechanisms. The direct mechanisms relate to the acute neurotoxicity and neuron death, as observed in...
Fig. 3. Levels of ubiquitinated and oxidized proteins are increased in a time-dependent manner following analog treatment. Rat primary cortical neurons and astrocyte cultures were analyzed for the levels of ubiquitinated (A) and oxidized (B) proteins following treatment with 5 mM AZC or canavanine for 3 hr. Results showed an increase in ubiquitination and oxidation levels with the increase in time of treatment. β-Actin was used to show equal loading of protein lysates. Data are presented as the mean and SEM of results from five independent experiments. *P < 0.05 and **P < 0.05 compared with the untreated neurons and astrocytes.

Fig. 4. Induction of heat shock proteins in neurons and astrocytes following treatment with amino acid analogs. Lysates from rat primary cortical neurons and astrocyte cultures were analyzed by Western blotting for the levels of heat shock proteins HSP70 (A,B) and HSP 40 (C,D) following amino acid analog treatment with 5 mM AZC or canavanine for different intervals. β-Actin was used to show equal loading of protein lysates. Data represent results from five independent experiments performed under similar conditions. See Supporting Information Figure 1 for quantification.

Fig. 5. Levels of TDP43 in neurons and astrocytes following treatment with amino acid analogs. A: Whole-cell lysates from rat primary cortical neurons and astrocyte cultures were analyzed by Western blotting for the levels of TDP-43 following treatment with different concentrations of amino acid analog AZC or canavanine for 3 hr. β-Actin was used to show equal loading of protein lysates. B: Insoluble fractions of lysates from rat primary cortical neurons and astrocyte cultures were analyzed by Western blotting for the levels of TDP-43 following treatment with 5 mM amino acid analog AZC or canavanine for 3 hr. Data represent findings from five independent experiments performed under similar conditions. See Supporting Information Figure 2 for quantification.
the present study. Alternatively, it is likely that the protein misfolding in the astrocytes in the current study is sufficient to promote the induction of chronic inflammatory signaling, which indirectly is sufficient to promote neurocognitive abnormalities based on the effects on neurons.

Neurons and astrocytes exhibited similar increases in Hsp70 and Hsp40 following amino acid analog treatment, even though neurons underwent preferential cell death in response to amino acid analog treatment. These data suggest that perturbations in Hsp levels are not the main modulators of cell death following acute increases in protein misfolding. Such data imply that Hsps are potentially overwhelmed or insufficient in this model of toxicity or conversely raise the possibility of protein misfolding being capable of initiating cell death independently of Hsp-regulated events. Studies are currently underway to elucidate which of these scenarios is most relevant to neuron death following amino acid analog treatment.

Interestingly, in the current study, it was observed that increases in TDP-43 protein occurred in neurons following amino acid analog treatment. Based on our survey of the literature, this appears to be the first report of amino acid analogs being sufficient to induce TDP-43 levels in any cell type. This study suggests that TDP-43 expression and the generation of the ~35-kDa TDP-43 fragment are rapidly induced in neurons following increases in protein misfolding. These data suggest that increases in protein misfolding in FTLD, ALS, and AD may be the basis for TDP-43 alterations observed in each of those conditions. Such increases may arise as the result of the activation of stress kinase pathways or other signal transduction cascades in response to misfolding or may result from the interactions of misfolded proteins with TDP-43. Studies are currently underway to elucidate the specific mechanism, particularly in light of the potential for TDP-43 to participate in neurodegenerative processes in a variety of pathological conditions. We do not believe that TDP-43 alterations are the primary source of toxicity in the present study but rather want to highlight our data demonstrating that amino acid analogs are capable of promoting TDP-43 alterations.

Our studies raise the possibility of specific proteins potentially playing an important role in the neurodegenerative process following elevations in protein misfolding. Specifically, the current study suggests a role for increased TDP-43 in mediating the toxicity of increased protein misfolding. The mechanisms responsible for TDP-43 neurotoxicity have not been firmly elucidated, although previous studies have provided data linking TDP-43 in cytoplasmic inclusions as mediators of neurodegeneration (Neumann et al., 2006; Arai et al., 2006; Sasaki et al., 2010; Barame et al., 2010). In the current study, we observed that analog treatment resulted in increased levels of insoluble TDP-43 (data not shown), consistent with the genesis of inclusions, although more data are needed to elucidate this aspect of TDP-43 pathology in the current model.

Previous studies in our laboratory have demonstrated that the ability of insults that increase protein misfolding (proteasome inhibitors) to induce neuron death is dependent on declines in protein synthesis (Ding et al., 2006). This has been extrapolated to generate a model in which protein synthesis is decreased in neurons during periods of increased protein misfolding as a means of protecting the cell (Ding et al., 2007). However, prolonged protein synthesis in this model transitions from being neuroprotective to being neurotoxic, as essential proteins are not generated or replaced because of the impairment in protein synthesis (Ding et al., 2007). Understanding the changes in protein synthesis that occur in the current model is currently being pursued, and when placed in the context of TDP-43 alterations this may help to increase our understanding of TDP-43 pathogenesis.

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