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N-ACETYL CYSTEINE BLUNTS PROTEOTOXICITY IN A HEAT SHOCK PROTEIN-DEPENDENT MANNER

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Abstract—*N*-Acetyl cysteine, a glutathione precursor, has been shown to benefit patients with Alzheimer's disease and reduce the symptoms of traumatic brain injury in soldiers. Parkinson's and Alzheimer's disease are both characterized by stress from protein misfolding, or proteotoxicity. We have developed a high-throughput model of proteotoxicity by treating neuroblastoma N2a cells with the proteasome inhibitor MG132 and performing three independent assays for viability. Our previous study showed that *N*-acetyl cysteine protects N2a cells against two sequential treatments of MG132 and raises glutathione levels in a two-hit model of synergistic neurodegeneration. In the present study, however, *N*-acetyl cysteine was found to reduce the toxicity of a single hit of MG132 independent of its effect on glutathione. All three viability assays confirmed this protection. We measured heat shock protein 70 (Hsp70) levels because Hsp70 is a protective chaperone that helps refold proteins or guides ubiquitinated proteins toward degradation by the proteasome. Hsp70 levels were higher in MG132-treated cells when *N*-acetyl cysteine was applied. No parallel change in heat shock cognate 70 (Hsc70) was elicited. Inhibition of Hsp70/Hsc70 activity with VER 155008 attenuated the protection afforded by *N*-acetyl cysteine in a dose–responsive manner. MG132 induced a large rise in ubiquitinated proteins and *N*-acetyl cysteine reduced this effect. Consistent with the chaperone functions of Hsp70, VER 155008 also prevented the reduction in ubiquitin-conjugated proteins by *N*-acetyl cysteine. These data reveal a new role for *N*-acetyl cysteine: this compound may reduce misfolded protein levels and ameliorate proteotoxicity through heat shock proteins. These findings broaden the potential mechanisms of action for this dietary supplement in neurodegenerative proteinopathies.

Key words: Parkinson's disease, Alzheimer's disease, heat shock protein 70, heat shock cognate 70, chaperone, *N*-acetylcysteine.

INTRODUCTION

Proteotoxic stress from protein misfolding is a hallmark of neurodegeneration and is further exacerbated in Alzheimer's and Parkinson's diseases by loss of proteasome activity (Keller et al., 2000; McNaught et al., 2003; McNaught, 2004). Inhibition of proteasome activity with toxins such as MG132 models proteotoxicity by increasing ubiquitin-conjugated proteins that cannot be degraded, causing protein aggregations, and eliciting cell death (Rideout et al., 2001, 2005; Rideout and Stefanis, 2002; Sawada et al., 2004; Sun et al., 2006; Xie et al., 2010). We recently developed a high-throughput model of proteotoxicity in which two sequential treatments of MG132 were applied to N2a cells (Unnithan et al., 2012). In accordance with the two-hit hypothesis of neurodegeneration, we found that exposure to toxic MG132 exacerbated the loss of viability in response to a second MG132 exposure. Two hits of MG132 were also found to cause synergistic loss of glutathione. The glutathione precursor *N*-acetyl cysteine prevented glutathione loss and almost completely protected against dual MG132 hits. Similarly, we have recently shown that *N*-acetyl cysteine raises glutathione and ameliorates MG132 toxicity in primary neurons (Posimo et al., 2013a). Other investigations have similarly revealed the protective impact of *N*-acetyl cysteine in experimental models of disease (for some examples, see Pocernich et al., 2000; Farr et al., 2003; Tucker et al., 2006; Clark et al., 2010). Furthermore, *N*-acetyl cysteine supplementation was found to improve cognitive performance in Alzheimer's patients (Adair et al., 2001) and is currently being tested on Parkinson's patients (Clinicaltrials.gov ID: NCT01470027). *N*-Acetyl cysteine also decreases mitochondrial oxidative stress in fibroblasts of Alzheimer's patients (Zhu et al., 2007). Recently, *N*-acetyl cysteine was shown to double the chances of recovery from symptoms of blast injury in soldiers during combat (Hoffer et al., 2013). These studies are largely based on the premise that *N*-acetyl cysteine is a glutathione precursor (Pocernich and Butterfield, 2012). However, in our previous study, *N*-acetyl cysteine also protected against single hits of MG132 without raising glutathione levels (Unnithan

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Abbreviations: ANOVA, analysis of variance; HO-1, heme oxygenase 1; HSF1, heat shock factor 1; Hsc70, heat shock cognate 70; Hsp70, heat shock protein 70; PBS, phosphate-buffered saline.

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et al., 2012). Only two MG132 hits elicited glutathione loss and *N*-acetyl cysteine was only able to raise glutathione in the dual-hit group. The present study therefore tested the hypothesis that *N*-acetyl cysteine protects against a single MG132 exposure in a glutathione-independent manner.

Heat shock proteins are defensive proteins that battle apoptosis, refold misfolded proteins, or escort irreparably damaged proteins to the proteasome (Ohtsuka and Hata, 2000; Beere, 2001; Garrido et al., 2001; Gabai and Sherman, 2002; Nollen and Morimoto, 2002; Morimoto, 2008; Kalia et al., 2010; Lanneau et al., 2010). Two well-known members of this protein family are the inducible heat shock protein 70 (Hsp70) and the constitutive heat shock cognate 70 (Hsc70). The present study reports for the first time that Hsp70 levels are higher in MG132-treated when *N*-acetyl cysteine is applied. No parallel effect on Hsc70 levels was observed. The second hypothesis of the present study was that inhibition of the ATPase activity of Hsp70/Hsc70 with VER 155008 (Massey et al., 2010; Chatterjee et al., 2013; Saykally et al., 2012) would attenuate the protective effect of *N*-acetyl cysteine. In addition to other functions, Hsp70 escorts ubiquitin-conjugated proteins to the proteasome for degradation (Gusarova et al., 2001; Zhang et al., 2001; Bailey et al., 2002; Adachi et al., 2003; Tsai et al., 2003; Kalia et al., 2004, 2010; Lanneau et al., 2010; Needham et al., 2011). Ubiquitinated proteins are expected to rise in response to proteasome inhibition because they cannot be degraded efficiently. Therefore, our third hypothesis was that *N*-acetyl cysteine would prevent the rise in ubiquitin-conjugated proteins normally seen in response to MG132. Finally, we also tested the fourth hypothesis that the Hsp70/Hsc70 inhibitor VER 155008 would prevent the impact of *N*-acetyl cysteine on ubiquitin-conjugated proteins because Hsp70 would no longer be able to refold proteins or escort them to the proteasome for degradation.

EXPERIMENTAL PROCEDURES

Cell maintenance and toxin treatments

N2a cells were maintained in 5% CO_2 in a humidified incubator at 37 °C. Cells were plated in high-glucose Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Carlsbad, CA, USA), supplemented with 0.37% sodium bicarbonate, 10% fetal clone III (ThermoScientific Hyclone, Logan, UT, USA), 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco, Life Technologies). Cells were plated at a density of 35,367 cells per cm^2 (10,000 cells/well in 96-well plates; Corning Incorporated, Corning, NY, USA). The day after plating, various concentrations of MG132 (EMD Millipore, Billerica, MA, USA) or dimethyl sulfoxide vehicle were applied as 10× stocks to existing media, either in the presence of *N*-acetyl cysteine (3 mM final concentration, Acros Organics, Somerset, NJ, USA) or an equal v/v of water. Media were exchanged the following day with fresh *N*-acetyl cysteine as a 1× stock or an equal v/v of water. In the inhibitor experiments, VER 155008 (12.5–100 μM; Tocris Bioscience, Bristol, UK) was applied in conjunction with vehicle, MG132, or *N*-acetyl cysteine in the same protocol. Plates were assayed on the following day for viability, 72 h after plating.

Primary and secondary antibodies

Antibodies used in the present report were purchased and diluted as follows: mouse anti-α-tubulin (1:10,000 for immunocytochemistry, 1:100,000 for Western blotting, Cat. No. T5168, Lot No. 078K4781, Sigma–Aldrich, St. Louis, MO, USA), mouse anti-heat shock protein 70 (Hsp70, 1:5000 for Western blotting, Cat. No. AB9920, Lot No. NG1571180, Millipore), mouse anti-ubiquitin-conjugated proteins (1:1000 for Western blotting, Cat. No. sc-8017 P4D1, Lot No. D0412, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rat anti-heat shock cognate 70 (1:5000 for Western blotting, Cat. No. SPA-815, Enzo Life Sciences, Farmingdale, NY, USA), mouse anti-ceruloplasmin (1:1000 for Western Blotting, Cat. No. 611488, Lot No. 80753, BD Biosciences, San Diego, CA, USA), rabbit anti-PA28α (1:1000 for Western blotting; Calbiochem, San Diego, CA, USA Cat. No. 539146, Lot No. D00092184), rabbit anti-PA700 10B (1:1000 for Western blotting; Calbiochem, San Diego, CA, Cat. No. 539147, Lot No. D00110930), mouse anti-catalase (1:5000 for Western blotting; Sigma–Aldrich Cat. No. C0979, clone CAT-505), rabbit anti-glutathione (1:300 for immunocytochemistry, Cat. No. AB5010, Lot No. NG1870405, EMD Millipore), mouse anti-β-actin (1:20,000 for Western blotting, Cat. No. A5441, Lot No. 028K4826, Sigma–Aldrich), rabbit anti-β-actin (1:1500 for Western blotting; Cat. No. A2103, Lot No. 051 M4767, Sigma–Aldrich), rabbit anti-GAPDH (1:1000 for Western blotting, Cat. No. 2118 (14C10), Lot No. 8, Cell Signaling Technology, Danvers, MA, USA), Alexa Fluor goat anti-mouse IgG 488 nm (1:1000 for immunocytochemistry, Cat. No. A11001, Molecular Probes, Life Technologies, Grand Island, NY, USA), donkey or goat anti-mouse IgG 800 nm (1:2000 for immunocytochemistry, 1:10,000 for Western blotting, Cat. Nos. 926-32212, 926-32210, Li-COR Biosciences, Lincoln, NV, USA), donkey or goat anti-rabbit IgG 800 nm (1:10,000 for Western blotting, Cat. Nos. 926-32213, 926-32211, Li-COR Biosciences, Lincoln, NV, USA), donkey anti-rabbit IgG 700 nm (1:10,000 for Western blotting; Cat. No. 926-32223, Li-COR Biosciences), and donkey or goat anti-mouse 700 nm (1:10,000 for Western blotting, Cat No. 926-32222, 926-32220, Li-COR Biosciences).

Viability assays

Three viability assays were used, as detailed previously (Unnithan et al., 2012). Briefly, cells were fixed in 4% formaldehyde, blocked in Odyssey Block (Li-COR Biosciences), and immunostained in the infrared wavelengths for the cytoskeletal protein α-tubulin (800 nm) followed by a combined solution of nuclear marker DRAQ5 and cytosolic marker Sapphire (700 nm; Li-COR Bioscience). α-tubulin immunostaining