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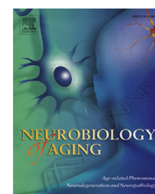

Hydroxyurea attenuates oxidative, metabolic, and excitotoxic stress in rat hippocampal neurons and improves spatial memory in a mouse model of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by accumulation of amyloid β-peptide (Aβ) plaques in the brain and decreased cognitive function leading to dementia. We tested if hydroxyurea (HU), a ribonucleotide reductase inhibitor known to activate adaptive cellular stress responses and ameliorate abnormalities associated with several genetic disorders, could protect rat hippocampal neurons against oxidative-, excitatory-, mitochondrial-, and Aβ-induced stress and if HU treatment could improve learning and memory in the APP/PS1 mouse model of AD. HU treatment attenuated the loss of cell viability induced by treatment of hippocampal neurons with hydrogen peroxide, glutamate, rotenone, and $A\beta_{1-42}$. HU treatment attenuated reductions of mitochondrial reserve capacity, maximal respiration, and cellular adenosine triphosphate content induced by hydrogen peroxide treatment. In vivo, treatment of APP/PS1 mice with HU (45 mg/kg/d) improved spatial memory performance in the hippocampus-dependent Morris water maze task without reducing Aβ levels. HU provides neuroprotection against toxic insults including Aβ, improves mitochondrial bioenergetics, and improves spatial memory in an AD mouse model. HU may offer a new therapeutic approach to delay cognitive decline in AD.

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1. Introduction

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder that affects more than 5 million Americans aged 65 y or older and is expected to affect nearly 14 million Americans by 2050 (Hebert et al., 2013). Individuals with AD experience episodic memory loss and dementia (Association, 2016). AD histology is characterized by the accumulation of amyloid β-peptide (Aβ) plaques and neurofibrillary tangles composed of hyperphosphorylated tau protein (Polanco et al., 2018). Brains from individuals with AD also exhibit an increase in inflammation characterized by activated astrocytes and microglial cells (Van Eldik et al., 2016). At the cellular level, neurons affected in AD exhibit mitochondrial dysfunction, impaired autophagy, increased oxidative stress, and an impaired ability to respond to and repair oxidative damage (Stranahan and Mattson, 2012).

Treatments are not available to prevent or slow the progression of AD. Currently, individuals with moderate-to-severe AD may be prescribed drugs such as memantine, an N-methyl-D-aspartate receptor antagonist, or acetylcholinesterase inhibitors, which have small effects on clinical symptoms (Aisen et al., 2012). However, there is currently no treatment available that substantially delays the onset or progression of AD. Due to a lack of prevention and an increase in prevalence, the current estimated cost of AD and other dementias in the United States alone is over 250 billion dollars annually. It is anticipated to rise to $1.1 trillion dollars by 2050 if an effective intervention(s) is not discovered (Association, 2016).

Previous studies in AD animal models have provided evidence that physiological and pharmacological interventions that activate adaptive cellular response signaling pathways, such as intermittent fasting and caloric restriction, can protect neurons against dysfunction and degeneration. For example, daily caloric restriction reduces Aβ pathology in amyloid precursor protein (APP) mutant

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mice and in APPswe695/PS1delE9 (APP/PS1) double-mutant transgenic mice (Patel et al., 2005). Intermittent fasting (alternate day fasting) and 30% daily caloric restriction ameliorate cognitive decline during aging in 3xTg AD mice (Halagappa et al., 2007). Intermittent fasting induces components of the adaptive cellular stress response and leads to increased neuronal resistance to stress and improved synaptic plasticity (Mattson et al., 2018). Treatment of APP mutant mice with the mechanistic target of rapamycin inhibitor rapamycin stimulated neuronal autophagy, reduced Aβ pathology, and lessened cognitive deficits (Spilman et al., 2010). Treatment of 3xTg AD mice with 2-deoxyglucose, which induces mild metabolic stress, bolstered mitochondrial function and reduced Aβ neuropathology (Yao et al., 2011). We previously found that hydroxyurea (HU), a ribonucleotide reductase inhibitor, improves cellular metabolic defects in fibroblasts from several genetic disorders and activates adaptive cellular stress responses in cultured human fibroblasts (Brose et al., 2012). These include upregulation of antioxidant defenses, protein chaperones and autophagy, and stimulation of mitochondrial biogenesis. Here we show that HU treatment protects cultured hippocampal neurons against oxidative, metabolic, and excitotoxic stress and improves memory in the APP/PS1 mouse model of AD.

2. Methods

2.1. Rat hippocampal neuronal cultures

Primary hippocampal neuron cultures were prepared from embryonic day 18 Sprague-Dawley rat brains as described previously (Kaech and Banker, 2006; Mattson et al., 1989). Dissociated neurons were plated at a density of 40,000 neurons per well (96-well plate) or 1×10^6 cells per well (6-well plate) on polyethyleneimine-coated plastic dishes in Minimum Essential Medium supplemented with 10% fetal bovine serum. Once the neurons attached to the plates, the cell plating medium was replaced with neurobasal (NB) medium with 5% B27 supplement, 1% GlutaMAX, and 1% antibiotic-antimycotic (Invitrogen, Waltham, MA) for 6–7 d. Before the addition of HU (5 μM unless otherwise specified; H8627, Sigma-Aldrich, St. Louis, MO), the neurons were washed with unsupplemented NB medium to remove traces of antioxidants in the B27 supplement, and the medium was replaced with NB medium supplemented with 5% B27 minus antioxidants (NB + B27 − AOX; Invitrogen), 1% GlutaMAX, and 1% antibiotic-antimycotic. Experiments were performed between days in vitro (DIV) 6 and 9.

2.2. Neurotoxicity assays

On DIV 6 or 7, neurons were treated with HU (5 μM; 2.5 μM for $A\beta_{1-42}$ experiment). The concentration of HU was titrated to allow 100% cell viability. After 24 h, H_2O_2 (15 μM), glutamate (50 μM; Sigma-Aldrich), rotenone (75 nM; Sigma-Aldrich), or oligomerized $A\beta_{1-42}$ peptide (5–10 μM; Bachem, Torrance, CA) was added and the neurons cultured for another 24 h. For the $A\beta_{1-42}$ peptide experiments, the neurons were incubated with HU (2.5 μM) in NB + B27 − AOX for the first 24 h. The medium was then changed to NB medium without any B27 supplement plus HU (2.5 μM); and, $A\beta_{1-42}$ peptide added for the last 24 h of incubation. Without B27 supplements, the neurons are more sensitive to perturbations. Therefore, the HU dosage was titrated to 2.5 μM to allow 100% cell viability. Cell viability was assessed using an MTS cell viability assay per manufacturer's instructions (CellTiter AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI). Briefly, 20 μL of CellTiter Aqueous One Solution was added to cells in 100 μL of NB medium + B27 − AOX. Cells were incubated at 37 °C in 5% CO_2, and the A_{490} measured with a Synergy H1 Microplate Reader (BioTek, Winooski, VT). Since metabolically active cells reduce the MTS tetrazolium compound to a colored formazan product, the amount of formazan measured at A_{490} was proportional to the number of metabolically active cells. Cell viability was normalized to the untreated control cells (100% viability).

2.3. Mitochondrial activity measurements

Mitochondrial activity was assessed using a Seahorse XF-96 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA) according to the manufacturer's instructions and as described previously (Divakaruni et al., 2014; Yao et al., 2017). Briefly, neurons were grown in Seahorse 96-well plates (40,000 cells/well). On DIV 6, the media was changed to NB + B27 minus AOX. Neurons were treated with HU (5 μM) on DIV 7. After 24 h, the neurons were treated with H_2O_2 (35 μM) for 1 h. The medium was changed to unbuffered XF assay medium (Seahorse Biosciences) supplemented with 5 mM glucose, 1 mM pyruvate, and 2 mM GlutaMAX (Invitrogen). The neurons were incubated in a non-CO_2 incubator at 37 °C for 1 h followed by measurement of basal oxygen consumption rate (OCR) and OCR after the sequential addition of 2 μM oligomycin, 1 μM FCCP, and 5 μM rotenone/antimycin A. Oligomycin inhibits mitochondrial adenosine triphosphate (ATP) synthase activity and facilitated the measurement of ATP-linked respiration. FCCP uncouples the mitochondrial proton gradient and disrupts the mitochondrial membrane potential allowing measurement of maximal respiration. Rotenone inhibits mitochondrial complex I, and antimycin inhibits mitochondrial complex III. The use of rotenone and antimycin together shuts down mitochondrial respiration and allows measurement of nonmitochondrial respiration.

2.4. ATP content

Cellular ATP content was determined using the ATP Determination Kit (#A22066; Molecular Probes, Eugene, OR) following the manufacturer's instructions. Briefly, on DIV 7, hippocampal neurons were treated with HU (5 μM). Approximately 24 h later, H_2O_2 peroxide was added (15 μM or 35 μM) for 1 h. All medium was removed, 11 μL boiling water was added to each well, and 110 μL of ATP reaction mixture was added and mixed with the lysed cells. ATP content was determined by measuring luciferase activity compared to a standard curve. The concentration of ATP per treatment condition was normalized relative to the untreated control neurons.

2.5. RNA isolation and microarray analyses

RNA was isolated from rat hippocampal neurons treated with HU (5 μM) for 2, 6, 12, or 24 h (n = 3 per time point) and from frozen mouse hippocampi isolated from 19- to 24-week-old nontransgenic (NTG) control littermates of APP/PS1 mice treated with HU (45 mg/kg/d; n = 4) or vehicle control (water; n = 4) for 2.5 mo using Trizol and following the manufacturer's instructions (Thermo Fisher Scientific). Animal housing conditions are listed below in Section 2.8. Total RNA was quality-controlled on the Agilent Bioanalyzer RNA 6000 Chip (Agilent, Santa Clara, CA) and 200 ng labeled using the Agilent Low-Input QuickAmp Labeling Kit, and purified and quantified according to the manufacturer's instructions. A total of 600 ng Cy3-labeled cRNA was hybridized for 17 h to Agilent SurePrint G3 Rat and Mouse GE 8x60K microarrays. Following posthybridization rinses, arrays were scanned using an Agilent SureScan microarray Scanner, and hybridization intensity data extracted from the scanned images using Agilent's Feature Extraction Software. Raw data were analyzed by Z-normalization as described previously (Cheadle et al., 2003). Principal component analysis was performed on the

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