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FREE RADICAL-MEDIATED NEUROTOXICITY MAY BE CAUSED BY INHIBITION OF MITOCHONDRIAL DEHYDROGENASES *IN VITRO* AND *IN VIVO*

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Abstract—We previously demonstrated that copper facilitated the formation of reactive oxygen species, and inhibited pyruvate dehydrogenase and α-ketoglutarate dehydrogenase *in vitro* and in animal models of Wilson's disease *in vivo*. However, direct Cu^{2+} toxicity has only been demonstrated for Wilson's disease. We now hypothesize that inhibition of these mitochondrial dehydrogenases might also contribute to many other injuries and disorders that are reactive oxygen species-mediated. We have modeled reactive oxygen species-mediated injuries using inducers of reactive oxygen species such as hydrogen peroxide, ethacrynic acid or menadione, or another redox active metal (Cd^{2+}). Here we demonstrated that these toxic exposures were accompanied by an early marked reduction in both pyruvate dehydrogenase and α-ketoglutarate dehydrogenase activities, followed by a decrease in neuronal mitochondrial transmembrane potential and ATP, prior to murine cortical neuronal death. Thiamine (6 mM), and dihydrolipoic acid (50 μM), required cofactors for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (thiamine as thiamine pyrophosphate), attenuated the reactive oxygen species-induced reductions in these enzyme activities, as well as subsequent loss of mitochondrial transmembrane potential and ATP, and neuronal death. We next tested the effect of thiamine supplementation on an *in vivo* model of reactive oxygen species-mediated injury, transient middle cerebral artery occlusion, and reperfusion in rats. Oral or i.p. thiamine administration reduced the middle cerebral artery occlusion-induced infarct. These data suggest that reactive oxygen species-induced neuronal death may be caused in part by reactive oxygen species-mediated inhibition of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase *in vitro* and *in vivo*, and that thiamine or dihydrolipoic acid may constitute potential therapeutic agents not just against Cu^{2+} neurotoxicity, but may reduce neuronal degeneration in the broader range of diseases mediated by free radical stress. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, hydroxyl free radicals, cadmium, focal ischemia, stroke.

Reactive oxygen species (ROS) have been implicated in CNS dysfunctions induced in numerous diseases and injuries. ROS have been suggested to play a role in neuronal death or disease etiology in brain ischemia (reviewed in Chan, 2001), Hemochromatosis (reviewed in Kang, 2001), Friedreich's ataxia (reviewed in Schulz et al., 2000), amyotrophic lateral sclerosis (Carri et al., 2003), Alzheimer's (reviewed in Honda et al., 2004; Huang et al., 2004), Parkinson's, and Wilson's diseases among others (Rossi et al., 2004). Furthermore, the redox-active metals (RAM) iron and copper have been suggested to be involved in this ROS generation for most of these neurodegenerative conditions (reviewed in Gotz et al., 2004; Mattson, 2004; Moos and Morgan, 2004; Todorich and Connor, 2004; Waggoner et al., 1999).

Cd^{2+}, in addition to Cu^{2+}, facilitates the formation of hydroxyl free radicals via Fenton reaction cycling with physiologic substances such as glutathione, cysteine, or ascorbic acid (Kachur et al., 1999; Ohta et al., 2000; Sokol, 1996). Excess copper in the plaques and inclusions has been suggested to interact with aggregated protein causing ROS generation in Alzheimer's (Huang et al., 1999a,b), Parkinson's (Lee et al., 2003; Paik et al., 1999; Uversky et al., 2001), and ALS (Carri et al., 2003). In each of these diseases, patients have increased markers of free radical stress. Furthermore, injection of beta-amyloid into rat brain was shown to induce oxidation of pyruvate dehydrogenase (PDH) by proteomic analysis (Boyd-Kimball et al., 2005).

ROS toxicity is suspected to be partly mediated by the oxidation of unknown target enzymes. ROS overload induces lipid peroxidation marked by formation of 4-hydroxy nonenal (HNE), and mitochondrial dysfunction (Zarkovic, 2003). HNE inhibits PDH and α-ketoglutarate dehydrogenase (KGDH), perhaps by covalently modifying the lipoic acid moiety of these enzymes (Brown et al., 1997; Humphries and Szweda, 1998). PDH has also been demonstrated to be preferentially sensitive to other insults that induce oxygen free radicals, such as ischemia, or exposure to hydrogen peroxide (Saris and Skulskii, 1991; Sims et al., 2000; Tabatabaie et al., 1996; Zaidan and Sims, 1997). Increased free radical generation, lipid peroxidation, HNE formation (Sokol et al., 1994; Yoshida et al., 2000; Zarkovic, 2003), and mitochondrial dysfunction occur in animal models and the affected organs of people

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Abbreviations: CCA, common carotid artery; CoA, coenzyme A; DHLA, dihydrolipoic acid; HNE, 4-hydroxy nonenal; KGDH, α-ketoglutarate dehydrogenase; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MEM, minimum essential medium; NBT, nitroblue tetrazolium; PDH, pyruvate dehydrogenase; RAM, redox active metals; ROS, reactive oxygen species; TMRE, Tetra-Methyl Rhodamine Methyl Ester; TPP, thiamine pyrophosphate; TTC, triphenyl tetrazolium chloride; $\Delta\Psi_M$, mitochondrial transmembrane potential.

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afflicted by some of the diseases mentioned above (Rego and Oliveira, 2003; Rossi et al., 2004; Zarkovic, 2003). Cu^{2+}, Fe^{3+}, and Cd^{2+}, are redox active and cause increased ROS generation (reviewed in Britton, 1996; Stohs and Bagchi, 1995). They also induce lipid peroxidation-dependent dysfunction of mitochondria *in vitro* or *in vivo* (Fujimoto et al., 1984; Kohno et al., 2000; Sheline and Choi, 2004; Sheline et al., 2002). H_2O_2 and ethacrynic acid have also been shown to cause lipid peroxidation and HNE formation (Joshi et al., 2005; Yamamoto et al., 2002). Environmental, and industrial exposures are also important risk factors for these RAM (Baddeley et al., 1983; Ellis et al., 1981). *In vitro* and animal models of exposure to Fe^{3+}, Ni^{2+}, Cu^{2+}, and Cd^{2+} have demonstrated an increased ROS production, lipid peroxidation, and in some cases mitochondrial dysfunction (Chakrabarti et al., 2001; Shukla and Chandra, 1989; Yiin et al., 1999).

We now hypothesize that mitochondrial dehydrogenase inactivation and dysfunction are crucial mechanisms in the broader setting of ROS-induced neurodegeneration encompassing many additional disorders. We postulate that pathophysiological concentrations of ROS induced in these many disorders (see above) kills neurons by: lipid peroxidation and oxidation or adduction of critical mitochondrial dehydrogenases causing dysfunction. The purpose of the present study was to test the hypothesis that neurons exposed *in vitro* or *in vivo* to excess ROS would develop impairment of energy metabolism associated with inhibition of PDH, and KGDH, and if so, whether the administration of the PDH and KGDH cofactors, thiamine or dihydrolipoic acid (DHLA) (Lehninger et al., 1993), might be neuroprotective. The mechanisms by which thiamine and DHLA reduce PDH and KGDH inhibition will be studied to aid in the development of further therapeutics. An abstract has appeared (Sheline and Choi, 2002).

EXPERIMENTAL PROCEDURES

Cell culture and toxicity studies

Near-pure cortical neuronal cell cultures were prepared from E15 mice as previously described (Sheline and Choi, 1998). Animals were handled in accordance with a protocol approved by our institutional animal care committee, and in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals and U.S. Department of Agriculture regulations. All efforts were made to minimize animal suffering and the number of animals used. Dissociated cortical neurons were plated in Eagle's minimum essential medium (MEM; Earle's salts, glutamine-free) containing 21 mM glucose, 5% fetal bovine serum, and 5% horse serum at a density of five hemispheres/plate onto poly-D-lysine/laminin (PDL)-coated 24-well plates. At 3 days *in vitro* (DIV) cytosine arabinoside was added to 10 μM to inhibit glial growth. HepG2 cultures were grown in Dulbecco's modified Eagle medium (DMEM)+10% fetal bovine serum and passaged every 2–3 days by trypsin/EDTA digestion. Cultures were grown to 50–75% confluence before exposure.

Toxicity was initiated by exposure to ROS inducing compounds, or $CdCl_2$ in MEM-defined media after thorough washing in the same medium. Cell death was assayed at varying times later. Cell death was assessed by lactate dehydrogenase efflux to the bathing medium, MTT staining, or staining with propidium iodide (Ying et al., 2000), and confirmed visually.

ATP measurements

For the ATP measurements, near-pure cortical neuronal cultures were lysed by addition of 0.1 M NaOH/1 mM EDTA after the indicated exposures and times, and the supernatant was neutralized and protein was precipitated by addition of a small amount of perchloric acid. These lysates were cleared and ATP was measured by the luciferin/luciferase luminescence assay (Lust et al., 1981).

Measurement of the mitochondrial transmembrane potential, $\Delta\Psi_M$

$\Delta\Psi_M$ Was assessed using the dyes Tetra-Methyl Rhodamine Methyl Ester (TMRE), and confirmed using the ratioable dye ApoAlert. Near-pure neuronal cultures were washed three times into MEM containing 21 mM glucose, loaded by bath application of 50 nM TMRE (Sigma, St. Louis, MO, USA; excitation λ: 560 nm, emission λ: 615 nm) for 30 min at 37 °C, and washed three times again prior to toxic exposure. Representative fluorescence photomicrographs were taken at identical exposures before and at various timepoints after ROS exposure using an SIS FluoViewII camera and AnalySIS 3.2 software connected to a Nikon TS-100 epifluorescence microscope. Neurons (25/well, eight wells) were chosen at random under brightfield, and fluorescence intensity was measured and normalized to sham wash control.

α-KGDH, and PDH assays

In situ α-KGDH and PDH assays were performed on near-pure cortical neuronal cells as described (Park et al., 2000). This method relies on the formation of formazan from nitroblue tetrazolium (NBT) upon electron transfer from NADH that is the product of the enzyme reaction of PDH and KGDH. Briefly, live cultures were incubated in buffer containing: 50 mM Tris–HCl (pH 7.6), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.05 mM EDTA, 0.3 mM thiamine pyrophosphate (TPP), 0.5 μg/ml rotenone, 0.2% Triton X-100, 3.5% polyvinyl alcohol, 3 mM α-ketoglutarate, 3 mM NAD^+, 0.75 mg/ml coenzyme A (CoA), 0.75 mM NBT, and 0.05 mM phenazine methosulfate for up to 60 min at room temperature. The NBT and phenazine methosulfate were added immediately before the reaction. The specificity of the reaction was demonstrated by leaving out CoA or the substrate, and the absorbance of sister cultures exposed in the absence of substrate was subtracted from each sample, and the absorbance of sham washed cultures was set as 100%. PDH was assayed in a similar fashion, except that pyruvate was substituted for α-ketoglutarate. The formazan was extracted in 10% Igepal CA-630 detergent by sonication, and the absorbance measured at 595 nm for quantitation.

Middle cerebral artery occlusion (MCAO) experiments

Long-Evans male rats (body weight: 300–350 g, Charles Rivers, Wilmington, DE, USA) were used in this study. Housing and anesthesia concurred with guidelines established by the institutional Animal Studies Committee, and were in accordance with the PHS Guide for the Care and Use of Laboratory Animals, USDA Regulations, and the AVMA Panel on Euthanasia guidelines. Rats were allowed free access to water and rat chow (Wayne, Chicago, IL, USA) until surgery.