

A zebrafish assay for identifying neuroprotectants in vivo

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Abstract

In this study, we developed an in vivo method to determine drug effects on oxidation-induced apoptosis in the zebrafish brain caused by treatment with L-hydroxyglutaric acid (LGA). We confirmed that LGA-induced apoptosis was caused by oxidation by examining the presence of an oxidative product, nitrotyrosine. Next, we examined the effects of 14 characterized neuroprotectants on LGA-treated zebrafish, including: D-methionine (D-Met), Indole-3-carbinol, deferoxamine (DFO), dihydroxybenzoate (DHB), deprenyl, L-NAME (*N(G)*-nitro-L-arginine methyl ester), *n*-acetyl L-cysteine (L-NAC), 2-oxothiazolidine-4-carboxylate (OTC), lipoic acid, minocycline, isatin, cortisone, ascorbic acid and α -tocopherol. Eleven of 14 neuroprotectants and 7 of 7 synthetic anti-oxidants exhibit significant protection in zebrafish. Buthionine sulfoximine (BSO), used as a negative control, exhibited no significant protective effects. In addition, three blood–brain barrier (BBB) impermeable compounds exhibited no significant effects. Our results in zebrafish were similar to results reported in mammals supporting the utility of this in vivo method for identifying potential neuroprotective anti-oxidants.

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1. Introduction

Oxidative stress occurs in response to an increase in oxidants, a decrease in anti-oxidants, or due to unrepaired oxidative damage induced by reactive oxygen species (ROS). ROS include free radicals, and reactive molecules containing oxygen such as nitric oxide (NO), superoxide (O_2^-), peroxynitrite ($ONOO^-$), and hydroxyl radical ($OH^\cdot$). Under normal conditions, ROS are cleared from cells by the action of superoxide dismutase (SOD), catalase, or peroxidases. ROS can modify proteins, lipids and nucleic acids and interfere with various biological processes, including impairing mitochondrial function, decreasing intracellular glutathione level (GSH), altering epithelial ion transport, increasing cell permeability, or altering calcium influx and gene expression, all of which eventually lead

to cell death and tissue damage [19]. Development of agents that modulate oxidation is a major focus of biopharmaceutical research; the ability to manipulate oxidation–reduction status could permit early therapeutic intervention in several major diseases.

Oxidation or ROS have been shown to induce pathologies such as cardiovascular disease, diabetes, dementia, macular degeneration, inflammation, hearing loss, various neurodegenerative diseases, stroke and aging [31,9]. Excessive production of NO following an oxidative insult has been shown to lead to cytotoxicity associated with neurodegenerative, cardiovascular and gastrointestinal diseases [1,20,28,30]. NO has been detected in Lewy bodies in brain sections of Parkinson's disease (PD) patients [8] by assessing nitrotyrosine immunoreactivity; an elevated level of nitrotyrosine indicates the presence of NO. In a mouse model of PD, increased nitration of tyrosine (Tyr) residues was also found in the striatum and ventral midbrain, supporting the theory that oxidation is involved in PD [24]. Experimental models of Alzheimer's disease (AD) have linked increased oxidative stress to disease progression [27] which is manifested by an increase in protein carbonyl content or lipid and DNA oxidation products [28]. In

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AD brain specimens, 3-nitrotyrosine (3-NT) levels were consistently five to eightfold higher in the hippocampus and neocortical regions than levels in the brains of normal subjects [11]. Results from several studies suggest that oxidative stress is involved in ALS pathophysiology, including a decreased level of glutathione (GSH) and an increased level of products of oxidative damage, such as protein carbonyls, 8-OHdG and 4-hydroxy-2-trans-nonenal (HNE) [28]. Increased levels of Tyr residue nitration have also been detected in ALS patients [35]. NO and oxygen free radicals appear to be involved in ischemic brain injury [5]. In a rat stroke model, 3-NT immunoreactivity was detected in neuronal cells in ischemic brain lesions [33]. It has been suggested that overproduction of NO contributes to stroke-related cerebral injury [32].

Conventional *in vitro* and *in vivo* methods for detecting oxidative stress using chemical reactions include: (1) reduction of oxidation (Redox) potential of the GSH/GSSH coupling ratio; (2) F2-isoprostane production for lipid peroxidation; (3) detection of superoxide (cytochrome *C* reduction, inhibition of aconitase, hydroethidine oxidation by superoxide, chemiluminescent reactions, electron spin resonance and spin trapping); (4) detection of hydrogen peroxide (dichlorofluorescein fluorescence, aminotriazole inhibition of catalase); (5) detection of NO and associated decomposition products (Griess reaction for determining nitrate and nitrite, fluorescence spectroscopy for determining NO, detecting *s*-nitrosothiols using colorimetric and fluorometric methods, quantitation of 3-nitrotyrosine) [6,34]. Because the level of reactive species is relatively low and the *in vivo* half-life is short, detection of changes in intra- and extra-cellular production of reactive species may require higher sensitivity and reproducibility than these methods provide. In addition, histochemistry or cell separation, used for cell or organ-specific assessment, is labor intensive.

Zebrafish has served as a useful sentinel for detecting environmental hazards and for risk assessment, which is one of the model organisms supported for the study of environmental toxicity by the National Institute of Environmental Health Sciences (NIEHS, USA) and Institute for Environment and Sustainability (IES, Europe). Genes and pathways involved in apoptosis, neuronal development, and oxidation are highly conserved [15,14,23,21,4] and the value of this model organism for drug screening, target validation and toxicological studies is increasingly recognized [10,29,7,22]. Because zebrafish larvae absorb small molecules diluted in the surrounding water through their skin and gills, drug delivery is straightforward. Simple delivery of agents that cause oxidative stress in specific tissues and organs, followed by rapid *in vivo* assessment of oxidative damage is not possible in other vertebrate models. In addition, compared to testing in other animal models, a statistically significant number of zebrafish can be used for each treatment condition and, due to their size, small amount (~1 µg) of drug is required for each dosing regimen. Therefore, zebrafish permit highly efficient phenotype analysis and rapid *in vivo* drug screening [23,26].

Here, we report that L-hydroxyglutaric acid (LGA), a biochemical hallmark of hydroxyglutaric aciduria (OHGA) [12,16], induces oxidative apoptosis in the zebrafish brain. In

addition, 11 of 14 neuroprotectants and 7 of 7 anti-oxidants we examined exhibited similar pharmacologic effects in zebrafish to effects reported in mammals. Three compounds which are BBB impermeable exhibited no protection. Our rapid, quantitative *in vivo* zebrafish assay can be used to identify potential anti-oxidants and to assess cell or organ-specific drug effects after cell-based evaluation and before expensive mammalian studies.

2. Materials and methods

2.1. Embryo handling

Zebrafish embryos were generated by natural pair-wise mating in our aquaculture facility [36]. 4–5 pairs were set up for each mating; on average 100–150 embryos per pair were generated. Embryos were maintained in fish water (5 g Instant Ocean Salt in 25 l of distilled water, pH 7.4), however, drug treatment was performed in embryo water (13.7 mM NaCl, 540 µM KCl, pH 7.4, 25 µM Na₂HPO₄, 44 µM KH₂PO₄, 300 µM CaCl₂, 100 µM MgSO₄, 420 µM NaHCO₃, pH 7.4, 5 g of Instant Ocean Salt with 3 g of CaSO₄ in 25 l of distilled water) at 28 °C. At 24 h, zebrafish were sorted for viability. Because the embryos receive nourishment from an attached yolk ball, no additional maintenance was required.

2.2. Treatment with LGA

3-dpf zebrafish were treated for 24 h with: 10, 30, 40, and 50 mM L-hydroxyglutaric acid (LGA, Sigma-Aldrich, St. Louis, MO). LGA was added directly to the embryo medium described above. Drug treatment was performed in 12-well plates. For each treatment, 15 zebrafish were used.

2.3. AO staining for apoptosis

At the end of drug treatment, 4-dpf zebrafish were washed with fish water three times and immersed in 1 µg/ml AO (acridinium chloride hemi-[zinc chloride], Sigma-Aldrich) in fish water for 60 min. Next, zebrafish were rinsed thoroughly in fish water three times (5 min/wash) and anaesthetized with MESAB (0.5 mM 3-aminobenzoic acid ethyl ester, 2 mM Na₂HPO₄). Zebrafish were then oriented on their ventral side and mounted with methylcellulose in a depression slide for observation by fluorescence microscopy.

2.4. Fluorescence microscopy and image analyses

All fluorescence microscopy studies were performed using a Zeiss M2Bio fluorescence microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY), equipped with a rhodamine cube and a green FITC filter (excitation: 488 nm, emission: 515 nm), and a chilled CCD camera (AxioCam MRM, Carl Zeiss Microimaging Inc.). Images were processed with Axiovision software Rel 4.0 (Carl Zeiss Microimaging Inc.) and Adobe Photoshop 7.0 software (Adobe, San Jose, CA). Particle analysis (Scion Image, Scion Co., Frederick, MD) was used to quantify fluorescence.

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