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CYP2E1 induction leads to oxidative stress and cytotoxicity in glutathione-depleted cerebellar granule neurons

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ABSTRACT

Increasing evidence suggests that brain cytochrome P450 (CYP) can contribute to the in situ metabolism of xenobiotics. In the liver, some xenobiotics can be metabolized by CYPs into more reactive products that can damage hepatocytes and induce cell death. In addition, normal CYP activity may produce reactive oxygen species (ROS) that contribute to cell damage through oxidative mechanisms. CYP2E1 is a CYP isoform that can generate ROS leading to cytotoxicity in multiple tissue types. The aim of this study was to determine whether CYP2E1 induction may lead to significant brain cell impairment. Immunological analysis revealed that exposure of primary cerebellar granule neuronal cultures to the CYP inducer isoniazid, increased CYP2E1 expression. In the presence of buthionine sulfoximine, an agent that reduces glutathione levels, isoniazid treatment also resulted in reactive oxygen species (ROS) production, DNA oxidation and cell death. These effects were attenuated by simultaneous exposure to diallyl sulfide, a CYP2E1 inhibitor, or to a mimetic of superoxide dismutase/catalase, (Euka). These results suggest that in cases of reduced antioxidant levels, the induction of brain CYP2E1 could represent a risk of in situ neuronal damage.

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1. Introduction

The cytochrome P450 (CYP) enzyme family is responsible for the oxidative metabolism of a vast array of endogenous and exogenous compounds. CYPs metabolize cholesterol, bile acid, steroids, arachidonic acid, eicosanoids, dietary constituents, clinical drugs, drugs of abuse and environmental toxins. Xenobiotics are often metabolized to polar hydrophilic molecules which facilitates their elimination from an organism; however, in some cases, CYP electrophilic products may produce cell toxicity through their interaction with macromolecules (Guengerich and MacDonald, 2007).

Xenobiotic metabolism occurs predominately in the liver, but it has also been demonstrated that functional CYPs are present in extra-hepatic sites, such as the gut, lung (Ding and Kaminsky, 2003) and brain (Ferguson and Tyndale, 2011).

CYP expression varies among specific brain regions and cell types, suggesting that the metabolism of xenobiotics capable of crossing the blood–brain barrier could occur in specific microenvironments (Dutheil et al., 2008). Since the discovery of CYP in the brain, several publications have demonstrated that these enzymes are inducible (Das et al., 1981) and that brain microsomes can metabolize environmental neurotoxins (Anandatheerthavarada et al., 1993; Cohn et al., 1977; Miller et al., 1986; Upadhya et al., 2001). Considering the well-known role of environmental toxins and the etiopathogenesis of certain neurodegenerative processes, it is crucial to determine whether CYP induction and neurotoxin metabolism can occur in various model systems, such as animal models or primary cell cultures. Notably, it has been reported that brain CYP2B and CYP2D can participate in the local metabolism and pharmacological effect of some drugs and neurotoxins in

Abbreviations: CGNs, cerebellar granule neurons; CYP2E1, cytochrome P450 2E1; ROS, reactive oxygen species; GSH, glutathione; DIV, days *in vitro*; INH, isoniazid; BSO, L-buthionine sulfoximine; DAS, diallyl sulfide; EUKA, Eukarion-134; 8-oxodG, 8-oxo-7,8-dihydro-2-deoxyguanosine.

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rodent models (Khokhar and Tyndale, 2011, 2012; Zhou et al., 2013).

One of the important CYP isoform involved in xenobiotic metabolism leading to chemical toxicities is CYP2E1 (Gonzalez, 2007). This isoform has been implicated in the bioactivation of many low-molecular-weight procarcinogens including nitrosamines and benzenes (Trafalis et al., 2010). Furthermore, CYP2E1 is partially responsible for the hepatotoxicity of ethanol (Lu and Cederbaum, 2008), acetaminophen (Lee et al., 1996) and isoniazid (Shen et al., 2006). CYP2E1 is able to generate reactive oxygen species (ROS) mostly superoxide anion and hydrogen peroxide in the absence of a substrate (Bell and Guengerich, 1997; Dai et al., 1993; Morgan et al., 1982), and its overexpression may cause oxidative stress and lipid peroxidation in hepatocytes (Chen and Cederbaum, 1998). The removal of glutathione (GSH) in human hepatoma cell line HepG2 overexpressing CYP2E1 resulted in apoptosis and necrosis (Wu and Cederbaum, 2001).

Although the hepatotoxic effect resulted from CYP2E1 over expression has been demonstrated, the possibility of irreversible damage and loss of neurons through in situ generation of reactive oxygen species by CYP2E1 in the nervous system requires to be deeply explored. Moreover all the information generated in xenobiotic bioactivation in neurons is valuable as the metabolites and reactive oxygen species generated by CYP2E1 activity could differ depending on the xenobiotic and cell type exposed, as well as the cellular environment conditions.

The aim of this study was to determine whether CYP2E1 induction may lead to brain cell impairment. We used cerebellar granule neurons (CGNs) because of their pronounced response among other brain structures toward CYP2E1 induction by xenobiotics (Joshi and Tyndale, 2006a; Yadav et al., 2006; Zhong et al., 2012) and to the abundance of this cell type in the cerebellum. CYP2E1 was induced by isoniazid and evaluated by immunocytochemistry and western blot. Consequences of this induction including the production of reactive oxygen species, DNA oxidation and cell viability were evaluated in the absence or presence of an inhibitor of GSH synthesis.

2. Materials and methods

2.1. Chemicals and materials

Fetal calf serum, penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA). Dihydroethidine and calcein AM were purchased from Invitrogen, Molecular Probes (Eugene, OR, USA). Poly-L-lysine (mol. wt. > 300,000), trypsin, DNAse, cytosine arabinoside, DMSO, glutathione reductase, propidium Iodide, GSH, GSSG, sulfosalicylic acid, dithiobis-2-nitrobenzoic acid, vinylpyridine, isoniazid (INH) and buthionine sulfoximine (BSO) were purchased from Sigma (St. Louis, MO, USA). Complete protease inhibitors were purchased from Roche (Mannheim, DE). Chemiluminescence system CDP-Star were purchased from New England BioLabs Inc. (Ipswich, MA, USA). Eukarion-134 (Euka) was from Cayman Chemicals (Ann Arbor, MI, USA). PVDF membranes and antibodies against CYP2E1 and GAPDH were obtained from Chemicon (Millipore, Billerica, MA, USA), and antibodies against 8-oxo-dG were obtained from TREVIGEN (Gaithersburg, MD, USA). CYP2E1 supersomes® were purchased form BD Biosciences (Franklin Lakes, NJ, USA). All other chemicals used were of the purest grade available from regular commercial sources.

2.2. Cerebellar granule neuronal cultures

All animals used were treated in accordance with the accepted standards of animal care and the procedures approved by the local Committee of Research and Ethics of the Instituto de Investigaciones Biomédicas and the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México.

Cerebellar granule neuronal cultures were prepared as previously described (Moran and Patel, 1989). Briefly, cell suspensions dissociated from 8-day-old rat cerebellum were plated at a density of 265×10^3 cells/cm^2 in plastic dishes or coverslips coated with poly-L-lysine (5 μg/mL). The culture medium contained basal Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 25 mM KCl, 50 U/mL penicillin and 50 mg/mL streptomycin. The culture dishes were incubated at 37 °C in a humidified, 5% CO_2 atmosphere. Cells were maintained under these conditions for 7–8 days *in vitro* (DIV). To induce CYP2E1, the cells were treated with 0.1 mM isoniazid (INH) for 12 h (Madan et al., 2003). In some experiments, 100 μM diallyl sulfide (DAS) and 10 μM Eukarion-134 (Euka) were added to the culture medium concurrently with isoniazid. Some cells were preincubated with BSO for 12 h.

2.3. Immunofluorescence

To visualize CYP2E1 in cultured neurons, cells were grown on glass coverslips. After treatment, cells were rinsed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and then washed 3 times with PBS. Cells were permeabilized and blocked with 0.3% Triton X-100 and 10% normal goat serum in PBS for 3 h before exposure to a rabbit polyclonal antibody against CYP2E1 (1:100 dilution) overnight at 4 °C. Cells incubated without primary antibody and unspecific IgG were used as negative controls. Cells were incubated for 1 h at room temperature with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (1:250 dilution). Next, the stained cells were mounted with medium for fluorescence and the nuclei were stained with DAPI (1.5 mg/ml). Fluorescence was examined with a confocal laser-scanning microscope (Olympus Fluoview FV1000).

2.4. Western blot

Cells were washed with PBS and homogenized with lysis buffer (Tris 25 mM, NaCl 50 mM, Igepal 2%, SDS 0.2% and Complete protease inhibitors, pH 7.4). Protein concentration was quantified by the method of Bradford (Bradford, 1976). 60 μg of protein homogenate were separated in SDS–PAGE gels (8%) and transferred to a PVDF membrane. Blots were blocked with Tris-buffered saline and incubated two days at 4 °C with the primary antibody raised against CYP2E1 (1:300 dilution,) or 1 h for GADPH (1:3000 dilution). After washing, blots were incubated with a secondary antibody coupled to alkaline phosphatase (anti-rabbit: 1:20,000 dilution; anti-mouse: 1:20,000) for 1 h at room temperature. Blots were processed for visualization using an enhanced chemiluminescence system according to the manufacturer's recommendations and exposed to Kodak film. Densitometric analysis was done using GelQuant.NET software.

2.5. Measurement of ROS generation

After treatment, neurons were incubated with DHE in the culture medium at 37 °C for 20 min. Following incubation, neurons were washed with PBS and then fixed in 4% fresh paraformaldehyde for 7 min. Neurons were examined with an epifluorescence Nikon Diaphot microscope using a rhodamine filter (546 nm excitation and 590 nm emission wavelengths). The results are expressed as the percentage of DHE-positive cells with respect to the total number of cells per field.

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