Reproductive Toxicology 33 (2012) 361–373

Contents lists available at SciVerse ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Protective role of endogenous catalase in baseline and phenytoin-enhanced neurodevelopmental and behavioral deficits initiated *in utero* and in aged mice☆

Julia P. Abramov[a], Audrey Tran[b], Aaron M. Shapiro[a], Peter G. Wells[a,b,*]

[a] *Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada*
[b] *Department of Pharmacology and Toxicology, University of Toronto, Toronto, Ontario, Canada*

ARTICLE INFO

Article history:
Received 11 May 2011
Received in revised form 13 January 2012
Accepted 31 January 2012
Available online 8 February 2012

Keywords:
Catalase
Oxidative stress
Hydrogen peroxide
Phenytoin
Neurotoxicity
Neurodevelopmental deficits

ABSTRACT

We used mutant catalase-deficient mice (acatalasemic, *aCat*) and transgenic mice expressing human catalase (*hCat*) to determine the neuroprotective role of catalase *in utero* and in aged animals treated with vehicle or the reactive oxygen species (ROS)-initiating drug phenytoin. Phenytoin-initiated postnatal death was enhanced in aCat mice and reduced in hCat mice. Catalase deficiency reduced postnatal surface righting, negative geotaxis and rotarod performances independent of drug treatment, and enhanced phenytoin-initiated negative geotaxis and rotarod deficits in aCat females. Untreated aged female but not male aCat mice exhibited reduced motor coordination. Conversely, hCat offspring showed treatment-independent increased surface righting, negative geotaxis, air righting and, in females, improved phenytoin-impaired rotarod performance. Gender dependencies were consistent with higher brain catalase activities in male than female neonatal and aged animals. Endogenous catalase plays an important gender-dependent neuroprotective role *in utero* and in aged mice, and reduces neurodevelopmental effects of phenytoin.

1. Introduction

Continuation of the antiepileptic drug phenytoin (5,5-diphenylhydantoin, Dilantin™) during pregnancy until recently has been a common practice among physicians due to the high risks to the mother and fetus associated with uncontrolled maternal seizures, as well as the proven efficacy of phenytoin in controlling seizures [1]. Evidence that *in utero* exposure to phenytoin may cause structural and behavioral teratogenesis was first reported by Hanson and Smith, who coined the term "fetal hydantoin syndrome" (*FHS*) [2]. Children diagnosed with FHS presented with a variety of symptoms, including craniofacial anomalies, growth deficiencies and cognitive impairment, shown by reduced IQ scores [2–6].

Numerous studies in pregnant rat or mouse models have demonstrated both the structural and developmental abnormalities associated with human *in utero* phenytoin exposure. Structural congenital abnormalities, including orofacial anomalies, abnormalities of the brain and kidney, skeletal defects and cardiac arrhythmia were shown to occur in a dose-dependent manner that was independent of maternal seizure disorder [7,8]. Similarly, some functional neurodevelopmental anomalies, such as air righting, have been shown to be dose-dependent [9]. Delayed air righting as well as an impaired rotarod performance were first observed in the offspring of pregnant rats exposed to 100 mg/kg of phenytoin in the drinking water or by gastric intubation during days 7–19 of pregnancy [10]. Subsequent studies in pregnant rats using phenytoin doses of 50–200 mg/kg by gavage also observed a delay in the development of the air righting reflex [9,11–13]. Numerous other neurodevelopmental deficiencies, such as impaired startle reflex, impaired maze performance and abnormal circling tendency, have been described in rat offspring exposed *in utero* to phenytoin [9,11–14]. Gender may exert a modulatory influence, as phenytoin exposure caused a greater delay in air righting and acceleration in the negative geotaxis test in females, while a greater acceleration in olfactory orientation was seen in males [13]. Despite the wealth of functional data in the literature, the mechanisms underlying phenytoin-initiated neurodevelopmental deficits and the modulatory influences of gender have not been determined.

Several mechanisms have been implicated in the teratogenicity of phenytoin [15], one of which involves embryonic oxidative stress

Abbreviations: aCat, acatalasemic; EGF, epidermal growth factor; FOX, ferrous oxidation in xylenol orange; FHS, fetal hydantoin syndrome; GD, gestational day; hCat, human catalase expressing; H_2O_2, hydrogen peroxide; •OH, hydroxyl radical; PHS, prostaglandin H synthase; PHT, phenytoin; PND, postnatal day; ROS, reactive oxygen species; VEH, vehicle; WT, wild-type.

☆ A preliminary report of this research was presented at the 2009 annual meeting of the Society of Toxicology (USA) [*The Toxicologist*, 108(1):454]. These studies were supported by a grant from the Canadian Institutes of Health Research.

* Corresponding author at: Faculty of Pharmacy, University of Toronto, 144 College St., Toronto, Ontario, Canada M5S 3M2. Tel.: +1 416 978 3221; fax: +1 416 267 7797.
E-mail address: pg.wells@utoronto.ca (P.G. Wells).

0890-6238/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2012.01.010

caused by reactive oxygen species (*ROS*), which include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) [16]. Teratogens can enhance embryonic ROS formation *via* several mechanisms, including their direct bioactivation to a free radical intermediate by embryonic enzymes like prostaglandin H synthases (*PHS*) and lipoxygenases (*LPO*) [16,17], and indirect ROS formation secondary to reperfusion following cardiac suppression [18]. If not cleared by embryonic antioxidative enzymes, such as superoxide dismutase (*SOD*) and catalase, ROS can oxidatively damage cellular macromolecules such as DNA, RNA, protein and lipids, and/or interfere with signaling pathways, adversely affecting developmental processes [16,19].

The embryo and fetus are particularly susceptible to ROS, as the embryonic activity of antioxidative enzymes like catalase, which detoxifies H_2O_2, may amount to only about 5% of maternal activity [20–23]. The embryopathic importance of ROS and the potential protective role of catalase have been implicated by studies in embryo culture and *in vivo*, where the administration of exogenous enzyme reduced phenytoin-initiated DNA oxidation and embryopathies [20,24]. Similarly, a role for endogenous catalase in protecting the embryo from structural embryopathies caused by endogenous and drug-enhanced oxidative stress in culture and *in vivo* was found in genetically altered mice with deficient or enhanced levels of embryonic catalase [22,23]. Oxidative stress also has been implicated in neurodegeneration in the aging brain [25–27], where catalase activity is similarly low [28,29]. However, it is not clear whether the low level of endogenous catalase in the embryo and adult brain are sufficient to protect against the respective neurodevelopmental and aging effects of baseline and drug-enhanced ROS formation in brain.

This study investigated the role that endogenous catalase in the developing and aging brain may play in detoxifying ROS generated by normal biological processes and phenytoin bioactivation. Offspring of mutant acatalasemic mice (*aCat*) (low catalase) and transgenic mice expressing human catalase (*hCat*) (high catalase) were exposed *in utero* to a single dose of phenytoin, or to its vehicle alone as a reflection of normal developmental oxidative stress. Pups were tested postnatally using a battery of tests to determine the presence of neurodevelopmental deficits. A similar study of motor coordination was conducted in untreated aged aCat and hCat mice. These studies provide the first evidence of a protective role for endogenous catalase against neurodevelopmental deficits caused by both developmental and phenytoin-enhanced ROS formation, and a related role in the modulatory effect of gender. A similar gender-dependent protective role for endogenous catalase against ROS-related neurodegeneration was observed in untreated aged mice.

2. Methods

2.1. Chemicals

Phenytoin (diphenylhydantoin sodium salt) (CAS # 630-93-3), catalase (CAS # 9001-05-2), xylenol orange (CAS # 1611-35-4), ammonium iron (II) sulfate hexahydrate (CAS # 7783-85-9), potassium permanganate (CAS # 7722-64-7) were purchased from Sigma Chemical (St. Louis, MO, USA). Hydrogen peroxide (CAS # 7722-84-1) and sucrose (CAS # 57-50-1) were from BDH Chemicals Inc. (Toronto, Ontario, Canada). Bio-Rad protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, CA). All other reagents and solvents were of analytical grade.

2.2. Animals

Breeding pairs of catalase-normal wild-type (C3HeB/FeJ) and mutant acatalasemic (*aCat*) (catalase-deficient) (C3Ga.Cg-*Cat*^{b}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Transgenic mice expressing human catalase (*hCat*) in addition to their intrinsic murine catalase (high catalase activity) (C57BL/6J) were donated by Dr. Arlan Richardson (University of Texas Health Science Center, San Antonio, TX) [30], and the wild-type (C57BL/6J) mice were purchased from The Jackson Laboratory. Same-sex animals were housed in plastic cages with ground cob bedding and were maintained in a temperature-controlled animal facility with a 12-hr light/dark cycle. Food (Purina Rodent Chow, Oakville, Ontario) and tap water were provided *ad libitum*. One male was housed with two females overnight between 17:00 and 09:00 hr. Pregnancy was ascertained the next morning by the presence of a vaginal plug, and this time was designated as gestational day (*GD*) 1.

For aging studies, adult aCat and hCat mice were housed 4 animals per cage in the same conditions as above, and tested at the age of 18–20 months.

2.3. Treatment

Homozygous aCat and hCat females and their respective wild-type (*WT*) controls were mated with males of the same genotype as described above. Dams were treated intraperitoneally (i.p.) at 10:00 am on GD 17 with vehicle alone or with phenytoin in saline containing 0.002 N NaOH, and allowed to deliver spontaneously. The doses of phenytoin were based upon previous studies of structural teratogenesis in mice, including a marginally teratogenic (55 mg/kg) or teratogenic dose (65 mg/kg) [17]. Gestational length was determined by checking the pregnant females every day starting on GD 19. Cages were inspected every morning, and the presence of a litter was designated as postnatal day (*PND*) 1. On that day the numbers of viable and dead pups were recorded, and the pups were left with their mothers until weaning, 21 days after birth.

For the aging studies, no treatments were administered to the mice.

2.4. Tests

For neonatal studies, all pups were subjected to the following battery of functional tests [10,13]:

Surface righting. Beginning on PND 4, each pup was placed on its dorsal surface and the time required for the pup to right on to all four feet was recorded, to a maximum of 30 s. The test was conducted three times daily until the pup was successful on all three trials.

Negative geotaxis. Beginning on PND 6, each pup was placed facing downward on an incline of 25° and the time required for the pup to turn 180 ± 45° (to face upward), to a maximum of 1 min, was measured. The test was conducted three times daily or until the pup was successful on all three trials.

Olfactory orientation. Beginning on PND 9, each pup was placed inside a 30 cm plastic tube at the halfway point between a dish of home bedding, and a dish of clean bedding. The time required for the pup to walk toward and recognize the home bedding, to a maximum of 2 min, was measured. This test was conducted once daily, or until the pup was successful.

Air righting. Beginning on PND 13, each pup was placed upside down, so that all four paws touched a plastic surface 30 cm above a padded surface. The pup was released and observed for the ability to land on all four feet. Each pup was scored as follows: 0, for landing on its back; 1, for an incomplete landing on its side or on a few feet; and 2, for landing on all four feet. Performance was calculated as the percent per group that received a score of 2 on their final trial on each day. This test was conducted three times daily until PND 22.

2.4.1. Post-weaning testing

Rotarod. Beginning on the 6th postnatal week, offspring were tested on a rotating rod. Mice were placed on the stationary rod for 90 s, and then allowed to run for 90 s at 5 rpm. This procedure was repeated following a 15-min break and subsequently, the speed was slowly increased to 20 rpm. Latency to fall was recorded. This test was repeated during weeks 8, 10 and 12.

For the aging studies, the same rotarod test was performed on the aged adult mice. The test was repeated one week later.

2.5. Catalase activity

Catalase activity was measured in brains of pups on PND 4, 9, 13, 17 and 31, as well as in aged adults older than 18 months. Samples were homogenized in phosphate-buffered saline and catalase activity was determined using the ferrous oxidation in xylenol orange (*FOX*) assay [31–33]. Catalase activity was determined using a standard curve with bovine liver catalase as the standard.

2.6. Genotyping

DNA was isolated from tail clips by heating the sample in 300 μL of a solution containing 10 mM NaOH/0.1 mM EDTA at 95 °C for 15 min. To determine the genotype of the acatalasemic mice and their WT controls, catalase was amplified by PCR using the following primers, CatF: TCCTTCCAATCCCGTCCTTTCT and CatR: AAATGCCAAACTCGGAGCCATC. PCR conditions for a Perkin Elmer 9600 thermal cycler were 94 °C for 5 min, 20 s at 94 °C, 20 s at 65 °C (−1° cycle), and 40 s at 72 °C for a total of 10 cycles, then 20 s at 94 °C, 20 s at 55 °C, and 40 s at 72 °C for a total of 30 cycles, with a 10 min extension at 72 °C and kept at 4 °C. Acatalasemic mice contain a point mutation, which produces an NdeI restriction site in the region amplified by the abovementioned primers. Following digestion at 37 °C for 16 hr, acatalasemic samples contain two bands at approximately 250 bp while the wild-type (undigested) products appear at 493 bp as determined on a 1.5% agarose gel. To genotype