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Original Contribution

Gender differences in brain susceptibility to oxidative stress are mediated by levels of paraoxonase-2 expression

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ABSTRACT

Paraoxonase 2 (PON2), a member of a gene family that also includes PON1 and PON3, is expressed in most tissues, including the brain. In mouse brain, PON2 levels are highest in dopaminergic areas (e.g., striatum) and are higher in astrocytes than in neurons. PON2 is primarily located in mitochondria and exerts a potent antioxidant effect, protecting mouse CNS cells against oxidative stress. The aim of this study was to characterize PON2 expression and functions in the brains of male and female mice. Levels of PON2 (protein, mRNA, and lactonase activity) were higher in brain regions and cells of female mice. Astrocytes and neurons from male mice were significantly more sensitive (by 3- to 4-fold) to oxidative stress-induced toxicity than the same cells from female mice. Glutathione levels did not differ between genders. Importantly, no significant gender differences in susceptibility to the same oxidants were seen in cells from PON2$^{-/-}$ mice. Treatment with estradiol induced a time- and concentration-dependent increase in the levels of PON2 protein and mRNA in male (4.5-fold) and female (1.8-fold) astrocytes, which was dependent on activation of estrogen receptor-α. In ovariectomized mice, PON2 protein and mRNA were decreased to male levels in brain regions and in liver. Estradiol protected astrocytes from wild-type mice against oxidative stress-induced neurotoxicity, but did not protect cells from PON2$^{-/-}$ mice. These results suggest that PON2 is a novel major intracellular factor that protects CNS cells against oxidative stress and confers gender-dependent susceptibility to such stress. The lower expression of PON2 in males may have broad ramifications for susceptibility to diseases involving oxidative stress, including neurodegenerative diseases.

Introduction

Paraoxonase 2 (PON2)[1] is a member of a multigene family of enzymes that also includes PON1 and PON3; the three genes share a high degree of identity and are located adjacent to one another on chromosome 7q21–q22, in humans, and on chromosome 6 in the mouse [1]. The name of these enzymes derives from the active metabolite of the organophosphorus insecticide parathion, paraoxon, which is a substrate of PON1, and has been applied to the other two PONs, despite their lack of esterase activity. All three PONs are lactonases and can hydrolyze a number of acyl-homoserine lactones (acyl-HCLs), molecules that mediate bacterial quorum-sensing signals and that are important in regulating the expression of virulence factors and inducing a host inflammatory response, with PON2 displaying the highest activity [2–5]. Two common polymorphisms have been found in human *PON2,* Ala147Gly and Ser311Cys [1,6]; the latter affects lactonase activity, with Cys311 displaying lower activity [7].

Whereas PON1 and PON3 are expressed primarily in liver and are associated with high-density lipoproteins in plasma [8], PON2 is a ubiquitously expressed intracellular enzyme, not present in plasma [6,9,10]. PON2 mRNA and/or protein has been detected in several tissues including liver, lung, kidney, heart, pancreas, small intestine, muscle, testis, endothelial cells, tracheal epithelial cells, and macrophages [3,6,9–15]. Prompted by limited information suggesting that PON2 is also expressed in the nervous system [1,6,9], we have recently characterized its presence and functions in the mouse CNS [15].

Abbreviations: CNS, central nervous system; $DCFH_2$-DA, 2′,7′-dichlorofluorescin diacetate; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; DMSO, dimethyl sulfoxide; GSH, glutathione (reduced); HCL, homoserine lactone; MPP, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1*H*-pyrazole dihydrochloride; MTT, 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide; PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo-(1,5-α)pyrimidin-3-yl)] phenol; PON, paraoxonase; ROS, reactive oxygen species; TNF, tumor necrosis factor.

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PON2 protein, mRNA, and lactonase activity were found in all mouse brain regions, with the highest levels in three dopaminergic regions (striatum, substantia nigra, nucleus accumbens). PON1 was detected at very low levels (30- to 40-fold less than PON2) and did not show any regional differences, whereas PON3 was not detected in brain [15]. In all regions, PON2 levels were higher in astrocytes than in neurons, and levels in cortical microglia were similar to those of neurons [15]. In both astrocytes and neurons, PON2 levels were highest in mitochondria, a finding that is corroborated by results in other tissues [16]. Mitochondria are a major source of free radical-related oxidative stress [17]; PON2 has been shown to bind to coenzyme Q_{10} that associates with complex III in mitochondria [16], thus reducing the release of superoxide from the inner mitochondrial membrane [18]. It is not surprising, therefore, that in several tissues and cell types, PON2 exerts an antioxidant effect [5,9,11–13,19]. In the CNS, the ability of two oxidants (hydrogen peroxide (H_2O_2) and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ)) to induce the formation of reactive oxygen species and to decrease viability of astrocytes and neurons was significantly enhanced in cells from PON2 knockout ($PON2^{-/-}$) mice [15]. As glutathione (GSH) levels do not differ between wild-type and $PON2^{-/-}$ mice, the observed 5- to 11-fold difference in susceptibility is probably due to the presence or absence of PON2 [15].

In the course of these studies [15], we discovered that in all peripheral tissues (lung, heart, small intestine, liver, kidney), PON2 expression (indicated by protein and mRNA levels and by lactonase activity) was significantly higher in female than in male mice. Similarly, in the CNS, a two to threefold difference between genders was seen in all brain regions, with the nucleus accumbens in female mice displaying the highest level of PON2 [15]. Given the antioxidant functions of PON2, these findings raised the possibility that the male's CNS might be intrinsically more susceptible to oxidative stress. Studies presented in this paper show that this is indeed the case; they also show that estrogens modulate PON2 expression and that PON2 confers increased resistance of female CNS cells to oxidative stress-mediate toxicity.

Materials and methods

Materials

Neurobasal-A medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), GlutaMAX, gentamycin, and SuperScript III first-strand synthesis system were from Invitrogen (Carlsbad, CA, USA). TaqMan Gene Expression Master Mix was from Applied Biosystems (Foster City, CA, USA). Anti-PON2 antibodies were from Abcam (Cambridge, MA, USA). The kit for measurement of serum estradiol levels was from Cayman Chemical (Ann Arbor, MI, USA). The estrogen receptor antagonists PHTPP (4-[2-phenyl-5,7-bis-(trifluoromethyl)pyrazolo-[1,5-α]pyrimidin-3-yl)] phenol), ICI 182,780 (7α,17β-[9-(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl estra-1,3,5(10)-triene-3,17-diol), and MPP dihydrochloride (1,3-bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1*H*-pyrazole dihydrochloride) were purchased from Tocris Bioscience (Ellisville, MO, USA). Dimethyl sulfoxide (DMSO), H_2O_2, DMNQ, mouse anti-β-actin antibody, reduced glutathione, 5-sulfosalicylic acid, naphthalene dicarboxaldehyde, dihydrocoumarin (3,4-dihydro-2*H*,1-benzopyran-2-one), actinomycin-D, 17β-estradiol, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma–Aldrich (St. Louis, MO, USA).

Animals

Male and female C57BL/6J mice (wild-type mice) and PON2 knockout ($PON2^{-/-}$) mice (kindly provided by Drs. A.J. Lusis, D.M. Shih, and S. Reddy at the University of California at Los Angeles) were used in this study. Animals were 2–3 months of age at the time of the study. $PON2^{-/-}$ mice were generated using the embryonic stem cell line XE661 (strain 129/Ola) and a gene-trap vector, as described in detail elsewhere [20]. After germ-line transmission, backcrossing was performed with C57BL/6J mice for six generations. Genotyping of mice was done as described by Ng et al. [20]. Mice were housed in a specific-pathogen-free facility with ad libitum access to food and water and a 12-h light/dark cycle. In some experiments, time-pregnant Sprague–Dawley rats were purchased from Charles River (Wilmington, MA, USA), and newborn pups were used for preparation of cortical astrocytes. All procedures for animal use were in accordance with the National Institutes of Health *Guide for the Use and Care of Laboratory Animals* and were approved by the University of Washington Institutional Animal Care and Use Committee.

Ovariectomy

Ovariectomized and sham-operated female mice were purchased from Charles River. One-month-old mice were anesthetized with ether and an incision was made in the lower abdomen. Ovaries were removed and the abdomen was sutured; sham-operated mice underwent an identical procedure without removal of the ovaries. Animals were sacrificed at 3 months of age; at sacrifice, lack of ovaries was confirmed by visual inspection. In addition to the various tissues, blood was also collected for measurement of serum estradiol levels with a commercial kit.

Primary cell cultures

Primary astrocytes were obtained from postnatal day (PND) 0.5 male and female mice, as previously described [15]. Gender was determined by inspecting the genital area and measuring the ano–genital distance. After tissue removal, gender identification was confirmed by inspecting internal organs for identification of the uterine horns or the testes. The striatum was dissected, mechanically dissociated, and incubated with trypsin, followed by trituration, repeated washing, and filtering. After being counted, the cells were plated at a density of 10^7 cells per flask in 75-cm^2 tissue culture flasks precoated with poly-D-lysine and grown in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2/95% (v/v) air. After 10 days in culture, the cells were plated in 24-well plates for the experiments at a density of 5×10^4 astrocytes/well. Striatal neurons were also prepared from PND 0.5 mice, as described by Giordano et al. [15,21]. Briefly, the striatum was collected in HBSS medium containing 0.02% (w/v) bovine serum albumin and 10 mM Hepes. The tissue was digested for 25 min in HBSS containing papain (1 mg/ml) and DNase (40 μg/ml) and centrifuged at 300 *g* (max *g* force is always indicated) for 5 min at room temperature. The supernatant (containing papain) was removed and the pellet was gently triturated in Neurobasal-A medium supplemented with B27, using a Pasteur pipette to dissociate larger aggregates. The cells were centrifuged at 200 *g* at 4 °C for 10 min and the cell pellet was gently resuspended. Neurons were then counted, seeded on poly-D-lysine-coated 48-well plates at a density of $5 \times 10^4/cm^2$, and cultured in Neurobasal medium supplemented with B27 (minus AO) AO antioxidants. Neurons were cultured for 8 days before experiments.

Rat astrocytes were obtained from 0.5-day-old pups from either gender, using a protocol essentially identical to that used for mice. For preparation of fetal human astrocytes, tissue was