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# Paraoxonase 2 (PON2) in the mouse central nervous system: A neuroprotective role?

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ABSTRACT

The aims of this study were to characterize the expression of paraoxonase 2 (PON2) in mouse brain and to assess its antioxidant properties. PON2 levels were highest in the lung, intestine, heart and liver, and lower in the brain; in all tissues, PON2 expression was higher in female than in male mice. PON2 knockout [$PON2^{-/-}$] mice did not express any PON2, as expected. In the brain, the highest levels of PON2 were found in the substantia nigra, the nucleus accumbens and the striatum, with lower levels in the cerebral cortex, hippocampus, cerebellum and brainstem. A similar regional distribution of PON2 activity (measured by dihydrocoumarin hydrolysis) was also found. PON3 was not detected in any brain area, while PON1 was expressed at very low levels, and did not show any regional difference. PON2 levels were higher in astrocytes than in neurons isolated from all brain regions, and were highest in cells from the striatum. PON2 activity and mRNA levels followed a similar pattern. Brain PON2 levels were highest around birth, and gradually declined. Subcellular distribution experiments indicated that PON2 is primarily expressed in microsomes and in mitochondria. The toxicity in neurons and astrocytes of agents known to cause oxidative stress (DMNQ and $H_2O_2$) was higher in cells from $PON2^{-/-}$ mice than in the same cells from wild-type mice, despite similar glutathione levels. These results indicate that PON2 is expressed in the brain, and that higher levels are found in dopaminergic regions such as the striatum, suggesting that this enzyme may provide protection against oxidative stress-mediated neurotoxicity.



## Introduction

The paraoxonase (PON) multigene family consists of three members (PON1, PON2 and PON3), whose genes share a high degree of identity, and are located adjacent to each other on chromosome 7q21–22 in humans and on chromosome 6 in mouse (Primo-Parmo et al., 1996). Phylogenetic analysis suggests that PON2 is the oldest PON family member, from which PON1 and PON3 have evolved (Draganov and La Du, 2004). The name of these enzymes derives from paraoxon, the active metabolite of the organophosphorus insecticide parathion, which is hydrolyzed by PON1 *in vitro*, though not efficiently *in vivo* (Li et al., 2000), and has been extended to the other two PONs, which do not have esterase activity. In contrast, all three PONs are lactonases, displaying overlapping but distinct substrate specificities for lactone hydrolysis (Draganov et al., 2005). All three PONs can hydrolyze a number of acyl-homoserine lactones (acyl-HCL), molecules which mediate bacterial quorum-sensing signals, important in regulating expression of virulence factors and in inducing a host inflammatory response; PON2 has the highest acyl-HCL hydrolytic activity of the three PON isozymes (Draganov et al., 2005; Stoltz et al., 2007; Teiber et al., 2008; Horke et al., 2010). Two common polymorphisms have been found in human *PON2*, an Ala/Gly substitution at position 147, and a Ser/Cys substitution at position 311 (Primo-Parmo et al., 1996; Mochizuki et al., 1998). The PON2 Ser311Cys polymorphism has been shown to affect lactonase activity, with PON2 Cys311 displaying lower activity (Stoltz et al., 2009).

*PON1* and *PON3* are expressed primarily in the liver, and their protein products are associated with high-density lipoproteins in the plasma, though more recent data suggest a wider expression pattern (Marsillach et al., 2008). In contrast, PON2 is a ubiquitously expressed intracellular enzyme, but is not present in plasma (Mochizuki et al., 1998; Ng et al., 2001; Marsillach et al., 2008). PON2 mRNA and/or protein have been detected in several tissues including the liver, lung, kidney, heart, pancreas, small intestine, muscle, testis, endothelial cells, tracheal epithelial cells, and macrophages (Mochizuki et al., 1998; Ng et al., 2001; Rosenblatt et al., 2003; Levy et al., 2007; Stoltz et al., 2007; Marsillach et al., 2008; Precourt et al., 2009). More limited information exists on PON2 expression in the nervous system. PON2 mRNA has been found in mouse and human brains (Primo-Parmo et al., 1996; Mochizuki et al., 1998; Ng et al., 2006), and PON2 protein has been detected in mouse brain (Ng et al., 2006; Marsillach et al., 2008).

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In several tissues, PON2 has been shown to exert an antioxidant effect (Ng et al., 2001). PON2 antagonizes oxidative stress induced by various sources in the intestine of humans and rats (Levy et al., 2007), in human vascular endothelial cells (Horke et al., 2007), in lung epithelial carcinoma cells (Horke et al., 2010), in Caco-2/15 cells (Precourt et al., 2009), and in mouse macrophages (Rosenblatt et al., 2003). Given that neurotoxicity and neurodegenerative disorders are often associated with increased oxidative stress (Reynolds et al., 2007; Sayre et al., 2008), a better understanding of the expression and function of PON2 in brain tissue seemed warranted.

## Materials and methods

*Materials.* Neurobasal-A medium, DMEM medium, fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), GlutaMAX, gentamycin, and SuperScript® III First-Strand Synthesis System were from Invitrogen (Carlsbad, CA). TaqMan Gene Expression Master Mix was from Applied Biosystems Inc. (Foster City, CA). Anti-PON2, PON1, and PON3 antibodies were from Abcam (Cambridge, MA, USA). Dimethylsulfoxide (DMSO), hydrogen peroxide ($H_2O_2$), 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), mouse anti-β-actin antibody, reduced glutathione, 5-sulfosalicylic acid, naphthalene dicarboxaldehyde, dihydrocoumarin (3,4-dihydro-2H,1-benzopyran-2-one), and 3-(4,5-dimethyltiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich (St. Louis, MO).

*Animals.* PON2 wild-type ($PON2^{+/+}$) and knockout ($PON2^{-/-}$) mice, kindly provided by Drs. A.J. Lusis, D.M. Shih and S. Reddy (UCLA) were used in this 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 (Ng et al., 2006). After germ line transmission, backcrossing was performed with C57BL/6J mice for six generations. Control mice were wild-type littermates. Genotyping of mice was done as described by Ng et al. (2006). For developmental studies, mice were euthanized at gestational day (GD) 20, and at postnatal days (PND) 1, 7, 14, 21, 30 and 60. The mice were housed in a specific pathogen-free facility with *ad libitum* access to food and water and a 12-h light cycle. All procedures for animal use were in accordance with the National Institute 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.

*Primary cell cultures.* Primary astrocytes from all brain regions were obtained from PND 0.5 mice, except for cerebellar astrocytes, which were obtained from PND 7 mice, as previously described (Giordano et al., 2008). Briefly, brain regions were dissected, mechanically dissociated and incubated with trypsin, followed by trituration, repeated washing, and filtering. After counting, cells were plated at a density of $10^7$ cells per flask in 75 $cm^2$ tissue culture flasks pre-coated 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 $5\times10^4$ astrocytes/well. Cultures of cerebellar granule neurons (CGN) were prepared from 7 day-old mice, as described by Giordano et al. (2006, 2008). Neurons were grown for 10–12 days before treatments. Neurons from other brain regions were prepared from PND 0.5 mice, as described by Giordano et al. (2008). Briefly, brain regions were collected in HBSS medium containing 0.02% (w/v) bovine serum albumin (BSA) and 10 mM HEPES. Tissues were 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 $5\times10^4/cm^2$, and cultured in Neurobasal medium supplemented with B27 (minus AO). Neurons were cultured 8 days before the experiments. Microglia were isolated from the cortical astrocyte cultures by shaking the flasks, collecting the medium and spinning at 300 ×*g* at 4 °C for 7 min. The cells were resuspended in a medium containing: DMEM-F12 (Invitrogen), 1% (v/v) G5 supplement (Invitrogen), and 0.1% (w/v) BSA, and plated on PDL-coated 24-well plates. After 3 days of incubation, the cells were collected and used for analysis. Cultures were determined to be at least 95% pure microglia by determining the number of β-III tubulin-positive neurons and GFAP-positive astrocytes relative to Iba-1-positive microglia (Klintworth et al., 2009).

*Western blots.* Immunoblots were carried out as described by Giordano et al. (2006). Twenty-five micrograms of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted using antibodies against PON1, PON2, PON3 and β-actin. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes that were incubated with antibodies using the following dilutions: 1:250 (for PON1, PON2, and PON3), and 1:1500 (for β-actin). After the transfer the blots were rinsed in Tris-buffered saline (pH = 7.5) and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody at the appropriate dilution of 1:750 (for PON2 and PON3), or incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody at the dilutions of 1:2500 for β-actin, or 1:750 (for PON1). Band intensity was measured by densitometry, and the intensity of the bands was normalized to β-actin content.

*Deglycosylation of PON2 with peptide-N-glycosidase F.* Peptide-*N*-glycosidase F (PNGase F) (Biolabs) digestion was performed as described by Marsillach et al. (2010) and Stoltz et al. (2009), and according to the manufacturer's directions. This enzyme releases asparagine-linked oligosaccharides from glycoproteins by hydrolyzing the amide group of the asparagine side chain. Briefly, cell lysates were collected in lysis buffer (50 mM Tris HCl, pH 7.5 (at 18 °C), 138 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin), and incubated with PNGase F (5 U) for 1 or 4 h at 37 °C. The reaction was stopped by adding SDS buffer and incubating at 100 °C for 5 min. Deglycosylation was then assessed with SDS-PAGE followed by immunoblot.

*RT-PCR.* Reverse transcription was performed according to the manufacturer's established protocol using total RNA and the SuperScript® III First-Strand Synthesis System. For gene expression measurements, 4 μl of cDNA was included in a PCR reaction (25 μl final volume) that also consisted of the appropriate forward (FP) and reverse (RP) primers at 360 nM each, 80 nM TaqMan probe, and TaqMan Gene Expression Master Mix. The PCR primers and the dual-labeled probes [6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA)] for all genes were designed using ABI Primer Express v.1.5 software (Applied Biosystems Inc., Foster City, CA). Amplification and detection of PCR amplicons were performed with the ABI PRISM 7900 system (Applied Biosystems Inc., Foster City, CA) with the following PCR reaction profile: 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 30 s and 62 °C for 1 min. β-Actin amplification plots derived from serial dilutions of an established reference sample were used to create a linear regression formula in order to calculate expression levels, and *β-actin* gene expression levels were utilized as an internal control to normalize the data.

*PON2 activity assay.* PON2 activity was measured as described by Rosenblatt et al. (2003). Briefly, tissues and primary cells were washed and resuspended in Tris buffer containing 25 mM Tris/HCl, pH 7.6 at 18 °C/1 mM $CaCl_2$. The homogenates were sonicated twice