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Paraoxonase 1 (PON1) Modulates the Toxicity of Mixed Organophosphorus Compounds

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Abstract

A transgenic mouse model of the human *hPON1*_{Q192R} polymorphism was used to address the role of paraoxonase (PON1) in modulating toxicity associated with exposure to mixtures of organophosphorus (OP) compounds. Chlorpyrifos oxon (CPO), diazoxon (DZO), and paraoxon (PO) are potent inhibitors of carboxylesterases (CaE). We hypothesized that a prior exposure to these OPs would increase sensitivity to malaoxon (MO), a CaE substrate, and the degree of the effect would vary among *PON1* genotypes if the OP was a physiologically significant PON1 substrate *in vivo*. CPO and DZO are detoxified by PON1. For CPO hydrolysis, *hPON1*_{R192} has a higher catalytic efficiency than *hPON1*_{Q192}. For DZO hydrolysis, the two alloforms have nearly equal catalytic efficiencies. For PO hydrolysis, the catalytic efficiency of PON1 is too low to be physiologically relevant. When wild-type mice were exposed dermally to CPO, DZO, or PO followed 4-h later by increasing doses of MO, toxicity was increased compared to mice receiving MO alone, presumably due to CaE inhibition. Potentiation of MO toxicity by CPO and DZO was greater in *PON1*^{-/-} mice, which have greatly reduced capacity to detoxify CPO or DZO. Potentiation by CPO was more pronounced in *hPON1*_{Q192} mice than in *hPON1*_{R192} mice due to the decreased efficiency of *hPON1*_{Q192} for detoxifying CPO. Potentiation by DZO was similar in *hPON1*_{Q192} and *hPON1*_{R192} mice, which are equally efficient at hydrolyzing DZO. Potentiation by PO was equivalent among all four genotypes. These results indicate that PON1 status can have a major influence on CaE-mediated detoxication of OP compounds.

Keywords

Paraoxonase; PON1; organophosphate; insecticide mixtures; chlorpyrifos; diazinon; parathion; malathion

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Introduction

Examining exposures to mixtures of insecticides is important for understanding the underlying mechanisms of organophosphorus (OP) compound toxicity and assessing aggregate risk. Numerous studies have examined additive and synergistic effects of combinations of OPs *in vitro* and *in vivo*. Early studies on OP mixtures in rodents found that exposure to malathion or malaoxon (MO), in combination with compounds that inhibit carboxylesterase (CaE), led to a potentiation of MO toxicity (Aldridge et al., 1954; Cook et al., 1957; Dubois, 1958; Murphy et al., 1959; Seume et al., 1960; Casida et al., 1961; Casida et al., 1963; Cohen and Murphy, 1971a; 1971b), even at doses below those required for biologically significant inhibition of cholinesterase activity (Dubois, 1969; Su et al., 1971).

The CaEs are members of a multigene family of enzymes that are widely distributed in the body with the highest activity levels being expressed in the liver, gastrointestinal tract, and the brain (Sato and Hosokawa, 2006). CaE activity is also present in the plasma of rodents, but not humans (Williams et al., 1989; Li et al., 2005). Significant individual variability in levels of CaE in human liver microsomes has been reported, with activities varying from 5.3- to 44.7-fold, depending on the substrate used for measuring CaE activity (Hosokawa et al., 1995). Inter-individual variability of OP detoxication and bioactivation has also been identified in cytochrome P450s (Tang et al., 2001; reviewed in Furlong et al., 2007). CaEs can catalytically hydrolyze the carboxylic esters of malathion and MO (March et al., 1956; O'Brien, 1957; Cook and Yip, 1958; Chen et al., 1969). The bioactivation of malathion to its oxon analog forms a potent direct inhibitor of AChE (DuBois et al., 1953; March et al., 1956; Murphy and DuBois, 1957; O'Brien, 1957). When MO and CaE are combined *in vitro*, MO can act as both a substrate for hydrolysis and as an irreversible inhibitor of CaE and the rate of each reaction is affected by the other (Main and Dauterman, 1967). In addition to catalytic hydrolysis of OPs by A-esterases, noncatalytic hydrolysis occurs when these compounds stoichiometrically phosphorylate serine esterases (B-esterases) that are inhibited by OPs but do not hydrolyze them catalytically (e.g., CaEs and butyrylcholinesterase). The irreversible binding of CaEs to some OPs [chlorpyrifos oxon (CPO), diazoxon (DZO), and paraoxon (PO)] allows the CaEs to act as scavengers, leaving less OP available to inhibit AChE at the target site (Chambers et al., 1990).

The importance of the HDL-associated enzyme paraoxonase 1 (PON1) in OP detoxication has been known for some time (reviewed in Costa, 2006; Furlong, 2008). PON1 exhibits broad substrate specificity with different rates of hydrolysis and substrate affinity for specific OP compounds (Furlong et al., 1989; Davies et al., 1996; Pond et al., 1998; Li et al., 2000). PON1 has two common amino acid polymorphisms (Q192R and L55M) (Hassett et al., 1991). The Gln (Q)/Arg (R) substitution at position 192 affects catalytic efficiency towards some OP substrates (Adkins 1993; Humbert et al., 1993; Davies et al., 1996; Li et al., 2000). Activity levels of plasma PON1 can vary by as much as 15-fold among individuals with the same *PON1* (192) genotype (Q/Q; Q/R; R/R), determined in part by polymorphisms in the promoter region of *PON1* (Furlong, 2007). PON1 "status" is a term used to encompass both the Q192R polymorphism and the level of PON1 activity (Li et al., 1993; Richter and Furlong, 1999; Li et al., 2000). An individual's PON1 status can be determined using a two-substrate assay that provides both PON1_{Q192R} functional phenotype and PON1 levels by plotting the plasma rates of DZO hydrolysis (at high salt) vs. PO hydrolysis (Li et al., 1993; Richter and Furlong, 1999; Costa et al., 1999; Jarvik et al., 2003). A recently-developed protocol for determining PON1 status makes use of the non-toxic substrates, phenyl acetate and 4-(chloromethyl)phenyl acetate (Richter et al., 2008; 2009).

PON1 knockout (*PON1*^{-/-}) mice have no detectable liver or plasma hydrolytic activity toward PO or DZO and only very limited activity toward CPO. Accordingly, these animals exhibit

dramatically increased sensitivity to the toxicity of CPO (Shih et al., 1998) and DZO (Li et al., 2000). Injection of purified PON1 increases the resistance of rats and mice to OP toxicity (Main, 1956; Costa et al., 1990; Li et al., 1995; Li et al., 2000). When *PON1*^{-/-} mice were injected with purified human PON1 alloforms (hPON1_{R192} or hPON1_{Q192}) to restore plasma PON1, hPON1_{R192} provided better protection than hPON1_{Q192} against CPO exposure, while both alloforms were equally effective in protecting against the toxicity of DZO (Li et al., 2000), and neither alloform provided protection against PO toxicity (Li et al., 2000). The extent of the protection provided by the hPON1_{Q192R} alloforms against OP exposure was dependent on the catalytic efficiency of hydrolysis of the specific OP compound (Li et al., 2000). A study utilizing “humanized” *PON1* transgenic mice provided further evidence of the role of PON1 in modulating OP toxicity. The genes of *PON1*^{-/-} mice were replaced with either human *hPON1*_{R192} or *hPON1*_{Q192}, and founders of each transgenic genotype were chosen that expressed *hPON1* at equivalent levels (Cole et al., 2003). When these mice were exposed to CPO or its parent compound chlorpyrifos (CPS), the mice expressing *hPON1*_{Q192} were found to be significantly more sensitive to CPO/CPS exposure than the mice expressing *hPON1*_{R192} (Cole et al., 2005).

The current study demonstrates that these differences in OP detoxication between the hPON1_{Q192} and hPON1_{R192} alloforms can have consequences beyond cholinesterase inhibition. In a combined or sequential exposure, the alloform differences can affect the subsequent toxicity (or efficacy) of compounds metabolized by CaE, even when the compound is not metabolized directly by PON1. We demonstrate that CPO, DZO, and PO inhibit CaE *in vitro* and *in vivo* and increase MO toxicity *in vivo*, and that PON1 status modulates the degree of MO potentiation by virtue of its impact on the metabolism of CPO and DZO.

Methods

Chemicals

Chlorpyrifos oxon (CAS 5598-15-2; 98% purity), diazoxon (diazinon-O-analog; CAS 962-58-3; 96% purity), paraoxon (O,O-diethyl-O-p-nitrophenylphosphate; CAS 311-45-5; 98.4% purity), malaoxon (CAS 1634-78-2; 88% purity) and tri-ortho-cresyl phosphate (CAS 1330-78-5; 98.5% purity) were purchased from Chem Service (West Chester, PA, USA). Acetylthiocholine, 5,5'-dithio-bis-nitrobenzoic acid (DTNB), p-nitrophenyl valerate, and phenyl acetate were from Sigma-Aldrich (St. Louis, MO). All other analytical grade chemicals were obtained from commercially available sources.

PON1^{+/+}, *PON1*^{-/-}, hPON1_{R192} and hPON1_{Q192} transgenic mice

Mice were 8–12 wk-old males and females, of congenic B6.129 strain background, >96% C57Bl/6J, backcrossed five times from the original C57Bl/6J×129/svEv strain. *PON1* knockout (*PON1*^{-/-}) mice (Shih et al., 1998) and mice expressing either the human *hPON1*_{R192} or *hPON1*_{Q192} transgene in place of endogenous mouse *PON1* (Cole et al., 2003; Cole et al., 2005) were kindly provided by Drs. Diana M. Shih, Aaron Tward and Aldons J. Lusis (UCLA, Los Angeles, CA). Mice with at least one copy of the transgene were crossed with same-genotype animals to produce both *PON1* knockout (*PON1*^{-/-}) mice and transgenic mice (*hPON1*_{R192} or *hPON1*_{Q192}) in the same litter. Wild-type (*PON1*^{+/+}) mice were bred from the same congenic B6.129 strain background. Equal numbers of males and females were used for all experiments. Presence of hPON1_{Q192} or hPON1_{R192} enzyme activity in heparinized saphenous-vein plasma was detected by measuring the rate of hydrolysis of either the alloform-neutral substrate, phenyl acetate (Furlong et al., 1989; Furlong et al., 1993; Furlong et al., 2006), or diazoxon, which is alloform-neutral at 0.5 M NaCl (Richter and Furlong, 1999) and has no background activity in *PON1*^{-/-} mice (Cole et al., 2003). PCR-based genotyping was used to determine the presence of the transgenes. Arylesterase (AREase)

and diazoxonase (DZOase) assays were carried out in a microtiter plate reader (SpectraMax Plus, Molecular Devices), using 0.5 μL (AREase) and 1.25 μL (DZOase) of plasma per well. Only the initial linear rates of hydrolysis were used for calculations.

Mice were housed in SPF (specific pathogen-free) or modified SPF facilities with a 12-h dark–light cycle and unlimited access to food and water. The animal use protocols used were approved by the Institutional Animal Care and Use Committee at the University of Washington. All animal experiments were carried out in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals, as adopted by the National Institutes of Health.

Exposure of mice to organophosphorus (OP) compounds

Chlorpyrifos oxon (CPO), diazoxon (DZO), paraoxon (PO), malaoxon (MO) and tri-ortho-cresyl phosphate (TOCP) were dissolved in acetone and applied (1 $\mu\text{L}/\text{g}$ body weight) to a shaved area (4 cm^2) on the upper back. Control mice received acetone only. For experiments involving mixtures, four hours following the first OP exposures (0.75 mg/kg CPO, 0.5 mg/kg DZO, or 0.35 mg/kg PO), MO was applied dermally (1 $\mu\text{L}/\text{g}$ body weight) at doses of 0, 30, 60, or 100 mg/kg. Mice were euthanized by cervical dislocation four hours following the second exposure, at the time of maximal AChE inhibition. During the time between the second dosing and euthanasia, animals were monitored for cholinergic symptoms. At the time of sacrifice, each mouse was assessed for signs of reduced body temperature, hypoactivity, eye secretions, and tremors. Following sacrifice, trunk blood was collected directly into a centrifuge tube, and serum was frozen at -80°C until analysis. Brains, diaphragms, livers, and tails were dissected, placed immediately on dry ice, and stored at -80°C until analysis. Blood was collected from the saphenous vein at least 24-h prior to the experiment, for the purpose of measuring PON1 activity (DZOase or AREase) in the plasma. For time course experiments, plasma CaE activity was monitored in individual mice by repeated blood draws ($\sim 50 \mu\text{L}$) from the saphenous vein at 4, 8, 12, and 24-hr following dermal exposure to the OP compounds.

Acetylcholinesterase (AChE) assays

Brain and diaphragm AChE activities were measured using a microtiter plate assay based on the method of Ellman et al. (1961), essentially as described (Cole et al., 2005) with minor modifications. Brain and diaphragm tissues were homogenized in 10 volumes (w/v) of ice-cold 0.1 M sodium phosphate buffer (PB), pH 8.0 using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) for 30 s (brain) or 45 s (diaphragm). The samples were then diluted in the same buffer to 4 mg/mL (brain) or 25 mg/mL (diaphragm). For triplicate assays, 35 μL of diluted tissue homogenate were added to 315 μL 0.1 M PB, pH 8.0, and 100 μL of the mixture were added to each of three wells of a 96-well plate. Following initiation of the kinetic assay by addition of 100 μL of freshly-prepared 2X substrate mix (2.0 mM acetylthiocholine, 0.64 mM 5,5'-dithio-bis-nitrobenzoic acid (DTNB), 0.1 M PB, pH 8.0), the formation of 5-thio-2-nitrobenzoate, the hydrolytic product of acetylthiocholine, was monitored continuously for 10 min at room temperature at 412 nm in a microtiter plate reader (SpectraMax Plus, Molecular Devices). A reagent blank and a previously-assayed standard sample were included in each plate to normalize the samples and ensure consistency among plate assays. The initial rates of 5-thio-2-nitrobenzoate formed during the assay (mOD/min) were converted to U/g of wet tissue using its extinction coefficient of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$. AChE activity is expressed as U/g of wet tissue (U = μmol of acetylthiocholine hydrolyzed per minute). Values are presented as percent of control (%C). Control values ($\pm$ SEM) for brain AChE and diaphragm AChE were 9.7 ± 0.8 U/g ($n = 48$) and 2.7 ± 0.1 U/g ($n = 48$), respectively.

Carboxylesterase (CaE) assays

Liver, plasma, and serum carboxylesterase (CaE) activities were measured using a microtiter plate assay based on the method of Munger et al. (1991) with minor modifications. Liver tissues were homogenized in 10 volumes (w/v) of ice-cold 0.1 M Tris-HCl, pH 8.0, then diluted in the same buffer to a final tissue concentration of 1.0 mg/ml. The liver homogenate was centrifuged at 10,000 g for 10 minutes at 4°C, and the supernatant was used for the assays. For triplicate assays, 20 µL of diluted tissue homogenate or 1:50 diluted serum or plasma were added to each of three wells of a 96-well plate. Following initiation of the kinetic assay by the addition of 200 µL of freshly-prepared *p*-nitrophenyl valerate (PNV) substrate mix (1 mM PNV, 0.1 M Tris-HCl, pH 8.0), the formation of *p*-nitrophenol was monitored continuously for 10 min (23°C) at 405 nm. The initial linear rates of *p*-nitrophenol formed during the assay were calculated using an extinction coefficient of 18.5 mM⁻¹ cm⁻¹. CaE activity is expressed as U/g of wet tissue or U/mL plasma or serum (U=µmol of PNV hydrolyzed per minute). Values are presented as percent of control (%C). Control values (± SEM) for liver CaE and plasma CaE were 167.0 ± 12.1 U/g (n = 8) and 8.1 ± 0.2 U/ml (n = 48), respectively.

In vitro inhibition of carboxylesterase (CaE) and acetylcholinesterase (AChE)

Brain and liver homogenates were prepared from three *PON1*^{+/+} mice by the methods described above. Liver and brain homogenates were pooled and diluted to final tissue concentrations of 1.0 mg/ml and 4.0 mg/ml, respectively. Commercial mouse plasma (Pel-Freez) was diluted 1:50. Mouse liver homogenate, brain homogenate, or plasma was added in triplicate to 96-well plates. The liver and plasma samples were diluted in 50 mM Tris-HCl, 1 mM EGTA buffer, pH 8.0 and the brain samples were diluted in 0.1 M PB, pH 8.0 as described above. The samples were then incubated with CPO (0–100 µM), DZO (0–100 µM), PO (0–100 µM), or MO (0–200 µM) at 23°C for 30 minutes. AChE and CaE assays were performed in triplicate as described above, in the presence or absence of inhibitor. Three separate inhibition curves were generated for each homogenate and with each compound. GraphPad Prism software was used for nonlinear regression curve fit analysis. Final IC₅₀ values are reported as the mean ± SD of the IC₅₀ values calculated from the three replicate experiments.

Statistical analysis

The data collected from the enzyme assays were compiled using Microsoft Excel and values beyond a 2X inter-quartile range (IQR) criteria for outliers were excluded. Only two data points were excluded based on this criterion. Statistical analysis of the data was performed using the STATA 8.0 software package (STATA Corporation, College Station, Texas). Multifactorial ANOVA was used to determine the statistical significance of potentiation for each genotype and to determine statistically significant effects of genotype on the relevant dependent variable (AChE or CaE activity). Values of *p* < 0.05 were considered to be significant. Results are presented as means ± SEM.

Results

The first objective of this study was to determine, through the use of *PON1*^{+/+} and *PON1*^{-/-} mice, whether PON1 modulates susceptibility to exposures of OP insecticide mixtures. The second objective was to determine the impact of PON1 status (*hPON1*_{Q192R} genotype and plasma PON1 level) on the toxicity of OP insecticide mixtures, through the use of humanized *hPON1*_{Q192} and *hPON1*_{R192} transgenic mice. The design involved measuring inhibition of AChE (brain and diaphragm) and CaE (liver and serum) in mice, resulting from acute exposures of specific combinations of OP insecticide metabolites (CPO, DZO, PO) with MO, and determining whether these effects were modulated by PON1 status. The study was designed to create a timed inhibition of CaE by exposure to one of the three tested OP compounds and then 4 hours later, at the time of maximal CaE inhibition, to challenge the mice with exposure

to malaoxon (MO), a CaE substrate. This protocol allowed determination of the degree to which PON1, based on genotype, was able to modulate the mixed exposure. First, inhibition of CaE by CPO, DZO, and PO was measured *in vitro*, then the “humanized” *PON1* transgenic mouse model was employed to evaluate the degree of potentiation *in vivo* and the effect of PON1 status on potentiation. We hypothesized, based on previous reports, that prior exposure to an OP that inactivates CaE would potentiate the effect of MO exposure, and that PON1 status would, in cases where the OP is a physiologically relevant PON1 substrate, be important in determining an individual’s sensitivity to the mixed exposure.

Organophosphorus compounds are potent inhibitors of CaE *in vitro*

OP inhibition of plasma CaE, liver CaE, and brain AChE was assessed by measuring IC₅₀ values, using different concentrations of CPO, DZO, PO, and MO incubated with tissues prepared from *PON1*^{+/+} (B6.129) mice (Table 1). CaE was inhibited *in vitro* by all four compounds (CPO, DZO, PO and MO), in both liver homogenates and plasma samples, following exposure to the compounds for 30 minutes at 23°C (Table 1, Fig. 1). Brain AChE was also inhibited *in vitro* by all four compounds (CPO, DZO, PO and MO) following exposure of homogenates for 30 minutes at 23°C (Table 1, Fig. 1). Liver CaE [mean IC₅₀ values (nM): 4.3 ± 0.8 (CPO); 3.9 ± 0.6 (DZO); 1.1 ± 0.3 (PO)] demonstrated a greater sensitivity to these three OP compounds than did plasma CaE [mean IC₅₀ values (nM): 33.7 ± 8.3 (CPO); 16.6 ± 4.2 (DZO); 18.7 ± 2 (PO)]. CPO and PO were more potent inhibitors [mean IC₅₀ values (nM): 14 ± 1.9 and 9.5 ± 1.3 respectively] of brain AChE than DZO [mean IC₅₀ value (nM): 97 ± 7.6]. MO was a rather weak inhibitor of plasma and liver CaE but a stronger inhibitor of brain AChE than DZO [mean IC₅₀ value (nM): 75 ± 7.8] (Table 1).

PON1 levels in *PON1*^{+/+}, *PON1*^{-/-}, *hPON1*_{Q192} and *hPON1*_{R192} mice

Plasma PON1 levels were determined for all mice used in this study by measuring either arylesterase (AREase) or diazoxonase (DZOase) activity (Fig. 2). The *hPON1*_{Q192} mice had the highest PON1 levels, ~50% higher than the levels in *hPON1*_{R192} mice and ~20% higher than the levels in *PON1*^{+/+} mice (Fig. 2). As seen previously, *PON1*^{-/-} mice had some background AREase activity that was not due to PON1 (Fig. 2A), most likely due to the presence of plasma CaE. In contrast, plasma DZOase activity was nearly zero in the *PON1*^{-/-} mice (Fig. 2B), indicating that the DZOase activity was due entirely to PON1.

Time course of CaE inhibition *in vivo*

Once *in vitro* inhibition of CaE was measured for each of the compounds, experiments were conducted to determine the time course of inhibition *in vivo*. Dermal exposure of mice (*PON1*^{+/+}, *hPON1*_{R192}, *hPON1*_{Q192}, *PON1*^{-/-}) to 0.75 mg/kg CPO (n=6–10 per genotype; male and female) revealed that maximal inhibition of plasma CaE occurred at four hours in all four genotypes (Fig. 3A). The time course of inhibition of plasma CaE in *PON1*^{-/-} mice exposed to 0.5 mg/kg DZO (n=4) or 0.35 mg/kg PO (n=4) was also maximal at 4 hours (Fig. 3B,C). Similar to what was predicted based on genotype, sensitivity to CPO 4-h after exposure was as follows (from least sensitive to most sensitive): *PON1*^{+/+}, *hPON1*_{R192}, *hPON1*_{Q192}, *PON1*^{-/-}. The *hPON1*_{Q192} mice were more sensitive than the *hPON1*_{R192} mice despite the presence of ~50% higher levels of PON1 in their plasma (Fig. 2). At 8-h, 12-h, and 24-h post-exposure, CaE inhibition was greater in *PON1*^{-/-} mice than in *PON1*^{+/+} mice, and was similar in the *hPON1*_{R192} and *hPON1*_{Q192} mice. Sensitivity to DZO 4-h after exposure was greatest in *PON1*^{-/-} mice, with equivalent sensitivities between the *hPON1*_{Q192} and *hPON1*_{R192} mice (Fig. 3B). The higher levels of plasma PON1 in the *hPON1*_{Q192} mice may have provided some additional protection against CaE inhibition (Fig. 3B). Sensitivity to PO 4-h after exposure was equivalent among all four genotypes (Fig. 3C). At the time of sacrifice, 24-h following exposure, plasma CaE in *PON1*^{+/+}, *hPON1*_{R192}, *hPON1*_{Q192}, and *PON1*^{-/-} mice had recovered

to 60–80% of control values (Fig. 3) and there was no detectable inhibition of liver CaE compared to controls (not shown). In a separate experiment, inhibition of CaE was measured in both serum and liver in *PON1*^{+/+}, *hPON1*_{Q192}, *hPON1*_{R192} and *PON1*^{-/-} mice 4-h after exposure to each of the OP compounds (0.75 mg/kg CPO, 0.5 mg/kg DZO, or 0.35 mg/kg PO). Again, no measureable inhibition of liver CaE was observed, with the exception of *PON1*^{-/-} mice exposed to CPO, which had CaE activities that were 17% lower than controls (not shown).

Dose response curves of CaE inhibition

The lack of inhibition of CaE in the liver was an unexpected result. Dose response curves for all three compounds were generated in the most sensitive mice (*PON1*^{-/-}) to determine whether higher doses might inhibit liver CaE (Fig. 4). Each dose group consisted of four mice and all animals were sacrificed at 4 h. Compared to serum CaE (Fig. 4A), liver CaE was resistant to OP inhibition in the *PON1*^{-/-} mice (Fig. 4B), perhaps due to interaction of the oxon forms of the OP compounds with plasma serine hydrolases before they reached the liver. Dermal exposure to 0.75 mg/kg CPO, 1.0 mg/kg DZO, or 0.35 mg/kg PO resulted in inhibition of liver CaE by 8%, 7%, and 4%, respectively (Fig. 4B). Exposure to higher doses resulted in greater inhibition of liver CaE at 4-h (Fig. 4B). OP concentrations sufficient to inhibit liver CaE by at least 20% produced substantially higher inhibition of brain AChE, with a range of inhibition by all three compounds between 80 and 95%. These higher concentrations of OP (e.g., exposure of *PON1*^{-/-} mice to 1 mg/kg CPO) were lethal when combined with MO doses as low as 30 mg/kg (data not shown).

Exposures to OP mixtures

Dermal exposures of *PON1*^{+/+} and *PON1*^{-/-} mice to CPO, DZO, and PO followed 4-h later by exposure to MO assessed the role of PON1 status in modulating these mixed exposures. Use of the transgenic mouse model, where mouse *PON1* was replaced by human *PON1*, addressed the relative importance of the two transgenic *hPON1*_{Q192R} alloforms in protecting against OP toxicity during sequential exposures. The dose of each compound was chosen based on its ability to maximally inhibit plasma CaE while minimally inhibiting brain AChE. Doses used for the initial exposure were 0.75 mg/kg CPO, 0.5 mg/kg DZO, and 0.35 mg/kg PO, while the doses of the CaE substrate, MO, administered four hours later were 0, 30, 60, 100 mg/kg. Animals were sacrificed eight hours following administration of the first OP.

Effects of OP compounds on malaoxon (MO) toxicity

Following dermal exposure of the mice, CaE inhibition by CPO, DZO, or PO was associated with increased MO-mediated inhibition of brain and diaphragm AChE (Figs. 5–7). The effect (*p* value) of potentiation for each genotype, as measured by the inhibition of brain and diaphragm acetylcholinesterase (AChE), is shown in Table 2. A significant increase (*p*<0.01) in the average inhibition of brain AChE across doses was observed in all genotypes and for all three compounds (Table 2; Figs. 5–7) with one exception: brain AChE inhibition was not significantly increased in the DZO + MO mixture in *PON1*^{+/+} mice (*p*<0.10; Fig. 6A vs. Fig. 6C). Diaphragm AChE inhibition was also significantly increased (*p*<0.01) with prior exposure to CPO, DZO, and PO (Figs. 5–7; Table 2) with a few exceptions: diaphragm AChE inhibition was not significantly increased (*p*<0.08) in the CPO + MO mixture in *PON1*^{+/+} or *hPON1*_{R192} mice (*p*<0.32; Fig. 5B vs. Fig. 5D,E; Table 2), nor was it increased (*p*<0.41) in the DZO + MO mixture in *PON1*^{+/+} mice (Fig. 6B vs. Fig. 6D; Table 2). Table 2 lists the *p*-values associated with potentiation in each of the four genotypes.

Effect of PON1 genotype

To assess the effect of *hPON1*_{Q192R} genotype on potentiation, it was necessary to first determine if the response to MO alone was affected by genotype. Because MO is detoxified

by CaE and is not a PON1 substrate, it was hypothesized that exposure to MO alone would not result in differences in AChE inhibition across *hPON1*_{Q192R} genotypes. Indeed, there were no significant differences in brain or diaphragm AChE inhibition among *hPON1*_{Q192R} genotypes for mice exposed to MO alone ($p=0.35$; Fig. 5A,B; Fig. 6A,B; Fig. 7A,B), nor were there differences in inhibition of serum CaE by MO alone ($p<0.99$; not shown). Exposures to the OP mixtures (CPO/MO, DZO/MO, and PO/MO) resulted in differential inhibition of brain and diaphragm AChE among mice of different *hPON1*_{Q192R} genotypes (Figs. 5–7; summarized in Table 3), as discussed below.

Effect of chlorpyrifos oxon (CPO) on MO toxicity

In the case of pre-exposure to CPO, genotype influenced the degree of potentiation of MO toxicity; brain and diaphragm AChE inhibition were significantly different between genotypes and across doses ($p<0.002$). Differences in the potentiation of MO toxicity followed our hypotheses based on the relative sensitivities of *PON1*^{-/-} and *PON1*^{+/+} mice (Fig. 5C,D), or *hPON1*_{Q192} and *hPON1*_{R192} mice (Fig. 5E,F; Table 3). Inhibition of brain AChE in *PON1*^{-/-} mice was significantly greater ($p<0.01$) than that seen in the *PON1*^{+/+} mice (Fig. 5C; Table 3), and *hPON1*_{Q192} mice had significantly greater ($p<0.02$) brain AChE inhibition than that seen in the *hPON1*_{R192} mice (Fig. 5E; Table 3). Diaphragm AChE inhibition in *PON1*^{-/-} mice was also significantly greater than in *PON1*^{+/+} mice ($p<0.001$; Fig. 5D; Table 3); however, inhibition of diaphragm AChE in *hPON1*_{Q192} mice was not significantly different from *hPON1*_{R192} mice ($p<0.19$; Fig. 5F; Table 3), possibly because of the higher PON1 levels present in the *hPON1*_{Q192} mice.

Effect of diazoxon (DZO) on MO toxicity

Brain and diaphragm AChE inhibition, due to the combined sequential exposure to DZO and MO, were significantly different between genotypes and across doses ($p<0.002$). Inhibition of brain and diaphragm AChE in the *PON1*^{-/-} mice was significantly greater than that seen in *PON1*^{+/+} mice [$p<0.001$ (brain); $p<0.001$ (diaphragm); Table 3], with *PON1*^{-/-} mice exhibiting higher sensitivity to DZO (Fig. 6C,D; Table 3). However, neither brain nor diaphragm AChE inhibition were significantly different between the *hPON1*_{Q192} and *hPON1*_{R192} mice [$p=0.13$ (brain); $p=0.62$ (diaphragm); Fig. 6E,F; Table 3], as expected based on their equivalent catalytic efficiencies of DZO hydrolysis (Li et al., 2000).

Effect of paraoxon (PO) on MO toxicity

PO, whose acute toxicity is not influenced by PON1 due to its low catalytic efficiency of hydrolysis (Chambers et al., 1994; Li et al., 2000), increased the toxicity of MO. It was hypothesized that exposure to PO followed by MO would produce levels of brain and diaphragm AChE inhibition that would be equivalent among all four *hPON1*_{Q192R} genotypes. Indeed, brain and diaphragm AChE inhibition were not significantly different between *PON1*^{-/-} mice and *PON1*^{+/+} mice ($p<0.88$; Fig. 7C,D; Table 3), nor between *hPON1*_{Q192} and *hPON1*_{R192} mice ($p<0.07$; Fig. 7E,F; Table 3).

Cholinergic symptoms

Cholinergic symptoms were recorded for all mice at 8 h post-exposure (Table 4). Mice that were exposed to 0.75 mg/kg CPO, 0.5 mg/kg DZO, or 0.35 mg/kg PO alone did not generally exhibit cholinergic symptoms. Mice that received MO alone did not exhibit any cholinergic signs for doses of 0, 30, or 60 mg/kg MO, despite inhibition of brain AChE by 5–50%. At doses of 100 mg/kg MO, brain AChE was inhibited 35–60% and slight hypoactivity and thermoregulatory problems (i.e., coolness to the touch) were observed. Following sequential exposure to CPO, DZO, or PO followed by MO, cholinergic symptoms were observed in the *PON1*^{-/-}, *PON1*^{+/+}, *hPON1*_{Q192} and *hPON1*_{R192} mice. Symptoms were (in the order of

occurrence with increasing dose): hypoactivity, difficulty with thermoregulation, muscle fasciculations, consistent fine tremor in forelimbs and/or head, mild lacrimation, and at higher doses of MO, tremor extending more caudally or whole body tremor that was usually associated with moderate to severe lacrimation (Table 4). At comparable doses of CPO and DZO, the *PON1*^{+/+} and *hPON1*_{R192} mice were generally more resistant to developing cholinergic symptoms than the *PON1*^{-/-} and *hPON1*_{Q192} mice. Following PO exposure, the dose at which the mice developed cholinergic symptoms was equivalent among genotypes (Table 4).

Effect of tri-ortho-cresyl phosphate (TOCP) on MO toxicity

The doses of CPO, DZO, and PO used above were not sufficient to inhibit liver CaE without severely compromising brain AChE. For example, prior exposure of mice to 1 mg/kg CPO inhibited liver CaE, but was lethal when combined with MO doses as low as 30 mg/kg (data not shown). TOCP has been shown previously to inhibit liver and serum CaE, and can potentiate the toxicity of MO while minimally inhibiting brain AChE (Casida et al., 1961; Cohen and Murphy, 1971a; 1971b). To assess the effect of inhibiting both liver and serum CaE, *PON1*^{+/+} mice (n=4 for each dose group) were exposed to 10 mg/kg TOCP followed 24-h later by MO exposure (0, 30, 60, 100 mg/kg).

Four hours after MO exposure, AChE activity was measured in brain and diaphragm and CaE activity was measured in liver and serum. Pretreatment with TOCP significantly increased ($p<0.001$) the inhibition of both brain and diaphragm AChE by MO at all doses (30, 60, 100 mg/kg), with a markedly greater potentiation at the 60 mg/kg dose of MO (Fig. 8B,C). TOCP caused significant inhibition ($p<0.001$) of both liver and serum CaE, with liver CaE being the more sensitive of the two (Fig. 8A). Prior exposure to TOCP at 10 mg/kg produced serum CaE inhibition similar to that produced from prior exposure to 0.75 mg/kg CPO, yet exposure to TOCP resulted in a greater potentiation of MO toxicity.

Discussion

This research focused on the relative importance of the two *hPON1*_{Q192R} alloforms in protecting against OP toxicity during sequential mixed OP exposures, using transgenic mouse models that included strains with different *PON1* status (*PON1*^{+/+}, *PON1*^{-/-}, *hPON1*_{R192} and *hPON1*_{Q192}). OP exposures are often multi-route and multi-chemical in nature. Based on a determination that OPs form a common mechanism group based on their shared ability to inhibit AChE in both the central and peripheral nervous systems (US EPA, 1999), the EPA carried out a cumulative risk assessment for this class of pesticides (US EPA, 2002), and recently reviewed and updated its cumulative assessment, eliminating or modifying many uses of these compounds (US EPA, 2006). While the assumption in these assessments was dose additivity of the OP compounds, we demonstrate here that greater-than-additive effects are possible when exposure to certain OP compounds precedes exposure to another OP compound, e.g., malaoxon. The endpoints where potentiation was observed were clinically relevant; that is, AChE inhibition in the brain and diaphragm. As CaE inhibition is known to influence the toxicity of malathion following pre-exposure to other AChE inhibitors (Casida, 1961; Cohen and Murphy, 1971a; 1971b; Verschoyle et al., 1982) we utilized specific combinations of OPs to explore our first hypothesis that prior exposure to a CaE inhibitor (CPO, DZO, PO) would increase toxicity of CaE substrates such as MO. In this study, the combined effects of the selected OP insecticides (CPO + MO, DZO + MO, and PO + MO) increased MO toxicity significantly.

The mechanism for this increased toxicity is most likely related to inhibition of carboxylesterase (CaE) enzymes in the blood and/or liver. In contrast to rodents, there is no CaE activity in human plasma (Williams et al., 1989; Li et al., 2005). In the current study, OP compounds inhibited CaE activity significantly in mouse plasma or serum, but not liver. This

did not appear to be due to differences in time for the OP compounds to reach the liver versus blood, because liver CaE was not inhibited at 4-h, 8-h, or 24-h after exposure at the doses used for the study of mixtures. A more likely explanation is that the oxon forms of the OP compounds used in this study encounter and bind to serum B-esterases before passing through the liver. Exposure of mice to higher doses of OP compounds resulted in inhibition of CaE activity in liver as well as serum; however, the resulting potentiation of MO toxicity was lethal.

Of particular importance is the demonstration that PON1 status (PON1 levels and *hPON1*_{Q192R} genotype) modulates the interactive toxicity of OP compounds. The results presented here show that PON1 status alters MO potentiation in a substrate-dependent manner, and that the potentiation is determined in part by the catalytic efficiency of the PON1 alloform present in serum. First, by comparing *PON1*^{-/-} and *PON1*^{+/+} mice, it was evident that the absence of PON1 significantly increased the potentiation of MO toxicity by both CPO and DZO, but not PO. This is consistent with the role of PON1 in the *in vivo* hydrolysis of CPO and DZO, but not PO. Our previous studies demonstrated that PON1 did not provide protection against PO exposure *in vivo* (Li et al., 2000). This is of particular importance given the large differences in PON1 levels among individuals and across development (Cole et al., 2003; Furlong et al., 2006).

Second, differences in potentiation were observed between mice expressing *hPON1*_{Q192} and *hPON1*_{R192} for CPO, but not DZO or PO, indicating that the *hPON1*_{Q192R} genotype is important for modulating the interactive toxicity of OP compounds in a substrate-specific manner. The differences in genotype-modulation of potentiation among OP compounds are entirely consistent with the known catalytic efficiency differences in the *hPON1*_{Q192} and *hPON1*_{R192} alloforms. Compared to the *hPON1*_{Q192} alloform, the *hPON1*_{R192} alloform has a higher catalytic efficiency of CPO hydrolysis [150 vs. 250 (Li et al., 2000)], and in the current study, the *hPON1*_{R192} alloform was more protective than the *hPON1*_{Q192} alloform against CPO-potentiation of MO toxicity. This was the case despite the 50% higher PON1 levels present in the plasma of *hPON1*_{Q192} mice compared to the *hPON1*_{R192} mice. The *hPON1*_{Q192} and *hPON1*_{R192} alloforms have similar catalytic efficiencies of DZO hydrolysis (Li et al., 2000) and in the current study provided similar protection against DZO-potentiation of MO toxicity. For PO, the catalytic efficiency of both alloforms is too low to be protective *in vivo* (Li et al., 2000), and in the current study neither *hPON1*_{Q192}, *hPON1*_{R192}, nor mouse PON1 provided any protection against the effects of PO on MO toxicity. In addition to brain and diaphragm AChE inhibition, cholinergic symptoms were differentially affected among mice of different genotypes. Following sequential exposure to CPO, DZO, or PO, followed by MO, cholinergic symptoms were observed in the *PON1*^{-/-} and *hPON1*_{Q192} mice. *PON1*^{+/+} and *hPON1*_{R192} mice were more resistant to developing cholinergic symptoms.

Third, the *hPON1*_{Q192} and *hPON1*_{R192} mice tended to have effects on potentiation that were intermediate between those observed in the *PON1*^{-/-} and *PON1*^{+/+} mice. This is somewhat paradoxical, since the *hPON1*_{Q192} mice had 20% higher levels of PON1 compared to *PON1*^{+/+} mice. The most likely explanation would be a higher catalytic efficiency of mouse PON1 compared to human PON1, a possibility that is addressable experimentally. An additional possibility is that the human PON1 protein is not as fully functional as the endogenous mouse PON1 protein in the context of mouse physiology.

In this study, the degree to which CPO, DZO, or PO inhibited CaE predicted the degree of potentiation of MO toxicity. Other studies have examined the potentiation of MO toxicity by OPs that inhibit CaE both *in vitro* and *in vivo*. In one study, CPO showed a high inhibitory potency toward CaE-mediated detoxication of malathion in human liver microsomes (Buratti and Testai, 2005). Concurrent exposure to a mixture of five OPs, including chlorpyrifos and diazinon, was found to potentiate malathion toxicity (AChE inhibition and behavioral changes)

in adult and pre-weanling rats, resulting in greater-than-additive effects for all endpoints measured (Moser et al., 2005; 2006). Previous work has reported both a lack of potentiation of the effects of malathion by pretreatment with parathion (Cohen and Murphy, 1971b) and a potentiation of effects (Su et al., 1971; Ramakrishna and Ramachandran, 1978). In addition, a study by Chambers and colleagues (1990) demonstrated that rat liver homogenates detoxified appreciable amounts of several organophosphates (including PO) stoichiometrically and this correlated well with aliesterase activity. A second study by this same group found that TOCP pretreatment potentiated PO toxicity, supporting the role of CaE in the detoxication of PO (Tang and Chambers, 1999). Timchalk and colleagues (2005) used a binary mixture of diazinon and chlorpyrifos to evaluate pharmacokinetic and pharmacodynamic interaction in the rat. At low doses (15 mg/kg), they found no apparent pharmacokinetic interactions, suggesting additive effects on AChE inhibition, whereas interactive effects between the two compounds were observed at a higher dose (60 mg/kg). The overall relative potency for AChE inhibition was chlorpyrifos + diazinon > chlorpyrifos > diazinon (Timchalk et al., 2005).

The data reported here indicate that the common *hPON1*_{Q192R} polymorphism, coupled with large differences in PON1 levels among individuals, can have impacts beyond those of simple OP-mediated cholinesterase inhibition. By modulating OP-mediated inhibition of carboxylesterase, differences in PON1 status can have effects on the toxicity of other compounds that are detoxified by carboxylesterases. Presumably this would include not only malaoxon or malathion, but also pyrethroid compounds (Abernathy and Casida, 1973; Gaughan et al., 1980; Godin et al., 2001; Choi et al., 2004; Wheelock et al., 2005) that have widespread residential uses. Of additional interest in this regard is the potential in aircraft for simultaneous exposure to tricresyl phosphate as a cabin air contaminant and pyrethroids that are sprayed in the cabin for insect control (Winder, 2002). Variability of PON1 status would also impact the metabolism of many drugs and pro-drugs, and insecticides detoxified by carboxylesterases. The data presented here indicate that differences in PON1 activity levels, but not *hPON1*_{Q192R} genotype, would be important for modulating mixed exposures involving diazinon. In contrast, individuals homozygous for the *hPON1*_{Q192} alloform would be the most susceptible for the interactive toxicity involving exposures to chlorpyrifos, particularly those with low plasma activity levels. These findings have particular relevance for newborns and young children, who have very low levels of PON1 (Cole et al., 2003).

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Figure 1. CPO, DZO, and PO inhibition of plasma carboxylesterase (A), liver carboxylesterase (B), and brain AChE (C) *in vitro*

OPs were incubated with tissue homogenates prepared from wild-type mice. Results represent the mean $\pm$ SEM (n=3). IC₅₀ values calculated from the data are listed in Table 1.

Figure 2. PON1 levels in $PON1^{+/+}$, $PON1^{-/-}$, $hPON1_{Q192}$ and $hPON1_{R192}$ mice

Plasma PON1 levels were determined for all mice (genotypes as indicated) by measuring (A) arylesterase (AREase) activity (n=32–42 mice) or (B) low-salt diazoxonase (DZOase) activity. Note the presence of AREase activity, but not DZOase activity, in the plasma of the $PON1^{-/-}$ mice. In contrast to AREase activity, DZOase activity is entirely due to PON1. Genotypes are Results represent the mean $\pm$ SEM (AREase, n=32–42; DZOase, n=16–78).

Figure 3. Time course of plasma CaE inhibition *in vivo*, following exposure to CPO, DZO, and PO
 Time course of plasma carboxylesterase (CaE) inhibition in *PON1*^{+/+}, *PON1*^{-/-}, *hPON1*_{Q192}, and *hPON1*_{R192} mice (genotypes as indicated) following dermal exposure to 0.75 mg/kg CPO (A), 0.5 mg/kg DZO (B), or 0.35 mg/kg PO (C). Maximal inhibition of CaE was at 4 hours. Results represent the mean ± SEM (n=5–10).

Figure 4. CPO, DZO, and PO inhibit serum CaE and liver CaE *in vivo*.

PON1^{-/-} mice were exposed dermally to CPO, DZO, or PO, and the dose response of (A) serum CaE and (B) liver CaE inhibition was measured 4-h after exposure. For PO, the highest dose given was 0.8 mg/kg. Because exposure at this dose caused lacrimation, tremors, and brain AChE inhibition of greater than 90% (not shown), higher doses would likely have been lethal. Results represent the mean $\pm$ SEM (n=4).

Figure 5. Effect of CPO exposure (0.75 mg/kg) on subsequent toxicity of malaoxon (MO)
Mice (genotypes as indicated) were exposed dermally to MO alone (A, B), or to CPO followed 4-h later by MO exposure (C, D, E, F). AChE was measured in the brain (A, C, E) and diaphragm (B, D, F) 4-h following the MO exposure. Results represent the mean $\pm$ SEM (n=4).

Figure 6. Effect of DZO exposure (0.5 mg/kg) on subsequent toxicity of malaoxon (MO)
Mice (genotypes as indicated) were exposed dermally to MO alone (A, B), or to DZO followed 4-h later by MO exposure (C, D, E, F). AChE was measured in the brain (A, C, E) and diaphragm (B, D, F) 4-h following the MO exposure. Results represent the mean $\pm$ SEM (n=4).

Figure 7. Effect of PO exposure (0.35 mg/kg) on subsequent toxicity of malaoxon (MO)
Mice (genotypes as indicated) were exposed dermally to MO alone (A, B), or to CPO followed 4-h later by MO exposure (C, D, E, F). AChE was measured in the brain (A, C, E) and diaphragm (B, D, F) 4-h following the MO exposure. Results represent the mean $\pm$ SEM (n=4).

Figure 8. Effect of TOCP exposure (10 mg/kg) on subsequent toxicity of malaoxon (MO)
PON1^{+/+} mice were exposed dermally to MO alone, or to TOCP followed 24-h later by MO exposure. (A) CaE inhibition in liver and serum was measured 24-h after TOCP exposure. Liver CaE was more sensitive than serum CaE to inhibition by TOCP. AChE was measured in the brain (B) and diaphragm (C) 4-h following the MO exposure. Results represent the mean $\pm$ SEM (n=4).

Table 1

In vitroIC₅₀ values of CPO, DZO, PO, and MO for serum and liver CaE and plasma AChE.

Tissue	Replicate	Organophosphorus Insecticide (nM) [†]			
		CPO	DZO	PO	MO
Plasma (CaE)	1	42.5	15.0	16.6	5662.2
	2	32.6	13.4	18.6	5719.0
	3	26.0	21.3	20.8	6547.0
	Mean	33.7 ± 8.3	16.6 ± 4.2	18.7 ± 2.1	5976.0 ± 495
Liver (CaE)	1	3.5	3.6	0.8	312.2
	2	4.4	4.5	1.2	336.1
	3	5.0	3.5	1.4	278.0
	Mean	4.3 ± 0.8	3.9 ± 0.6	1.1 ± 0.3	308.8 ± 29.2
Brain (AChE)	1	12.6	98.8	11.0	74.2
	2	13.1	88.5	8.6	67.1
	3	16.1	103.3	8.8	82.6
	Mean	14.0 ± 1.9	96.9 ± 7.6	9.5 ± 1.3	74.6 ± 7.8

[†]IC₅₀ values (mean ± SEM).

Table 2Statistical significance of OP potentiation for each *PONI* genotype.

		OP Exposure (<i>p</i> value) [†]		
Tissue	Genotype	CPO + MO	DZO + MO	PO + MO
Brain (AChE)	<i>PONI</i> ^{+/+}	0.0002	NS (0.0971)	0.0003
	<i>PONI</i> ^{-/-}	0.0001	0.0001	0.0001
	<i>hPONI</i> _{Q192}	0.0001	0.0024	0.0001
	<i>hPONI</i> _{R192}	0.0141	0.0001	0.0001
Diaphragm (AChE)	<i>PONI</i> ^{+/+}	NS (0.0811)	NS (0.4050)	0.0025
	<i>PONI</i> ^{-/-}	0.0001	0.0001	0.0078
	<i>hPONI</i> _{Q192}	0.0001	0.0048	0.0001
	<i>hPONI</i> _{R192}	NS (0.3172)	0.0021	0.0029

[†] Potentiation was tested for statistical significance by multifactorial ANOVA. NS = not significant (*p* value > 0.05).

Table 3Statistical significance of *PON1* genotype effects on potentiation by OPs.

Tissue	Genotype	OP Exposure (<i>p</i> value) [†]		
		CPO + MO	DZO + MO	PO + MO
Brain (AChE)	<i>PON1</i> ^{+/+} vs. <i>PON1</i> ^{-/-}	0.0141	0.0001	NS (0.8744)
	<i>hPON1</i> _{Q192} vs. <i>hPON1</i> _{R192}	0.0178	NS (0.1346)	NS (0.0700)
Diaphragm (AChE)	<i>PON1</i> ^{+/+} vs. <i>PON1</i> ^{-/-}	0.0001	0.0015	NS (0.8782)
	<i>hPON1</i> _{Q192} vs. <i>hPON1</i> _{R192}	NS (0.1932)	NS (0.6169)	NS (0.0709)

[†] Differences in potentiation among genotypes were tested for statistical significance by multifactorial ANOVA. NS = not significant (*p* value > 0.05).

Table 4

Cholinergic symptoms[†].

	Genotype	Malaoxon Dose (mg/kg)			
		0	30	60	100
0.75 mg/kg CPO	<i>PONI</i> ^{+/+}	0	0	0.3 ± 0.3	0.8 ± 0.5
	<i>PONI</i> ^{-/-}	0	1.3 ± 0.3	2.3 ± 0.5	2.0 ± 0.6
	<i>hPONI</i> _{Q192}	0.3 ± 0.3	0.5 ± 0.3	1.5 ± 0.6	2.0 ± 0.6
	<i>hPONI</i> _{R192}	0	0.5 ± 0.3	0.5 ± 0.3	1.5 ± 0.3
0.5 mg/kg DZO	<i>PONI</i> ^{+/+}	0	0	0	1.0 ± 0.0
	<i>PONI</i> ^{-/-}	0	0.8 ± 0.3	1.3 ± 0.3	1.5 ± 0.5
	<i>hPONI</i> _{Q192}	0	0.5 ± 0.3	0.8 ± 0.5	1.5 ± 0.3
	<i>hPONI</i> _{R192}	0	0	1.0 ± 0.0	2.0 ± 0.4
0.35 mg/kg PO	<i>PONI</i> ^{+/+}	0	0	1.0 ± 0.0	0.8 ± 0.3
	<i>PONI</i> ^{-/-}	0	0	0.3 ± 0.3	0.8 ± 0.3
	<i>hPONI</i> _{Q192}	0	0.3 ± 0.3	1.5 ± 0.3	0.8 ± 0.5
	<i>hPONI</i> _{R192}	0	0	1.3 ± 0.3	1.8 ± 0.3

[†]Cholinergic symptoms recorded 8-h after exposure: (0) No symptoms; (1) Hypoactivity/Coolness to touch/Muscle fasciculations; (2) Consistent fine tremor in forelimbs and/or head/mild lacrimation; (3) Tremor extending more caudally or whole body tremor/moderate to severe lacrimation. Results are presented as mean ± SEM (n=4).