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The Toxicity of Mixtures of Specific Organophosphorus Compounds is Modulated by Paraoxonase 1 Status

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Abstract

Most chemical exposures involve complex mixtures. The role of paraoxonase 1 (PON1) and the Q192R polymorphism in the detoxication of individual organophosphorous (OP) compounds has been well-established. The extent to which PON1 protects against a given OP is determined by its catalytic efficiency. We used a humanized transgenic mouse model of the Q192R polymorphism to demonstrate that PON1 modulates the toxicity of OP mixtures by altering the activity of another detoxication enzyme, carboxylesterase (CaE). Chlorpyrifos oxon (CPO), diazoxon (DZO), and paraoxon (PO) are potent inhibitors of CaE, both in vitro and in vivo. We hypothesized that exposure of mice to these OPs would increase their sensitivity to the CaE substrate, malaoxon (MO), and that the degree of effect would vary among PON1 genotypes if the OP was a physiologically relevant PON1 substrate. When wild-type mice were exposed dermally to CPO, DZO, or PO and then, after 4 h, to different doses of MO, the toxicity of MO was increased compared to mice that received MO alone. The potentiation of MO toxicity by CPO and DZO was higher in PON1 knockout mice, which are less able to detoxify CPO or DZO. Potentiation by CPO was higher in Q192 mice than in R192 mice due to the decreased ability of $PON1_{Q192}$ to detoxify CPO. Potentiation by DZO was similar in the Q192 and R192 mice, due to their equivalent effectiveness at detoxifying DZO. PO exposure resulted in equivalent potentiation of MO toxicity among all four genotypes. These results indicate that PON1 status modulates the ability of CaE to detoxicate OP compounds from specific mixed insecticide exposures. PON1 status can also impact the capacity to metabolize drugs or other CaE substrates following insecticide exposure.

Keywords

Mixed exposures; Chlorpyrifos oxon; Chlorpyrifos oxon; Diazinon; Diazoxon; Malathion; Malaoxon; Parathion; Paraoxon; Pyrethroids; Tricresyl phosphate; Carboxylesterase; Paraoxonase 1 (PON1)

1 PON1 and Detoxication of OP Compounds

The involvement of paraoxonase 1 (PON1) in the detoxication of organophosphorus (OP) compounds has been well-documented (reviewed in Costa, 2006; Furlong, 2008). *PON1* knockout ($PON1^{-/-}$) mice are dramatically more sensitive than wild-type ($PON1^{+/+}$) mice to the toxicity of chlorpyrifos oxon (CPO) and diazoxon (DZO) and to a lesser extent the parent phosphorothioates, chlorpyrifos and diazinon (Shih et al. 1998; Li et al. 2000; Cole et al. 2005). Injection of purified plasma PON1 protein (Main, 1956; Costa et al. 1990; Li et al. 1995, 2000) or, more recently, recombinant PON1 (Stevens et al. 2008), increased the resistance of rats and/or mice to OP toxicity. The extent of protection was shown to be

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dependent on the catalytic efficiency of PON1 hydrolysis for the respective OP compounds (Li et al. 2000). The Q192R amino acid polymorphism of PON1 ($hPON1_{Q192R}$) affects the catalytic efficiency of hydrolysis for some substrates, but not others (Hassett et al. 1991; Adkins et al. 1993; Davies et al. 1996; Li et al. 2000). For diazoxon (DZO), the $hPON1_{Q192}$ and $hPON1_{R192}$ alloforms had equivalent catalytic efficiencies measured in vitro and injection of the $hPON1_{Q192}$ and $hPON1_{R192}$ alloforms provided equivalent protection in vivo (Li et al. 2000). For chlorpyrifos oxon (CPO), the $hPON1_{R192}$ alloform had a higher catalytic efficiency of hydrolysis than the $hPON1_{Q192}$ alloform in vitro and also provided better protection than $hPON1_{Q192}$ in vivo (Li et al. 2000). For paraoxon (PO), the catalytic efficiency measured in vitro was very low, and PON1 did not provide any protection in vivo (Li et al. 2000). Further evidence came from a study of humanized PON1 transgenic mice, in which the endogenous mouse *PON1* gene was removed and human *PON1* transgenes were inserted that encoded either $hPON1_{Q192}$ or $hPON1_{R192}$ (Cole et al. 2003). The $hPON1_{Q192}$ mice were much more sensitive than $hPON1_{R192}$ mice to CPO and, to a lesser extent, chlorpyrifos (Cole et al. 2005).

2 PON1 Status

In addition to the $hPON1_{Q192R}$ amino acid polymorphism, activity levels of plasma PON1 vary tremendously, as much as 15-fold among individuals of the same $hPON1_{Q192R}$ genotype (Furlong et al. 2006; Furlong, 2007). PON1 levels are also very low and variable in newborns, and reach adult levels between 6 months and 2 years of age (Cole et al. 2003; Eskenazi et al. 2008). “PON1 status” is a term that was introduced to take into account both the $hPON1_{Q192R}$ polymorphism and the level of plasma PON1 activity (Li et al. 1993; Richter and Furlong, 1999). PON1 status has been determined primarily through the use of a two-substrate assay that compares plasma rates of DZO hydrolysis in the presence of high salt to plasma rates of PO hydrolysis (Li et al. 1993; Richter and Furlong, 1999; Costa et al. 1999; Jarvik et al. 2003; Huen et al. 2009). More recently, Richter et al. (2008, 2009) developed a protocol that uses the non-toxic substrates phenyl acetate and 4-(chloromethyl)phenyl acetate to determine an individual’s PON1 status.

3 Toxicity of OP Mixtures

Chemical exposures are likely to involve multiple different types of compounds and different routes (e.g., oral and dermal). The assumption of the EPA cumulative risk assessment for the OPs as a class of compounds was dose additivity, with the rationale that the OPs share a common mechanism of action (US EPA, 1999, 2002, 2006). However, numerous studies have reported greater-than-additive effects of combinations of OP compounds. Of particular relevance are early studies demonstrating that toxicity associated with exposure to malathion or its oxon metabolite, malaoxon (MO), was potentiated when the exposure occurred in combination with compounds that inhibit carboxylesterases (CaEs) (Aldridge, 1954; Cook et al. 1957; Dubois, 1958; Murphy et al. 1959; Seume and O’Brien, 1960; Casida et al. 1961, 1963; Cohen and Murphy, 1971a;b; Verschoyle et al. 1982). Malathion is converted in the liver to MO, which can be a potent inhibitor of AChE (DuBois et al. 1953; March et al. 1956; Murphy and DuBois, 1957; O’Brien, 1957), yet potentiation of malathion/MO toxicity was observed even at doses that would not normally inhibit acetylcholinesterase activity (Dubois, 1969; Su et al. 1971). CaEs hydrolyze the carboxylic esters of malathion and MO (March et al. 1956; O’Brien, 1957; Cook and Yip, 1958; Chen et al. 1969). In vitro, MO can undergo hydrolysis by CaEs, but can also bind to CaEs resulting in irreversible inhibition (Main and Dauterman, 1967). Other OP compounds, most notably CPO, DZO, and PO, are not hydrolyzed by CaEs, but instead bind to CaEs and other serine esterases (B-esterases) stoichiometrically and irreversibly, allowing the CaEs to act as stoichiometric scavengers of OP compounds and inhibiting the CaEs in the process (Su et al.

1971; Ramakrishna and Ramachandran, 1978; Chambers et al. 1990; Buratti and Testai, 2005). Tang and Chambers (1999) also found that triorthocresyl phosphate (TOCP) pretreatment potentiated PO toxicity, supporting the role of CaE in the detoxication of PO. CaE activity is highest in the liver, gastrointestinal tract, and brain, with interindividual variability as high as 44-fold among samples of human liver microsomes (Hosokawa et al. 1995; Satoh and Hosokawa, 2006). Rodents, but not humans, possess significant plasma CaE activity (Williams et al. 1989; Li et al. 2005).

Several more recent studies examined the toxicity of OP mixtures. Moser et al. (2005,2006), using concurrent exposure to a mixture of five OP compounds, found greater-than-additive effects on the potentiation of malathion toxicity, using AChE inhibition and behavioral changes as endpoints. Timchalk et al. (2005) used a binary mixture of diazinon and chlorpyrifos in the rat, and reported additive effects on AChE at low doses (15 mg/kg), and interactive effects at a higher dose (60 mg/kg).

4 Effect of PON1 on the Interactive Toxicity of OP Mixtures

We performed a series of experiments to demonstrate that differences in OP detoxication between the hPON1$_{Q192}$ and hPON1$_{R192}$ alloforms can affect the interactive toxicity of chemical mixtures (Jansen et al. 2009). As reported below, the OP compounds CPO, DZO, and PO bind to CaE and inhibit its activity. By virtue of their differential detoxication of CPO, DZO, and PO, hPON1$_{Q192}$ and hPON1$_{R192}$ modulate the degree of this OP-mediated CaE inhibition. As a result, in a combined or sequential exposure PON1 status can modulate the interactive toxicity of OP compounds, even when one of the compounds is not metabolized directly by PON1. We demonstrated this to be the case for the toxicity of MO, which is not a physiologically-relevant PON1 substrate, when combined with exposure to DZO and CPO, which are physiologically-relevant PON1 substrates.

5 Inhibition of CaE by OP Compounds In Vitro

Inhibition of CaE and AChE by OP compounds was measured in liver and brain homogenates and plasma prepared from wild-type (*PON1*$^{+/+}$; B6.129) mice. Liver, plasma, and brain samples were incubated with CPO, DZO, PO, or MO for 30 minutes at 23°C, followed by measurement of CaE or AChE activity. CPO, DZO, and PO were relatively potent inhibitors of CaE and AChE, with IC$_{50}$ values in the low nM range (Table 1).

6 Inhibition of CaE by OP Compounds In Vivo

Transgenic and knockout mice were used to address whether OP compounds inhibited CaE in vivo and whether PON1 is involved in modulating the toxicity of mixtures of OP compounds. *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, 2005) were provided by Drs. Diana M. Shih, Aaron Tward and Aldons J. Lusis (UCLA, Los Angeles, CA). *PON1*$^{+/+}$ mice were bred from the same congenic B6.129 strain background. Mice were housed in SPF (specific pathogen-free) facilities with a 12-h dark–light cycle and unlimited access to food and water. 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, and were approved by the Institutional Animal Care and Use Committee at the University of Washington.

To determine plasma PON1 levels in the mice, saphenous-vein plasma was used to measure the rates of hydrolysis of the alloform-neutral substrates, phenyl acetate (Furlong et al. 1989, 1993, 2006) or DZO, which is alloform-neutral at physiological salt concentrations (Richter and Furlong, 1999). Arylesterase (AREase) and diazoxonase (DZOase) assays were carried

out in a microtiter plate reader (SpectraMax Plus, Molecular Devices), and the initial linear rates of hydrolysis were used to calculate units of activity per ml plasma. As seen previously (Cole et al. 2003, 2005), plasma from the $PON1^{-/-}$ mice had some background AREase activity that was not due to PON1, whereas DZOase activity was essentially absent from $PON1^{-/-}$ mouse plasma (Table 2). Plasma PON1 levels were about 50% higher in $hPON1_{Q192}$ mice compared to $hPON1_{R192}$ mice (Table 2; Jansen et al. 2009).

To determine the time course of CaE inhibition by OP compounds in vivo, mice were exposed dermally (1 μl/g body weight) to 0.5 mg/kg DZO or 0.75 mg/kg CPO, or to 0.35 mg/kg PO, which inhibits CaE but is not a physiologically-relevant PON1 substrate. Plasma CaE inhibition was maximal 4 hours after exposure (Fig. 1; Jansen et al. 2009). For CPO exposure, the order of sensitivity to plasma CaE inhibition at 4 hours (from greatest to least inhibition) was $PON1^{-/-} > hPON1_{Q192} > hPON1_{R192} > PON1^{+/+}$ (Fig. 1a), as expected based on their different catalytic efficiencies of CPO hydrolysis (Li et al. 2000). For DZO exposure, $PON1^{-/-}$ mice were more sensitive than $PON1^{+/+}$ mice to CaE inhibition, and $hPON1_{Q192}$ and $hPON1_{R192}$ mice had similar sensitivities to CaE inhibition (Fig. 1b) as expected based on their equivalent catalytic efficiencies of hydrolysis (Li et al. 2000). For exposure to PO, which is not a physiologically-relevant PON1 substrate, there were no differences in CaE inhibition among genotypes (Fig. 1c). At these concentrations of OPs, there was minimal to no inhibition of liver CaE (Jansen et al. 2009). Exposure to higher doses of OPs (0.50–0.75 mg/kg PO; 1.5–2.0 mg/kg DZO; 1.5–3.0 mg/kg CPO) resulted in inhibition of liver CaE and even more substantial inhibition of serum CaE (Jansen et al. 2009).

7 Effect of PON1 Status on the Toxicity of OP Compound Mixtures

The effects of CPO, DZO, and PO on subsequent toxicity of MO were determined by exposing mice dermally to 0.75 mg/kg CPO, 0.5 mg/kg DZO, or 0.35 mg/kg PO, followed 4 hours later (at the time of maximal CaE inhibition) by dermal exposure to MO (Jansen et al. 2009). Pre-exposure to CPO, DZO, or PO inhibited plasma CaE, and was associated with a significant ($p < 0.01$; multifactorial ANOVA) increase in MO-mediated inhibition of brain and diaphragm AChE (Figs. 2, 3, and 4, compare a, b vs. c, d and e, f). To assess whether the presence of PON1 affected this potentiation of MO toxicity, $PON1^{+/+}$ mice were compared to $PON1^{-/-}$ mice for their sensitivity to mixed OP exposures (Figs. 2c, d, 3c, d, and 4c, d). With pre-exposure to CPO (Fig. 2c, d) or DZO (Fig. 3c, d), but not PO (Fig. 4c, d), AChE inhibition by MO was significantly greater in $PON1^{-/-}$ mice than in $PON1^{+/+}$ mice ($p < 0.02$, CPO/MO; $p < 0.0001$, DZO/MO; $p = 0.87$, PO/MO), consistent with the known roles of PON1 in detoxication of CPO and DZO, but not PO (Li et al. 2000).

To assess whether the $hPON1_{Q192R}$ polymorphism affected the potentiation of MO toxicity, $hPON1_{Q192}$ and $hPON1_{R192}$ transgenic mice were compared for sensitivity to mixed OP exposures (Figs. 2e, f, 3e, f, and 4e, f). The results were consistent with the different catalytic efficiencies of hydrolysis of $hPON1_{Q192}$ and $hPON1_{R192}$ for CPO, DZO, and PO. Specifically, with pre-exposure to PO, AChE inhibition by MO was not affected by either the presence of PON1 (Fig. 4c, d) or by $hPON1_{Q192R}$genotype (Fig. 4e, f. With pre-exposure to DZO, AChE inhibition by MO was affected by the presence of PON1 (Fig. 3c, d), but there was no difference ($p = 0.13$) in modulation between the $hPON1_{Q192}$ and $hPON1_{R192}$ alloforms (Fig. 3e, f). In contrast, with pre-exposure to CPO, AChE inhibition by MO was affected by both the presence of PON1 (Fig. 2c, d) and by the $hPON1_{Q192R}$ polymorphism (Fig. 2e, f). With CPO pre-exposure, $hPON1_{Q192}$ mice had significantly ($p < 0.02$) greater inhibition of AChE by MO than did the $hPON1_{R192}$ mice (Fig. 2e, f). These results are consistent with a higher catalytic efficiency of CPO hydrolysis by the $hPON1_{R192}$ alloform

compared to the $hPON1_{Q192}$ alloform, and with their equivalent catalytic efficiencies of DZO hydrolysis (Li et al. 2000).

8 Conclusions

Clearly, PON1 status modulates the interactive toxicity of OP compounds. We demonstrated 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. The degree to which CPO, DZO, or PO inhibited CaE was predictive of their degree of potentiation of MO toxicity. Whereas PON1 had no affect on the potentiation of MO toxicity by PO, the absence of PON1 significantly increased the potentiation of MO toxicity by both CPO and DZO. These data indicate that interindividual differences in plasma PON1 levels would be important for determining sensitivity to mixed exposures involving diazinon/DZO and pesticides detoxified by the CaEs. Plasma PON1 levels are highly variable not just among individuals, but during development as well (Cole et al. 2003; Furlong et al. 2006). For mixed exposures involving chlorpyrifos/CPO, both plasma PON1 levels and $hPON1_{Q192R}$ genotype would be important determinants of sensitivity. Differences in potentiation of MO toxicity were observed between mice expressing $hPON1_{Q192}$ and $hPON1_{R192}$ with pre-exposure to CPO, but not DZO or PO.

The differences in genotype-modulation of potentiation among OP compounds are consistent with the catalytic efficiencies of the $hPON1_{Q192}$ and $hPON1_{R192}$ alloforms. Thus, PON1 status can have impacts on the detoxication of chemicals that are not direct PON1 substrates. Presumably, this modulation of OP mixture toxicity by PON1 would be relevant for not only MO, but also for other compounds that are detoxified or bioactivated by CaEs, including drugs, pro-drugs, pyrethroid insecticides, and other OP compounds (Abernathy and Casida, 1973; Gaughan et al. 1980; Godin et al. 2007; Choi et al. 2004; Wheelock et al. 2005).

Of particular relevance to the toxicity of OP mixtures is a study by Lu et al. (2006) that measured pesticide metabolites in the urine of children, with the most commonly-occurring metabolites being malathion dicarboxylic acid (MDA, a metabolite of malathion), and 3,5,6-trichloro-2-pyridinol (TCPY, a metabolite of chlorpyrifos). Newborns have very low levels of PON1 (Cole et al. 2003), and would be particularly susceptible to the interactive toxicity of OP mixtures. In the case of co-exposure to malathion and chlorpyrifos, children homozygous for $hPON1_{Q192}$ represent a particularly susceptible population for both AChE inhibition by chlorpyrifos and for the interactive effects on malathion toxicity. Children of farm workers face additional risk due to multiple pathways of exposure and proximity to sources of OPs (Fenske et al. 2005).

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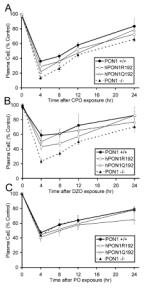

Fig. 1.

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). Reproduced from Jansen et al. (2009) with permission

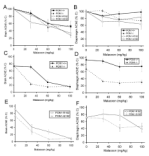

Fig. 2.

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 hours later by MO exposure (**c**, **d**, **e**, **f**). AChE was measured in the brain (**a**, **c**, **e**) and diaphragm (**b**, **d**, **f**) 4 hours following the MO exposure. Results represent the mean ± SEM (n = 4). Reproduced from Jansen et al. (2009) with permission

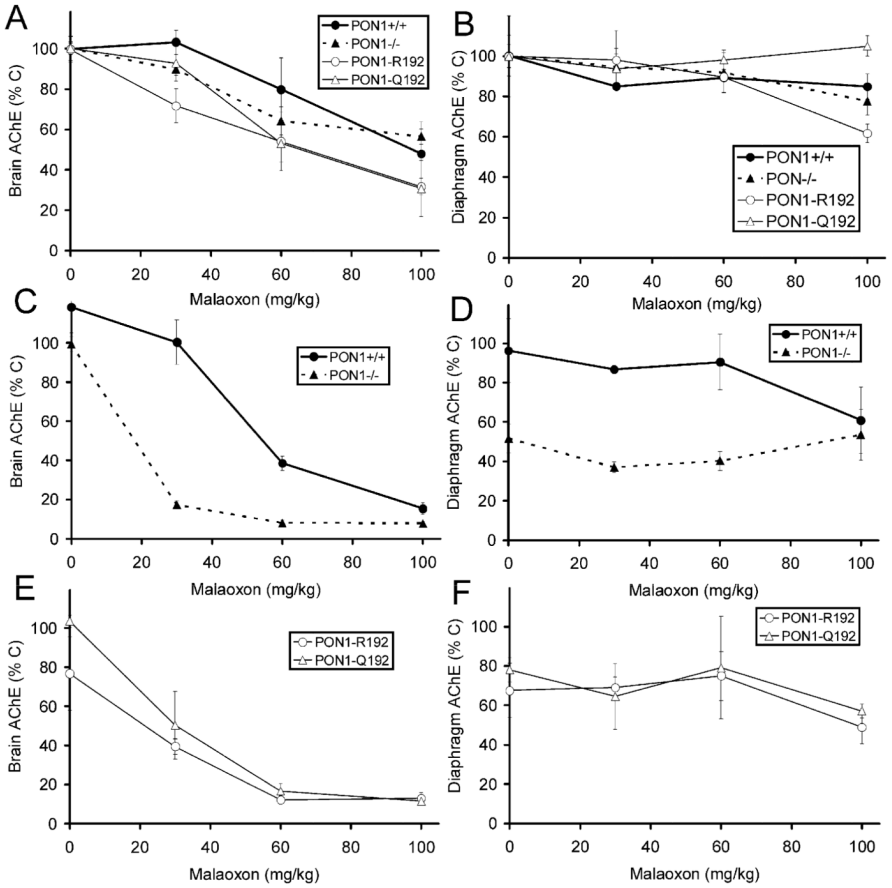

Fig. 3.

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 hours later by MO exposure (**c**, **d**, **e**, **f**). AChE was measured in the brain (**a**, **c**, **e**) and diaphragm (**b**, **d**, **f**) 4 hours following the MO exposure. Results represent the mean ± SEM (n = 4). Reproduced from Jansen et al. (2009) with permission

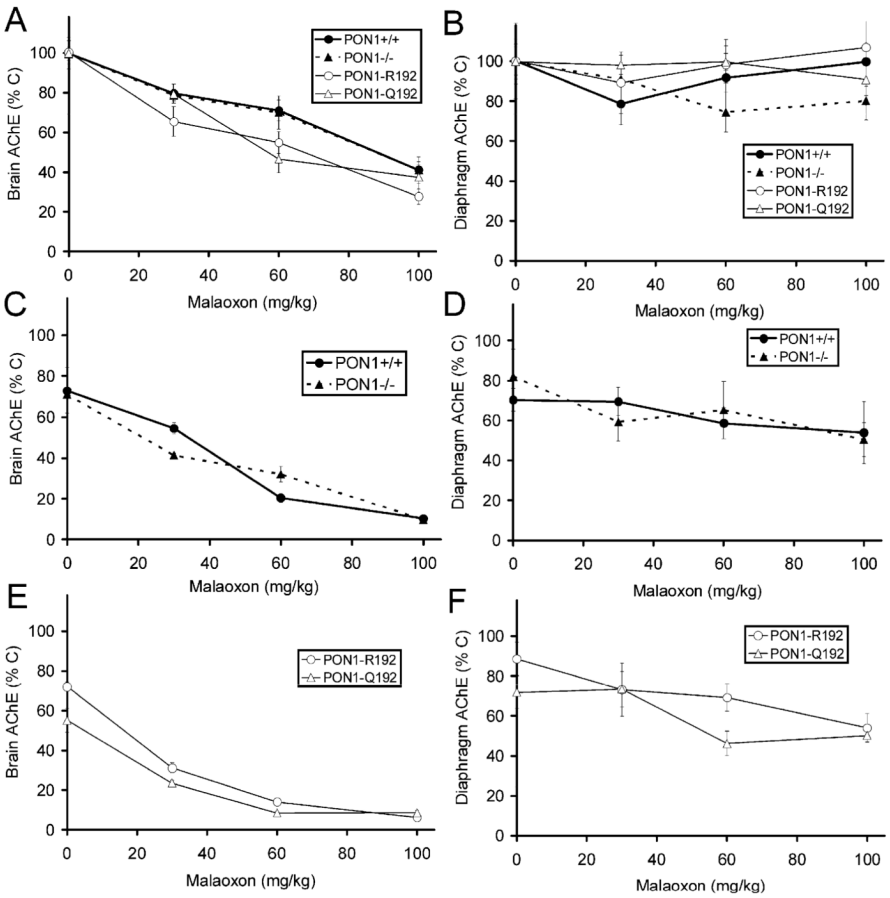

Fig. 4.

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 hours later by MO exposure (**c**, **d**, **e**, **f**). AChE was measured in the brain (**a**, **c**, **e**) and diaphragm (**b**, **d**, **f**) 4 hours following the MO exposure. Results represent the mean ± SEM (n = 4). Reproduced from Jansen et al. (2009) with permission

Table 1

In vitro IC_{50} values of CPO, DZO, PO, and MO for plasma and liver CaE and brain AChE

	Organophosphorus insecticide (nM)[a]			
Tissue	**CPO**	**DZO**	**PO**	**MO**
Plasma (CaE)	33.7 ± 8.3	16.6 ± 4.2	18.7 ± 2.1	5976.0 ± 495
Liver (CaE)	4.3 ± 0.8	3.9 ± 0.6	1.1 ± 0.3	308.8 ± 29.2
Brain (AChE)	14.0 ± 1.9	96.9 ± 7.6	9.5 ± 1.3	74.6 ± 7.8

[a] IC_{50} values (mean ± SEM). Data from Jansen et al. (2009) with permission

Table 2

Serum PON1 levels in the experimental mice [a]

	hPON1$_{Q192}$	**hPON1$_{R192}$**	***PON1*$^{+/+}$**	***PON1*$^{-/-}$**
AREase (Units/ml)	76.49 ± 3.65	53.07 ± 3.50	65.42 ± 3.00	13.60 ± 1.01
DZOase (Units/ml)	10.95 ± 0.60	4.93 ± 0.19	6.42 ± 0.61	0.06 ± 0.02

[a] Data from Jansen et al. (2009) with permission