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Catalytic efficiency determines the *in-vivo* efficacy of PON1 for detoxifying organophosphorus compounds

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Human paraoxonase (PON1) is a polymorphic, high-density lipoprotein (HDL)-associated esterase that hydrolyzes the toxic metabolites of several organophosphorus (OP) insecticides and nerve agents. The activity polymorphism is determined by a Gln/Arg (Q/R) substitution at position 192. Injection of purified PON1 protects animals from OP poisoning. In the present study, we investigated the *in-vivo* function of PON1 for detoxifying organophosphorus insecticides in *PON1*-knockout mice that were challenged via dermal exposure with diazoxon, diazinon and paraoxon. *PON1*-knockout mice were extremely sensitive to diazoxon. Doses (2 and 4 mg/kg) that caused no cholinesterase (ChE) inhibition in wild-type mice were lethal to the knockout mice, which also showed slightly increased sensitivity to the parent compound diazinon. Surprisingly, these knockout mice did not show increased sensitivity to paraoxon. *In-vitro* assays indicated that the $PON1_{R192}$ isoform hydrolyzed diazoxon less rapidly than did the $PON1_{Q192}$ isoform. *In-vivo* analysis, where *PON1*-knockout mice received the same amount of either $PON1_{192}$ isoform via intraperitoneal (i.p.) injection 4 h prior to exposure, showed that both isoforms provided a similar degree of protection against diazoxon, while $PON1_{R192}$ conferred better protection against chlorpyrifos–oxon than $PON1_{Q192}$. Injection of purified rabbit PON1 or either human $PON1_{192}$ isoform did not protect *PON1*-knockout mice from paraoxon toxicity, nor did over-expression of the human $PON1_{R192}$ transgene in wild-type mice. Kinetic analysis of the two human $PON1_{192}$ isoforms revealed that the catalytic efficiency (V_{max}/K_m) determines the *in-vivo* efficacy of PON1 for organophosphorus detoxication. The results indicate that PON1 plays a major role in the detoxication of diazoxon and chlorpyrifos oxon but not paraoxon. Pharmacogenetics 10:767–779 © 2000 Lippincott Williams & Wilkins

Keywords: PON1, diazinon, diazoxon, paraoxon, chlorpyrifos.

Introduction

Organophosphorus insecticides act by inhibiting acetylcholinesterase (AChE) in the central and peripheral nervous systems. Most organophosphorus insecticides are produced in the form of phosphorothioates, which are weak inhibitors of AChE, and must be activated to the toxic oxygen analogs (oxons) by cytochromes P450 (Murphy, 1980; Ecobichon, 1996). There are several potentially important pathways for detoxifying organophosphorus compounds. First, a number of toxic oxons are hydrolyzed by paraoxonase (PON1) in liver and plasma (Aldridge, 1953; Sultatos & Murphy, 1983; Butler *et al.*, 1985). Second, the toxic oxons can be inactivated by binding to non-target enzymes such as carboxylesterase (Clement, 1984; Chambers *et al.*, 1990; Maxwell, 1992) and butyrylcholinesterase (Broomfield *et al.*, 1991; Wolfe *et al.*, 1992). Third, cytochrome P450s also detoxify phosphorothioates as well as their oxon forms by removing their aryl or alkyl groups (Kamataki *et al.*, 1976). The relative importance of these detoxication pathways may differ among organophosphorus compounds, tissues, species and individuals. These differences probably contribute to the

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differential toxicity of each organophosphorus compound.

Paraoxonase (PON1) is an arylesterase predominantly synthesized in liver (Hassett *et al.*, 1991). It circulates in plasma as a component of HDL particles (Blatter *et al.*, 1993). *In-vitro* studies have shown that PON1 hydrolyzes arylesters and the toxic oxons of several organophosphorus insecticides, including parathion, chlorpyrifos and diazinon, as well as the nerve agents soman and sarin (Furlong *et al.*, 1989; Smolen *et al.*, 1991; Davies *et al.*, 1996). Despite its broad substrate specificity, PON1 exhibits different rates of hydrolysis and substrate affinity for specific organophosphorus compounds (Furlong *et al.*, 1989; Davies *et al.*, 1996; Pond *et al.*, 1998). Several lines of evidence have suggested that PON1 is important for *in-vivo* organophosphorus detoxication. Species such as birds with little or no plasma PON1 activity are very susceptible to organophosphorus poisoning (Brealey *et al.*, 1980). Rabbits that have the highest PON1 activity among mammals are quite resistant to organophosphorus toxicity (McCollister *et al.*, 1974; Costa *et al.*, 1987; Furlong *et al.*, 1989). More direct evidence comes from animal model studies. Injection of purified PON1 into rodents provided protection against exposure to toxic oxons as well as some protection against exposure to the parent compounds (Main, 1956; Costa *et al.*, 1990; Li *et al.*, 1993, 1995). More recent studies with *PON1*-knockout mice demonstrated that PON1 is critical for the *in-vivo* detoxication of chlorpyrifos–oxon (Shih *et al.*, 1998). Preliminary evidence showed that *PON1*-knockout mice also demonstrate an increased sensitivity to diazoxon but surprisingly, not to paraoxon (Li, 1999).

Human plasma PON1 displays a large variation in activity levels between individuals. Both *PON1* genotype and phenotype (i.e. PON1 status) contribute to this large degree of variability. The genotype of *PON1* responsible for the substrate-dependent polymorphism is determined by a Gln/Arg (Q/R) substitution at position 192. *In-vitro* assays indicated that the $PON1_{R192}$ isoform hydrolyzed paraoxon more rapidly but diazoxon more slowly than the $PON1_{Q192}$ isoform (Humbert *et al.*, 1993; Davies *et al.*, 1996; Richter & Furlong, 1999). There is also at least a 13-fold variation in enzyme levels among individuals with the same genotype (Furlong *et al.*, 1989; Davies *et al.*, 1996; Richter & Furlong, 1999). The activity polymorphism at position 192, together with the large variability in PON1 levels between individuals, results in large potential individual differences in the ability to detoxify organophosphorus compounds metabolized via the cytochrome P450/PON1 pathway. It should be noted, however, that the different rates of hydrolysis of specific substrates by the human $PON1_{192}$ isoforms have only been determined *in vitro* under non-physiological conditions. The question of whether the reported *in-vitro* rate differences translate into differences of *in-vivo* sensitivities has not yet been explored. The *PON1*-knockout mice provide an important animal model with which to address this question. The lack of endogenous *PON1* expression, both in the plasma and liver, in these animals allows for the determination of the significance of plasma PON1 in organophosphorus detoxication *in vivo*. Moreover, it allows the reconstitution of plasma PON1 activity in the knockout mice by injection of each of the human $PON1_{192}$ isoforms, providing an *in-vivo* evaluation of the effects of the $PON1_{192}$ polymorphism on detoxication of organophosphorus compounds.

Materials and methods

Chemicals

Diazinon (*O,O*-diethyl-*O*-[2-isopropyl-4-methyl-6-pyrimidyl]-phosphorothioate; 98% purity), diazoxon (diazinon-*O*-analog; 96% purity) and chlorpyrifos–oxon (98% purity) were purchased from Chem Service (West Chester, PA, USA). Paraoxon (*O,O*-diethyl-*O*-*p*-nitrophenylphosphate) was obtained from Sigma (St Louis, MO, USA; 90% purity) or Chem Service (West Chester, PA, USA) (98.4% purity). All chemicals were potent neurotoxicants, thus, were handled with caution.

Animals

PON1-knockout mice of 96% C57BL/6J and 4% 129/SvJ genetic background were generated as described (Shih *et al.*, 1998). Their genotype was determined based on polymerase chain reaction (PCR) analysis and the phenotype was confirmed by PON1 activity assay. $PON1_{R192}$-transgenic (*HPON1R-Tg*) mice with C57BL/6J background were generated by microinjection of an 80 kb human genomic DNA fragment containing the human $PON1_{R192}$ allele with 12 kb of 5′ flanking and 43 kb of 3′ flanking sequences. Potential transgenic mice were identified by PCR analysis using a pair of primers specific for exon 4 of the human *PON1* gene. Mice homozygous for the normal *PON1* allele served as controls. All animals were housed in modified SPF (specific pathogen free) facilities with a 12 h dark–light cycle and free access to food and water. All protocols were approved by the Animal Care Committee at the University of Washington.

Purification of human PON1 Isoforms

Human PON1 isoforms were purified from pooled plasma of individuals homozygous for the $PON1_{R192}$

or $PON1_{Q192}$ allele. The *PON1* genotype of each unit of plasma was accurately inferred by two-dimensional enzyme activity assays (Richter & Furlong, 1999). Each $PON1_{192}$ isoform was purified following the described procedure (Furlong *et al.*, 1991) with modifications. Human plasma pretreated with $CaCl_2$ (final concentration, 10 mM) was mixed with Cibacron Blue-agarose resin (Sigma) in 3 M NaCl, 50 mM Tris-HCl, pH 8, 1 mM $CaCl_2$ and 5 μM EDTA. The volume of resin used is 1.5 times the volume of plasma used. The mixture was allowed to bind at room temperature. Unbound proteins were washed away with sufficient equilibration buffer to bring the A_{280} of the eluate to less than 0.05. The Blue-agarose column was then washed with two resin bed volumes of 20 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$ to reduce the salt concentration. The PON1 activity was eluted with the same buffer containing 0.1% deoxycholate. The fractions containing PON1 activity were collected for the following DEAE chromatography steps. A DEAE Bio-Gel A (Bio-Rad Laboratories, Hercules, CA, USA) column was equilibrated with 20% glycerol, 25 mM Tris-HCl, pH 8, 1 mM $CaCl_2$, 5 μM EDTA and 0.1% Emulgen 911 (Kao Co., Japan). The volume of DEAE resin used was 200 ml/l of plasma. The pooled fractions were adjusted to equilibration buffer conditions and loaded onto the column. The absorbed protein was washed with three resin bed volumes of equilibration buffer. The column was then eluted batchwise, first with 45 mM NaCl, then 200 mM NaCl in equilibration buffer. The 200 mM eluate contained PON1. The next DEAE Bio-Gel A (100 ml per starting litre of plasma) was equilibrated without detergent in 25 mM Tris-HCl, pH 7.5, 1.0 mM $CaCl_2$. The sample was diluted in two volumes of equilibration buffer, and loaded onto the column. The column was then washed with five volumes of equilibration buffer. The column was eluted batchwise with first 90 mM NaCl, then 400 mM NaCl in equilibration buffer. The 400 mM elution fractions contained PON1. Fractions with highest PON1 activity were pooled and concentrated to the desired arylesterase activity level.

Treatments

To determine the dose–response curves for organophosphorus compounds, the chemicals were dissolved in acetone (0.67 μl per g of body weight) and applied on the shaved (4 cm^2) back of the mice. Control animals received acetone only. Four hours after treatment, mice were sacrificed and their brains and diaphragms collected and stored at −70 °C before assaying for cholinesterase (ChE) activity. To determine the effect of the human $PON1_{192}$ polymorphism on organophosphorus toxicity, mice were injected 4 h prior to organophosphorus exposure with either the $PON1_{R192}$ or PON_{Q192} (100–120 μl, i.p.) isoform, and then sacrificed 4 h after exposure. The amount of each isoform was standardized by the rate of hydrolysis of phenylacetate, a 'non-polymorphic' substrate. The 4-h time frames were chosen based on the time course of increased plasma PON1 activity in the knockout mice, which showed a peak increase in enzyme activity between 4 and 8 h after the injection of the human PON1 isoforms.

PON1 activity assays

Plasma PON1 activities towards phenylacetate, paraoxon, diazoxon and chlorpyrifos–oxon were determined as described previously (Furlong *et al.*, 1989; Richter & Furlong, 1999) at 2 M or 0.15 M NaCl solution. PON1 activity was expressed as U/l (U = μmol of substrate hydrolyzed per minute).

ChE activity assay

Brain and diaphragm ChE activities were assayed based on the method of Ellman *et al.* (1961) with minor modifications. Tissues were homogenized in ice-cold 0.1 M sodium phosphate buffer, pH 8. A fraction of tissue homogenate equal to 0.8 mg of brain or 5 mg of diaphragm was added to a 5 ml assay containing 0.1 M sodium phosphate, pH 8 and 0.1 mM of DTNB (5,5′-dithio-bis-nitrobenzoic acid). The kinetic assay was initiated by addition of acetylthiocholine (final concentration = 1 mM) and 1 ml of the reaction mix was continuously monitored for 10 min at room temperature. Absorbances were read at 412 nm in a Beckman DU-70 spectrophotometer. The amount of 5-thio-2-nitrobenzoate formed during the assay was calculated using an extinction coefficient of 13 600 M^{-1}/cm. ChE activity was expressed as U/g of wet tissue (U = μmol of acetylthiocholine hydrolyzed per minute).

Statistical analysis

Data were analysed by Student's *t*-test or ANOVA using Microsoft Excel Software to determine statistical significance. Results are given as mean ± SD.

Results

The consequence of low PON1 levels was examined by comparing organophosphorus sensitivities in *PON1*-knockout and *PON1*-hemizygous mice with wild-type mice. Plasma from *PON1*-knockout mice (*PON1*−/−) had no detectable hydrolytic activity towards paraoxon and diazoxon, and very limited chlorpyrifos–oxonase activity (Table 1). *PON1*-hemizygous mice (*PON1*+/−) had approximately 40% of plasma PON1 activity compared to wild-type mice (*PON1*+/+). In *PON1*+/+ and *PON1*+/−mice, there was a minor but statistically significant difference in

Table 1. Plasma PON1 levels in wild-type, *PON1* hemizygous and *PON1* null mice

	PON1+/+		*PON1*+/−		*PON1*−/−	
	Male (n = 23)	*Female* (n = 25)	*Male* (n = 24)	*Female* (n = 24)	*Male* (n = 25)	*Female* (n = 24)
Paraoxonase	232.4 ± 64.1	269.6 ± 48.8	99.9 ± 17.6	111.8 ± 20.0	Not detectable	Not detectable
Diazoxonase	3568.9 ± 550.5	4211.0 ± 964.4	1325.1 ± 326.1	1512.5 ± 273.2	Not detectable	Not detectable
Chlorpyrifos-oxonase	1921.8 ± 499.6	1863.1 ± 514.6	730.8 ± 220.3	744.2 ± 239.1	75.4 ± 24.5	81.0 ± 28.2

Paraoxonase, diazoxonase, and chlorpyrifos-oxonase activities were determined as described in Methods. PON1 levels are given in units/litre and values are expressed as mean ± SD. Units = μmol of hydrolysis product formed per min.

paraoxonase and diazoxonase activity between males and females; however, there was no significant gender difference in chlorpyrifos–oxonase activity. The knockout mutation of the *PON1* gene also resulted in the elimination of PON1 activity in liver as previously shown (Furlong *et al.*, 2000). *PON1*−/− mice had neither paraoxonase nor diazoxonase, but a small amount of chlorpyrifos–oxonase activity in liver. *PON1*+/− mice had 40% of the liver PON1 activity found in *PON1*+/+ mice (data not shown).

Due to the lack of both plasma and liver PON1 activity, the knockout mice showed dramatically increased sensitivity to diazoxon. The lowest dose of diazoxon (1 mg/kg) resulted in 80% inhibition of brain ChE in *PON1*−/− mice but had no effect on *PON1*+/+ mice (Fig. 1a). The higher doses (2 and 4 mg/kg) were lethal to *PON1*−/− mice, killing five animals out of eight, but caused no significant inhibition of brain ChE in *PON1*+/+ mice. Diazoxon exposure resulted in greater inhibition of ChE activity in diaphragm than in brain. *PON1*+/+ mice showed no inhibition in brain while their diaphragm ChE levels were decreased by 50% at the doses of 1 and 2 mg/kg, and by 84% at the highest dose (Fig. 1b). Diaphragm ChE activity of *PON1*−/− mice was inhibited severely (up to 94–97%) by diazoxon exposure. There were significant differences in inhibition of diaphragm ChE activity by diazoxon between *PON1*+/+ and *PON1*−/− mice in all dose groups. *PON1*-hemizygous mice showed an intermediate sensitivity to diazoxon exposure. *PON1*+/− mice in all treatment groups were more resistant to diazoxon than *PON1*−/− mice (Fig. 1a,b). Compared to wild-type mice, *PON1*+/− mice had an increased sensitivity in brain when challenged with 4 mg/kg of diazoxon but showed no differences at lower doses.

Although *PON1* null mice were extremely sensitive to diazoxon, they showed only slightly increased sensitivity to the parent compound diazinon. When *PON1*−/− mice were treated with three different doses of diazinon, only the group that received 8 mg/kg of diazinon exhibited a higher sensitivity than *PON1*+/+ and *PON1*+/− mice in both brain and diaphragm (Fig. 1c,d). The highest dose (16 mg/kg) of diazinon caused a significantly higher ChE inhibition in the diaphragm of *PON1*−/− mice but not the brain, although one animal in this group died at 160 min after exposure.

The most surprising observation was that *PON1*-knockout mice did not show an increased sensitivity to paraoxon, the substrate for which the enzyme was named. *PON1*+/+, *PON1*+/− and *PON1*−/− mice were challenged with a wide range of doses of paraoxon, from a low dose (0.15 mg/kg) which inhibited brain ChE by only approximately 15% to a

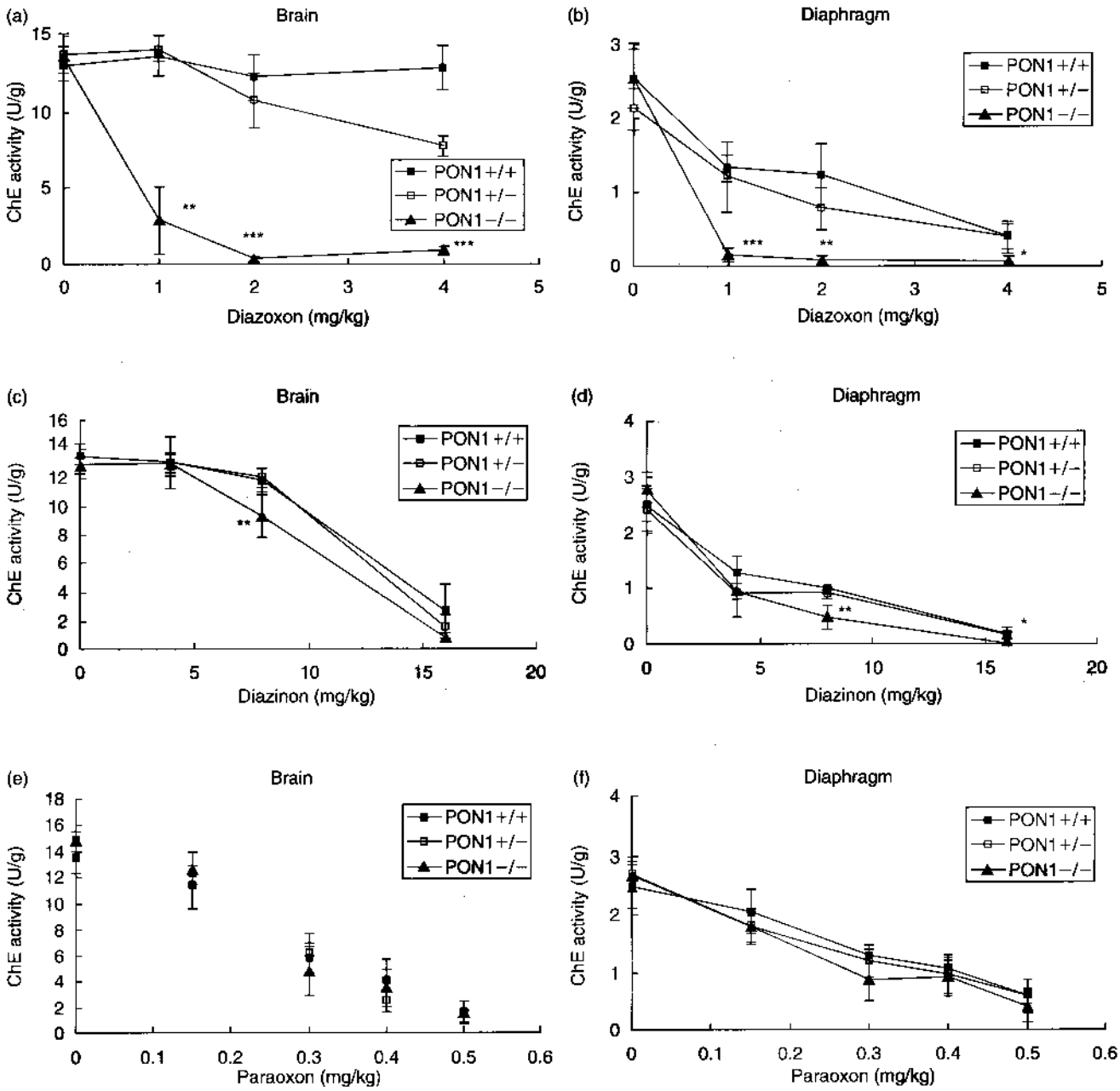

Fig. 1. Effects of diazoxon (a,b), diazinon (c,d) and paraoxon (e,f) on cholinesterase activity in *PON1* null mice. Cholinesterase (ChE) activity in brain (a,c,e) and diaphragm (b,d,f) samples from wild-type (*PON1*+/+), *PON1* hemizygous (*PON1*+/−) and *PON1* null mice (*PON1*−/−) exposed to the indicated doses of the organophosphorus compound. The organophosphorus compound was given by dermal exposure and animals were sacrificed 4 h after treatment. Results (a–d) represent the mean ± SD ($n = 4$; two males and two females). For paraoxon toxicity test (e,f), results were obtained from two independent experiments ($n = 4$–8; with equal numbers of each sex). Data were analysed by ANOVA for statistical significance. Asterisks indicate significant difference from both *PON1*+/+ and *PON1*+/− mice: $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$.

high dose (0.5 mg/kg) which resulted in over 90% reduction of ChE activity (Fig. 1e,f). In all dose groups, however, there were no significant differences in ChE activity levels among the three genotypes of mice. *PON1* null mice did not show any increased sensitivity to paraoxon, in spite of the fact that they had no paraoxonase activity in either the plasma or liver compartments.

To determine whether *PON1* null mice are more sensitive to the toxicity of general organophosphorus insecticides, we treated the animals with demeton-S-methyl (DSM), which is not a substrate for PON1.

DSM is an organophosphorus insecticide with a structure similar to malathion. Unlike paraoxon, diazoxon or chlorpyrifos–oxon, DSM is a mercapto ester (-P-S-R) instead of an oxygen ester (-P-O-R) and therefore, it cannot be hydrolyzed by PON1 (Geldmacher-von Mallinckrodt & Diepgen, 1988). We found no differences in ChE activity between the knockout and wild-type mice (Fig. 2) when exposed to DSM. The results indicated that *PON1* null mice were not susceptible to general organophosphorus compounds but only to organophosphorus compounds that are good substrates for PON1.

The availability of the *PON1*-knockout mice, devoid of PON1 activity in both the plasma and liver compartments, provided the opportunity to reconstitute the plasma compartment alone with injected, purified PON1 from different sources. This approach allows for the determination of the efficacy of the two human $PON1_{192}$ isoforms or other PON1s (e.g. rabbit) in organophosphorus detoxication under physiological conditions.

First, we restored plasma PON1 activity in the knockout mice by injection of purified rabbit enzyme (Furlong *et al.*, 1991) prior to exposure. The enzyme injection gave the knockout mice a plasma paraoxonase activity level that was eight-fold higher than wild-type mice (2088 ± 647 versus 252 ± 59 U/l). In contrast to the protection previously reported in rats (Main, 1956; Costa *et al.*, 1990), injected purified rabbit PON1 did not protect the *PON1*-knockout mice from paraoxon poisoning (Fig. 3a) despite the high activity of the rabbit enzyme for

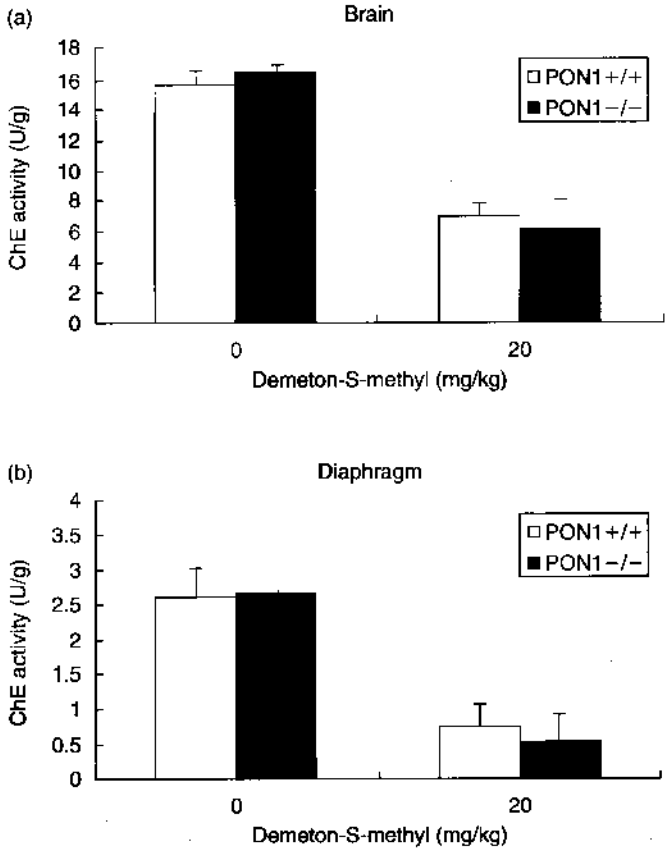

Fig. 2. Cholinesterase (ChE) activity in brain (a) and diaphragm (b) samples from wild-type (*PON1*+/+) and *PON1* null mice (*PON1*−/−) treated with demeton-*S*-methyl. Demeton-*S*-methyl is a toxic organophosphorus-oxon that is not hydrolyzed by PON1. The organophosphorus was given by dermal exposure and animals were sacrificed 4 h after treatment. Results represent the mean ± SD ($n = 3$–10). Data were analysed by Student's *t*-test for statistical significance at the level of $P < 0.05$.

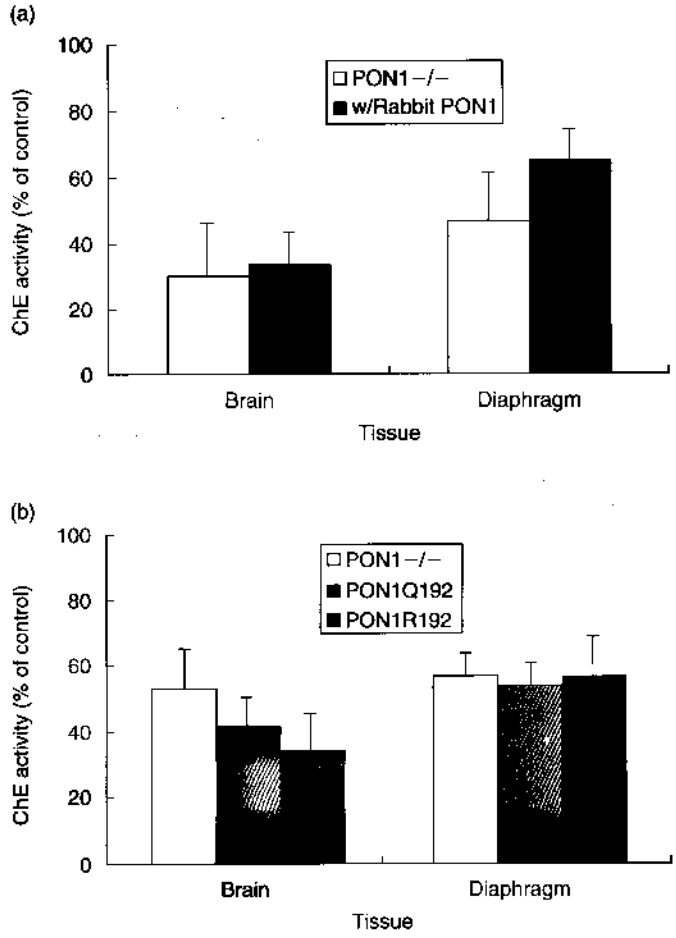

Fig. 3. Effects of paraoxon on ChE activity in *PON1* null mice that received (a) purified rabbit PON1 or (b) purified human $PON1_{192}$ isoforms. Mice were injected i.p. with (a) 8.5 U (paraoxonase activity) of purified rabbit plasma enzyme; (b) 3 U of $PON1_{Q192}$ or 15 U of $PON1_{R192}$ isoform before paraoxon treatment (0.3 mg/kg; dermal). The amount of injected $PON1_{Q192}$ and $PON1_{R192}$ isoforms was standardized by arylesterase activity (732 U of $PON1_{Q192}$ and 720 U of $PON1_{R192}$). Results are expressed as percentage of control animals (acetone-treated only) and represent the mean ± SD ($n = 4$–5).

paraoxon hydrolysis.

We next injected *PON1*-knockout mice with each of the purified human $PON1_{192}$ isoforms. *In-vitro* assays have shown that $PON1_{R192}$ isoform has much higher rates of paraoxon hydrolysis (approximately five-fold) compared to the $PON1_{Q192}$ isoform (reviewed in Geldmacher-von Mallinckrodt & Diepgen, 1988) and it has been assumed for many years that the human $PON1_{R192}$ isoform would be much more protective against parathion/paraoxon toxicity than the $PON1_{Q192}$ isoform. However, neither injected human $PON1_{192}$ isoform increased the resistance to paraoxon in the *PON1*-knockout mice (Fig. 3b). In addition, a newly established transgenic mouse line that carries the human $PON1_{R192}$ allele was tested for its sensitivity to paraoxon. The $PON1_{R192}$-transgenic (*HPON1R-Tg*) mice express human $PON1_{R192}$ isoform over the endogenous mouse PON1 as shown by immunoblotting (Fig. 4), resulting in a 3.5-fold increase in plasma paraoxonase activity level (862 ± 149 versus 246 ± 22 U/l). The *HPON1R-Tg* mice showed a similar sensitivity as wild-type mice to paraoxon exposure (Fig. 5).

Our earlier studies with *in-vitro* assays showed that individuals homozygous for the $PON1_{Q192}$ allele have higher average diazoxonase activity than individuals homozygous for the $PON1_{R192}$ allele, leading to the hypothesis that the two isoforms may provide different degrees of protection against diazoxon (Davies *et al.*, 1996; Richter & Furlong, 1999). We therefore tested the *in-vivo* efficacy of the purified human $PON1_{192}$ isoforms for diazoxon detoxication. *PON1* null mice received the same amount of each of the purified human $PON1_{192}$ isoforms by i.p. injection 4 h before exposure to diazoxon. The amount of each isoform was standardized by arylesterase activity (i.e. the rate of phenylacetate hydrolysis, a non-polymorphic substrate). Both isoforms increased the plasma diazoxonase activity to a similar level in the knockout mice (Fig. 6a). After exposure to diazoxon, mice that received human PON1

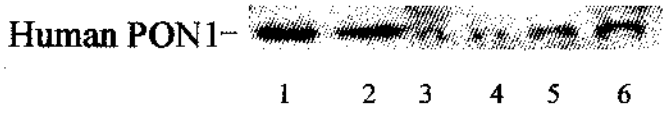

Fig. 4. Human PON1 levels in $PON1_{R192}$-transgenic (*HPON1R-Tg*) mice as determined by immunoblotting. Two hundred ng (lane 1), 100 ng (lane 2) of purified human PON1, and 1 µl of each of plasma samples from wild-type (lanes 3 and 4) and *HPON1R-Tg* (lanes 5 and 6) mice were fractionated by polyacrylamide gel electrophoresis under reducing condition and transferred onto nitrocellulous membrane. The blot was probed with a rabbit polyclonal antibody against human PON1 (1 : 500 dilution).

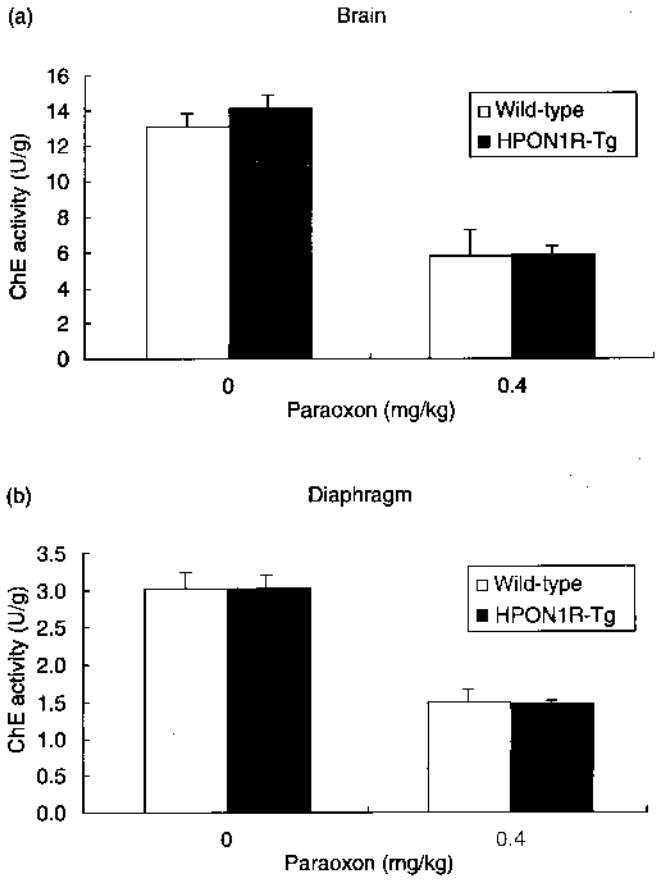

Fig. 5. Inhibition of brain (a) and diaphragm (b) ChE activity in $PON1R_{192}$-transgenic (*HPON1R-Tg*) mice following dermal exposure to paraoxon (0.4 mg/kg). The hemizygous transgenic mice carry the human $PON1R_{192}$ allele over the endogenous mouse *PON1* gene. Paraoxonase activity was 246 ± 22 U/l ($n = 3$) in wild-type mice and 862 ± 149 U/l ($n = 4$) in transgenic mice.

were significantly protected from ChE inhibition. However, there was no significant difference in the extent of protection provided by either of the $PON1_{192}$ isoforms (Fig. 6b). Although the $PON1_{Q192}$ isoform hydrolyses diazoxon faster than the $PON1_{R192}$ isoform *in vitro*, the latter provided the same, if not better, protection *in vivo*.

A major discrepancy between the conditions of the *in-vitro PON1* diazoxonase assays and *in-vivo* injection studies was the high concentration NaCl buffer used in the *in-vitro* assays. For population studies, the high salt condition (2 M NaCl) provides a better resolution of $PON1_{192}$ phenotypes than assays run at lower salt concentrations. The conditions of the *in-vitro* assays do not reflect physiological conditions, however, in which the concentration is approximately 150 mM for Na^+ and 110 mM for Cl^-. To examine why the $PON1_{Q192}$ isoform had a higher activity against diazoxon *in vitro* but did not offer more protection *in*

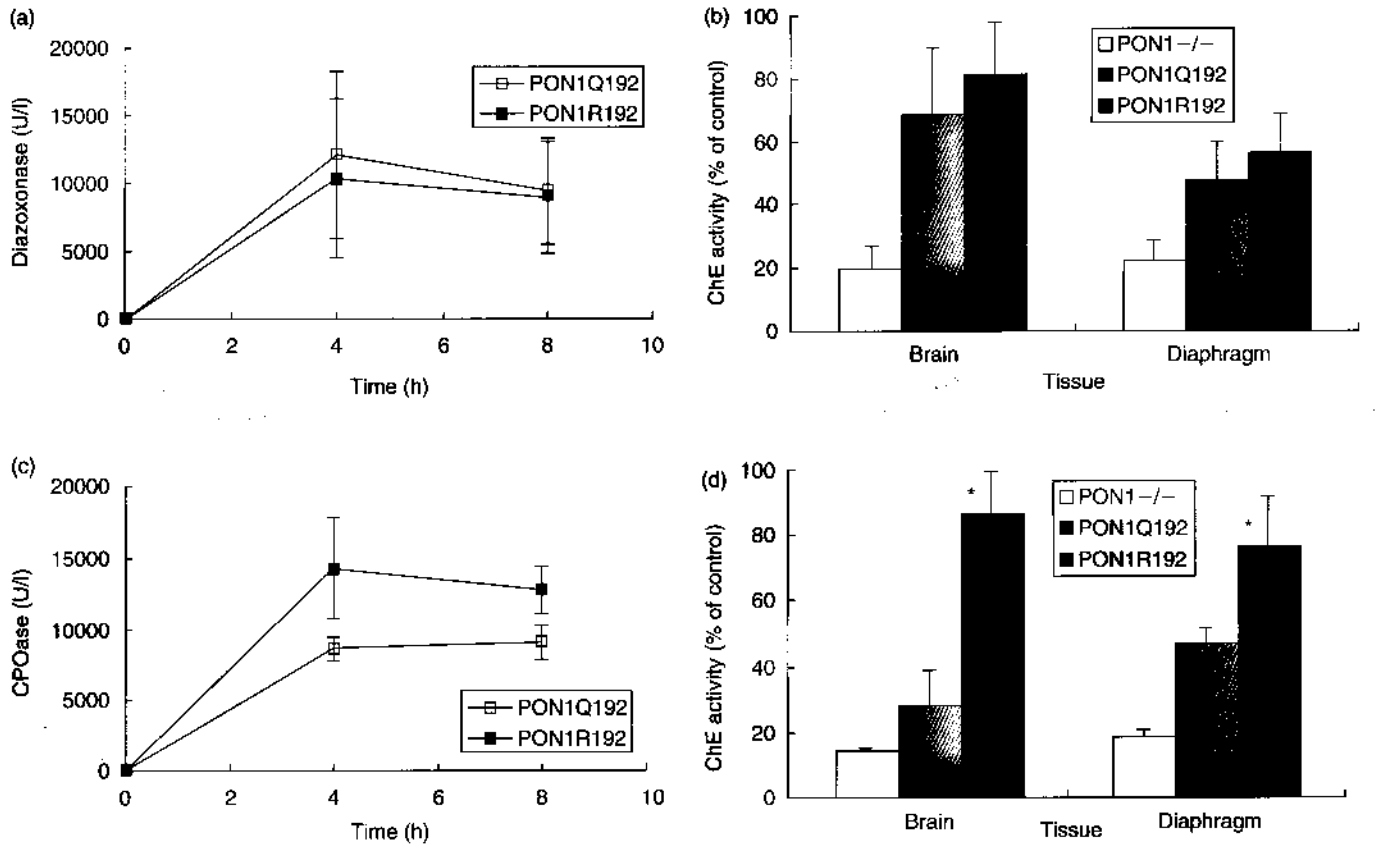

Fig. 6. Protection against diazoxon (a,b) and chlorpyrifos–oxon (c,d) in *PON1* null mice by injection of purified human PON1 isoforms. The amount of the two purified PON1 isoforms was first standardized by arylesterase activity. Mice were injected i.p. with an equal amount (a, 850 U; b, 916 U of arylesterase activity) of each of the human PON1 isoforms. Four hours following the injection, mice were challenged with diazoxon (1 mg/kg; dermal) or chlorpyrifos–oxon (2 mg/kg, dermal) and sacrificed 4 h after exposure. Time course of plasma diazoxonase (a) and chlorpyrifos–oxonase (c) activity in *PON1* null mice was measured following the injection of human PON1 isoforms. Inhibition of brain and diaphragm ChE activity was determined and shown in (b) and (d). Results are expressed as percentage of control animals (acetone-treated only) and represent the mean ± SD (n = 5–8, for diazoxon exposure; n = 4, for chlorpyrifos–oxon exposure). *Represents a significant difference ($P < 0.01$) between the $PON1_{R192}$ and $PON1_{Q192}$-treated groups.

vivo, we examined the effects of NaCl on in-vitro diazoxon hydrolysis by the two human $PON1_{192}$ isoforms. The $PON1_{Q192}$ isoform was stimulated by high NaCl concentrations whereas the $PON1_{R192}$ isoform was differentially inhibited by high salt (Fig. 7a). Thus, although the $PON1_{Q192}$isoform hydrolyzed diazoxon faster than the $PON1_{R192}$ isoform at 2 M NaCl, under more physiological conditions (approximately 150 mM), both $PON1_{192}$ isoforms exhibited more similar rates of diazoxon hydrolysis.

We next examined the *in-vivo* effects of the two isoforms on chlorpyrifos–oxon detoxication. Our previous studies had shown that the $PON1_{R192}$ isoform hydrolyzed chlorpyrifos–oxon at a slightly higher rate than the $PON1_{Q192}$ isoform (Furlong *et al.*, 1989; Davies *et al.*, 1996; Richter & Furlong, 1999). Unlike the case with diazoxon, we found a consistent result between *in-vitro* hydrolysis rates and *in-vivo* detoxication of chlorpyrifos–oxon by the two isoforms. *PON1* null mice that received the $PON1_{R192}$ isoform had a 1.7-fold higher plasma chlorpyrifos–oxonase activity than those that received the $PON1_{Q192}$ isoform (Fig. 6c). The $PON1_{R192}$ isoform also provided more protection against chlorpyrifos–oxon (Fig. 6d). Two of the mice that received the $PON1_{Q192}$ isoform developed clinical signs of organophosphorus toxicity after exposure, including weakness and hypothermia, whereas no signs were observed in mice that received the $PON1_{R192}$ isoform. Analysis of salt dependence of the two isoforms showed that regardless of NaCl concentration used in assay, $PON1_{R192}$ hydrolyses chlorpyrifos–oxon more rapidly than $PON1_{Q192}$ (Fig. 7b).

The varied results obtained with the PON1 injection experiments prompted us to examine the catalytic efficiency of each purified human plasma $PON1_{192}$

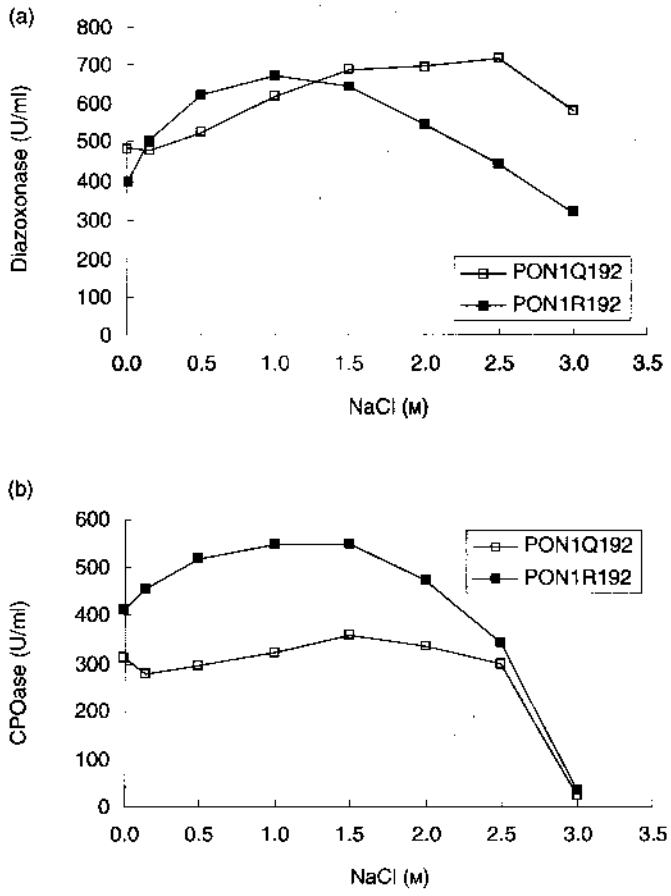

Fig. 7. Effects of NaCl concentration on rate of diazoxon hydrolysis (a) and chlorpyrifos–oxon hydrolysis (b) by the two human $PON1_{192}$ isoforms. Enzyme activity was determined using the purified human $PON1_{R192}$ and $PON1_{Q192}$ isoforms as indicated.

isoform under more physiological conditions than used for the population activity distribution assays. Since we had previously observed some differences in determined K_m values with different lots of substrate, we assayed both $PON1_{192}$ isoforms at the same time with the same substrate solutions. The substrate dependency of hydrolysis rates of paraoxon, diazoxon and chlorpyrifos–oxon are shown in Fig. 8 along with reciprocal plot determinations of V_{max} and K_m values. The data are summarized in Table 2. The catalytic efficiencies for hydrolysis of paraoxon by either $PON1_{192}$ isoform was significantly lower than those for hydrolysis of diazoxon or chlorpyrifos–oxon, even though the efficiency for $PON1_{R192}$ was more than eight times greater than that of the $PON1_{Q192}$ isoform. The higher rate of diazoxon hydrolysis by the $PON1_{Q192}$ isoform is compensated by the better affinity of the $PON1_{R192}$ isoform for diazoxon, resulting in nearly identical catalytic efficiencies of the two isoforms for diazoxon hydrolysis, in agreement with the protection observed in the *in-vivo* injection experiments. The better catalytic efficiency of $PON1_{R192}$ for chlorpyrifos–oxon hydrolysis is also in agreement with the increased *in-vivo* protection provided by injection of this isoform into the *PON1* knockout mice.

Discussion

The finding that *PON1* null mice are extremely sensitive to diazoxon (Fig. 1a,b) is consistent with our previous study on chlorpyrifos–oxon (Shih *et al.*, 1998). The very high sensitivity to both oxons indicates that other detoxifying pathways, such as binding to carboxylesterase or butyrylcholinesterase, do not provide adequate protection against diazoxon and chlorpyrifos–oxon in the knockout mice. These results lead us to conclude that hydrolysis by PON1 is a significant route for detoxication of both of these oxons *in vivo*. The different kinetic constants and catalytic efficiencies of PON1 for hydrolysis of these two substrates are in good agreement with the degree of protection provided to the *PON1* null mice by injection of the purified human $PON1_{192}$ isoforms.

PON1 null mice also show an increased sensitivity when exposed to certain doses of the parent compound diazinon (Fig. 1c,d); however, the increased sensitivity is much less than that observed for diazoxon. The parent thioate compound diazinon is metabolized through two major pathways involving the cytochrome P450 systems (Shishido *et al.*, 1972). One activates diazinon into diazoxon while the other degrades diazinon by cleavage of its aryl phosphate bond releasing pyrimidinol. Thus, the ratio of the oxon and pyrimidinol produced by cytochrome P450s is a key factor in determining the degree of toxicity of diazinon. The lower the rate of oxon produced, the less critical PON1 will be in the detoxication mechanism. Several P450 isozymes are involved in the metabolism of diazinon in rat liver, including constitutive CYP2C11 and CYP3A2, which catalyse more production of pyrimidinol than diazoxon (Fabrizi *et al.*, 1999). Together with our observations, it appears that more diazinon may be directly detoxified by the cytochrome P450s before being activated to the oxon. However, the phenobarbial inducible CYP2B1/2 is also involved in diazinon metabolism with preferential production of diazoxon (Fabrizi *et al.*, 1999). The importance of PON1 in diazoxon toxicity depends on P450 status as well as the level of oxon in the exposure. Oxon percentage in actual exposures is not insignificant and may range from relatively low values to as much as 80% or more of total residue (Yuknavage *et al.*, 1997).

It has been observed that PON1 is not efficient at

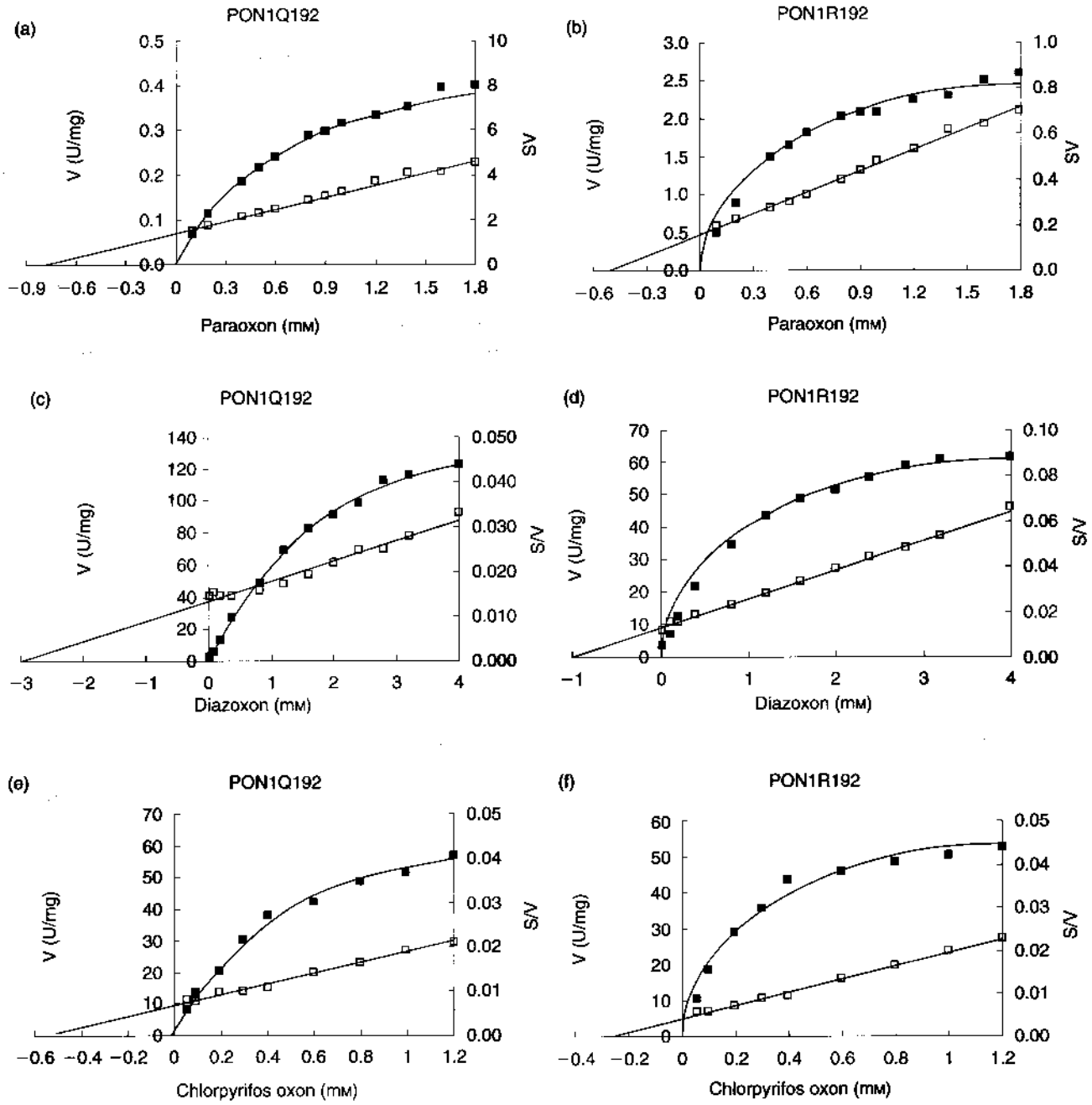

Fig. 8. Substrate dependence of each $PON1_{192}$ isoform as indicated with reciprocal plots (S/V versus S) for determinations of K_m and V_{max} values. (a,b) for paraoxon hydrolysis (c,d) for diazoxon hydrolysis and (e,f) for chlorpyrifos–oxon hydrolysis. All assays were run at 0.15 M NaCl as described in the Methods section in a Molecular Devices SPECTRAmax *PLUS* Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

hydrolyzing paraoxon at low, toxicologically relevant concentrations (Chambers *et al.*, 1994; Pond *et al.*, 1995), suggesting that PON1 may not degrade paraoxon efficiently *in vivo*. The evidence that *PON1* null mice are not more sensitive to paraoxon toxicity (Fig. 1e,f) supports this hypothesis. In addition, our previous studies showed that although mouse PON1 has a relatively poor efficiency for paraoxon hydrolysis, rabbit PON1 and the human $PON1_{R192}$ isoform have much better catalytic efficiencies for paraoxon (Furlong *et al.*, 2000). In fact, rats that were pretreated with purified rabbit PON1 became more resistant to paraoxon toxicity (Main, 1956; Costa *et al.*, 1990). The injection of rabbit PON1, however, did not protect the knockout mice from paraoxon poisoning (Fig. 3a). The results indicate that, after

Table 2. Kinetic analysis of substrate hydrolysis by purified human plasma $PON1_{192}$ isoforms

	Paraoxon hydrolysis		*Diazoxon hydrolysis*		*Chlorpyrifos-oxon hydrolysis*	
	$PON1_{Q192}$	$PON1_{R192}$	$PON1_{Q192}$	$PON1_{R192}$	$PON1_{Q192}$	$PON1_{R192}$
K_m (mM)	0.81	0.52	2.98	1.02	0.54	0.25
V_{max} (U/mg)	0.57	3.26	222	79	82	64
V_{max}/K_m	0.71	6.27	75	77	152	256

Paraoxonase, diazoxonase and chlorpyrifos-oxonase activities were determined at 0.15 M NaCl as described in the methods section. Catalytic constants were determined from a Woolf plot (concentration/velocity versus concentration) as shown in Fig. 8 and calculated from three independent measurements. Protein concentration of purified human $PON1_{192}$ isoforms were measured using the Pierce BCA Protein Assay (Rockford, IL, USA).

depletion of both plasma and liver paraoxonase activity, the reconstitution of the activity with rabbit PON1 only in the plasma compartment does not contribute significantly to paraoxon detoxication. The evidence that neither the injection of human $PON1_{R192}$ isoform (Fig. 3b), nor the expression of the *PON1*$_{R192}$ allele (Fig. 5) could protect animals from paraoxon poisoning, indicates the inability of the $PON1_{R192}$ isoform to detoxify paraoxon *in vivo*. It also suggests that other pathways, rather than hydrolysis by PON1, are primarily responsible for detoxifying paraoxon *in vivo*. One likely route is non-target binding to carboxylesterase in serum and liver (Chambers *et al.*, 1994; Pond *et al.*, 1995). This is not a highly efficient mechanism, however, since the B-type esterases are inactivated in this process and continued resistance is dependent on synthesis of new enzyme.

The results of our earlier studies had also indicated that the $PON1_{Q192}$ isoform hydrolyzes diazoxon more rapidly than does the $PON1_{R192}$ isoform (Davies *et al.*, 1996), suggesting that it might provide better *in-vivo* protection against exposure to diazoxon. In developing the assay conditions for clear resolution of the three PON1 genotypes, we ran the assays at 2 M NaCl. At this concentration, the $PON1_{R192}$ isoform is stimulated for paraoxon hydrolysis but inhibited for diazoxon hydrolysis. In contrast, 2 M NaCl has no effect on paraoxon hydrolysis by the $PON1_{Q192}$ isoform but stimulates its rate of diazoxon hydrolysis. The totally opposite response to NaCl of the two human isoforms makes it possible to clearly distinguish the three $PON1_{192}$ genotypes by plotting paraoxonase versus diazoxonase activity (Richter & Furlong, 1999). However, it is also important to understand how well PON1 hydrolyzes organophosphorus substrates under physiological conditions. The present results show that the $PON1_{R192}$ isoform provides equivalent protection against diazoxon compared to the $PON1_{Q192}$ isoform when injected into *PON1* null mice (Fig. 6b). When we examined the catalytic efficiencies of the two $PON1_{192}$ isoforms for diazoxon hydrolysis, it became clear that despite differences in K_m and V_{max} values, the overall efficiencies of the two isoforms for diazoxon hydrolysis was essentially equivalent, in agreement with the protection provided by injection of either isoform. On the other hand, the $PON1_{R192}$ isoform has a higher catalytic efficiency for the hydrolysis of chlorpyrifos–oxon and therefore, provides more protection *in vivo* (Fig. 6d).

It has been long proposed that PON1 may represent a risk factor for identifying individuals susceptible to organophosphorus toxicity. Using *PON1*-knockout mice as a model system, we were able to bridge the gap between the previous *in-vitro* studies on the $PON1_{192}$ polymorphism and their implications for in-vivo effects on organophosphorus detoxication. Our results show that both $PON1_{192}$ isoforms provide similar protective effects against diazoxon *in vivo*, suggesting that the level of PON1 expression in individuals, rather than *PON1* genotype, should be considered as a risk factor for diazoxon susceptibility. In the case of chlorpyrifos–oxon, however, both genotype and phenotype should be taken into account. Considering the large variability of human PON1 hydrolytic activities for diazoxon and chlorpyrifos–oxon (Davies *et al.*, 1996), as well as the fact that the oxon forms can be present at significant levels in actual exposure (Yuknavage *et al.*, 1997), our data also point out the importance of including realistic levels of oxon forms in safety testing protocols for organophosphorus pesticides.

Overall, the present study emphasizes the significance of *in-vivo* animal models for verification of assumptions based on *in-vitro* assays. The availability of *PON1*-knockout mice that express different levels of the human $PON1_{192}$ isoforms in both their livers and plasma, together with the *PON1*-knockout mice injected with each $PON1_{192}$ isoform as described here, will allow us to better characterize the *in-vivo*

function of the human $PON1_{192}$ isoforms in both organophosphorus detoxication and cardiovascular disease (Shih *et al.*, 1998).

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