Engineered recombinant human paraoxonase 1 (rHuPON1) purified from *Escherichia coli* protects against organophosphate poisoning

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The high-density lipoprotein-associated enzyme paraoxonase 1 (PON1) hydrolyzes lactones, aromatic esters, and neurotoxic organophosphorus (OP) compounds, including insecticide metabolites and nerve agents. Experiments with mice lacking PON1 (*PON1*$^{-/-}$ mice) have established that plasma PON1 protects against chlorpyrifos/chlorpyrifos-oxon and diazinon/diazoxon (DZO) exposure but does not protect against parathion/paraoxon or nerve agents. The catalytic efficiency of PON1 determines whether or not it will protect against a given OP exposure. Expression of active recombinant human PON1 (rHuPON1) in *Escherichia coli* provides a system in which PON1 can be engineered to achieve a catalytic efficiency sufficient to protect against or treat specific OP exposures. Here, we describe the generation of highly purified engineered rHuPON1$_{K192}$ that protects against DZO exposure when injected into *PON1*$^{-/-}$ mice. The injected rHuPON1 is nontoxic, persists in serum for at least 2 days after injection, and provides protection against DZO exposures of at least three times the median lethal dose value.

Escherichia coli-produced human PON1 | engineered PON1 | therapeutic PON1

The World Health Organization estimates that ≈3 million pesticide poisonings occur each year worldwide, resulting in 220,000 deaths (1, 2). In addition, there is the potential for accidental or intentional release of insecticides or highly toxic nerve agents. Organophosphorus (OP) compounds, which include both pesticides and nerve agents, inhibit brain and diaphragm acetylcholinesterase and cause accumulation of acetylcholine to excessive levels, resulting in cholinergic crisis (3). In addition to respiratory failure, OP exposure can result in temporary or permanent damage to neurologic, cognitive, and motor functions. Current treatments for OP intoxication include anticholinergic drugs and an oxime, which can reactivate acetylcholinesterase provided that the adducted OP has not undergone the "aging reaction" (4). These treatments are often supplemented with the anticonvulsant atropine (5). These treatment protocols are associated with negative side effects, however (6).

In an effort to avoid these problems, investigators have examined the possibility of using proteins capable of detoxifying OPs. Stoichiometric bioscavengers, such as butyrylcholinesterase, have been demonstrated to provide some protection against OP intoxication (7, 8); however, the fact that each very large protein molecule can inactivate only a single OP molecule limits their usefulness due to the large quantities of enzyme required (9). Catalytic scavengers may be more efficient therapeutic agents, because each molecule of enzyme can inactivate many thousands of OP molecules per injected protein molecule. Such treatments would be useful not only in cases of deliberate OP poisoning and large-scale chemical terrorism, but also in treating the many individuals accidentally exposed to commonly used insecticides. Human paraoxonase 1 (PON1) is a leading candidate for development as a therapeutic catalytic scavenger. PON1 can hydrolyze a wide range of substrates, including the OP pesticide products paraoxon, diazoxon (DZO), and chlorpyrifos-oxon, as well as the nerve agents sarin, soman, and VX (10–12).

The ideal therapeutic protein for treating OP exposure should be nonimmunogenic or minimally immunogenic, nontoxic, and persistent in the circulation after injection, and should have a high catalytic efficiency for specific OP hydrolysis. Previously, we suggested PON1 as an excellent candidate to develop for treating OP exposures and demonstrated PON1's therapeutic potential by injecting either rabbit or human PON1 purified from serum into rats and mice both before and after OP exposure (13–16). Our early studies were extended to *PON1*$^{-/-}$ mice that were completely devoid of plasma PON1 activity. We demonstrated that injected purified human plasma PON1 restored plasma PON1 levels and conferred resistance to DZO and chlorpyrifos-oxon exposure, but not to paraoxon exposure (15). These studies clearly demonstrated the importance of catalytic efficiency in determining whether PON1 can protect against a specific OP exposure.

Here, we report the first expression and purification of native and variant recombinant human PON1s (rHuPON1) from an *Escherichia coli* (*E. coli*) system. The rHuPON1$_{K192}$ variant, analogous to rabbit PON1 at this position, exhibits greater hydrolysis of DZO, chlorpyrifos-oxon, and paraoxon compared with PON1$_{R192}$, the more efficient of the two natural human PON1$_{192}$ alloforms PON1$_{Q192}$ and PON1$_{R192}$ (15). Purified rHuPON1$_{K192}$ (192 μg) injected into *PON1*$^{-/-}$ mice was found to be nontoxic, to persist in the circulation for >2 days, and to protect against up to three median lethal doses (LD_{50}) of DZO.

Results

Expression and Purification of rHuPON1. Expression of active rHuPON1 in *E. coli* was achieved through the Staby plasmid system (Eurogentec), in which the *E. coli* genome carries the *ccdB* gene that encodes a stable topoisomerase inhibitor and the plasmid carries the *ccdA* gene that encodes a labile inhibitor of *ccdB* expression (17), thereby forcing the cells to retain the plasmid even at high cell densities. The expression levels were enhanced between twofold and sevenfold by the addition of a second plasmid (pRARE; Invitrogen) that encodes tRNAs for codons most commonly found in mammalian transcripts.

Cell growth and rHuPON1 extraction are described in *Materials and Methods*. rHuPON1 was purified from the extracts through a series of column chromatography steps. Because the expression strain had no endogenous arylesterase (AREase)

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The authors declare no conflict of interest.

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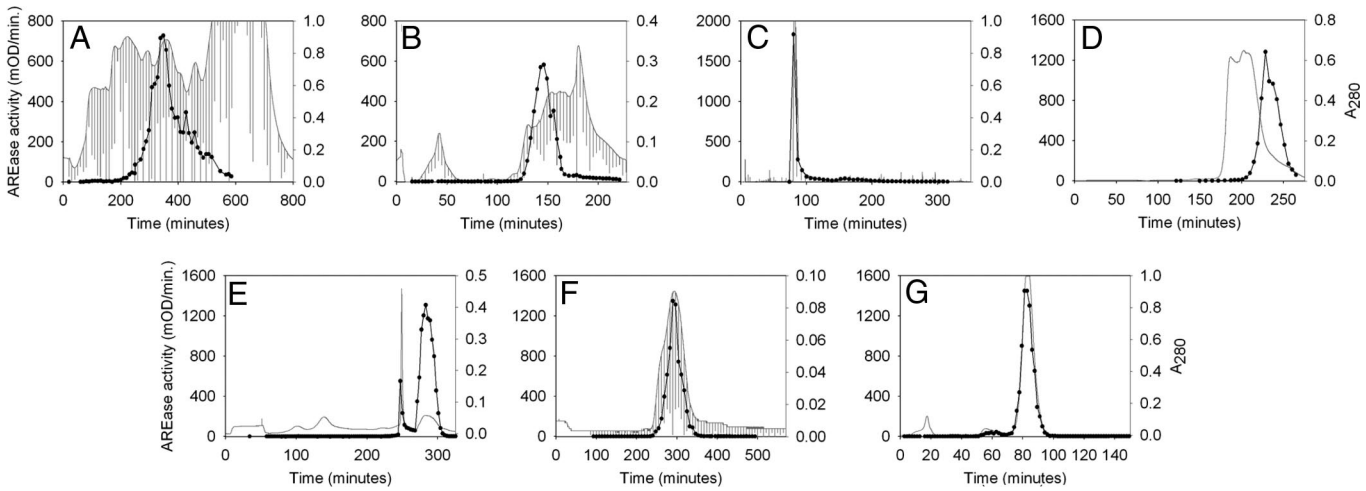

Fig. 1. Protein (A_{280}) and activity monitoring during chromatographic purification of rHuPON1$_{K192}$. Absorbance measurements at 280 nm (gray lines) allowed for continuous monitoring of protein elution from chromatography media, whereas AREase activity (black data points and line) measurements of column fractions allowed monitoring of rHuPON1$_{K192}$. (*A*) First diethylaminoethyl (DEAE) column. (*B*) Second DEAE column. (*C*) hydroxyapatite (HA) column. (*D*) Third DEAE column. (*E*) Hydrophobic interaction chromatography (HIC) column. (*F*) Gel filtration column. (*G*) Fourth DEAE column.

activity (i.e., hydrolysis of phenyl acetate, a nontoxic PON1 substrate), AREase was used to monitor the column fractions containing PON1. Fig. 1 shows the elution profile of each column fractionation step in the purification of rHuPON1$_{K192}$. The conditions for these chromatographic steps are described in *Materials and Methods*. Table 1 summarizes the purification of rHuPON1$_{K192}$. Here, rHuPON1$_{K192}$ was purified 90-fold, with a yield of 17%. As noted in *Materials and Methods*, a slightly modified protocol was used to purify rHuPON1$_{R192}$ 88-fold, with a 19% yield. Fig. 2 gives the results of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE) analysis of each step of rHuPON1$_{K192}$ and rHuPON1$_{R192}$ purification, showing a high degree of purity for each protein. The purified rHuPON1 variants were stable for at least 1 month at 4°C. Most of the higher-molecular-weight minor contaminants reacted with anti-rHuPON1 antibodies on Western blot analysis, whereas the lower-molecular-weight minor contaminants did not (data not shown).

Kinetic Analysis of rHuPON1 Variants. The kinetics of substrate hydrolysis for paraoxon, DZO, chlorpyrifos-oxon, and phenyl acetate were determined for the two PON1 variants. The kinetic data are summarized in Table 2. rHuPON1$_{K192}$ had higher catalytic efficiency than rHuPON1$_{R192}$ for all of the OP substrates examined; however, the two variants had similar kinetic values for phenyl acetate hydrolysis.

Therapeutic Potential of rHuPON1$_{K192}$. To test the therapeutic potential of the engineered rHuPON1$_{K192}$ variant, we carried out a series of experiments to examine the persistence of injected rHuPON1$_{K192}$. The first experiment demonstrated that rHuPON1$_{K192}$ (1.12 units) injected intraperitoneally (i.p.) entered the plasma compartment, was not toxic, and persisted for at least 48 h after injection (Fig. 3*A*). There were no obvious adverse reactions to the rHuPON1$_{K192}$ injections in the three mice followed for >4 months after injection. Plasma PON1 levels, measured by diazoxonase (DZOase) activity, were maximal at ≈8 h after injection and were roughly half those seen in wild-type mice. Because *PON1*$^{-/-}$ mice lack DZOase activity in their plasma (Fig. 3), all of the plasma DZOase activity can be attributed to the injected rHuPON1. The second experiment involved administering combined i.p. and intramuscular (i.m.) injections of a larger quantity of rHuPON1$_{K192}$ (3.91 units) to two *PON1*$^{-/-}$ mice, followed by monitoring plasma DZOase activity for 48 h and then challenging the mice with 1 mg/kg dermal DZO exposure at 48 h. Fig. 3*B* shows that plasma PON1 levels were maximal at ≈8 h, reaching about twice those found in wild-type mice. Fig. 3*C* shows that even the lower level of plasma rHuPON1$_{K192}$ remaining after 48 h was sufficient to protect brain cholinesterase (ChE) activity from inhibition by DZO.

The final experiment addressed the question of whether injection of rHuPON1$_{K192}$ after an OP exposure would protect against high levels of exposure. *PON1*$^{-/-}$ mice were exposed dermally to either 3 or 7 mg/kg of DZO. [The LD_{50} of dermal DZO exposure for *PON1*$^{-/-}$ mice is 1–2 mg/kg (15).] Ten minutes after exposure, the mice were given rHuPON1$_{K192}$ (3.91 units) through combined i.p. and i.m. injections. Fig. 4 shows the

Table 1. Summary of rHuPON1$_{K192}$ purification

Purification step	Volume, ml	Total protein, mg	Total AREase activity, units*	Specific activity, units/mg	Fold purification	Yield, %
Cell extraction	900	2,880	14,835	5.15	–	100
DEAE I	190	850	7,673	9.00	1.8	52
Desalting	390	700	6,944	9.92	2.0	48
DEAE II	146	175	5,627	32.2	6.3	38
HA	62	27	4,282	159	31	29
DEAE III	54	9.4	3,325	354	69	22
HIC	36	6	2,762	460	89	19
Gel filtration	42	6	2,762	460	89	19
DEAE IV	10	5.5	2,558	465	90	17

*Units of arylesterase activity are μmol phenol produced per minute.

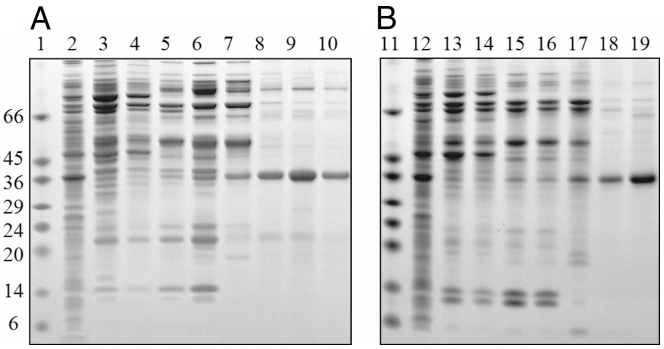

Fig. 2. SDS/PAGE gel analysis of the pooled column fractions from the purification of rHuPON1s. (*A*) rHuPON1$_{K192}$ variant. Lane 1, molecular weight markers (kDa); lane 2, starting extract; lane 3, DEAE I; lane 4, G-25 desalting column; lane 5, DEAE II; lane 6, HA; lane 7, DEAE III; lane 8, HIC; lane 9, gel filtration; lane 10, DEAE IV. (*B*) rHuPON1$_{R192}$ alloform. Lane 11, molecular weight markers; lane 12, starting extract; lane 13, DEAE I; lane 14, G-25 desalting column; lane 15, DEAE II; lane 16, G-25 desalting column; lane 17, DEAE III; lane 18, HIC; lane 19, DEAE IV. Six micrograms of protein were added to each gel lane. The gels were stained with Imperial protein stain (Pierce).

time course of the appearance of rHuPON1$_{K192}$ in the plasma compartment of the injected mice. Control mice that received i.p./i.m. injections of saline were exposed to lower doses of DZO (1, 1.5, or 2 mg/kg), because high doses would have killed them. DZOase activity was absent in the plasma of the saline-injected *PON1*$^{-/-}$ mice (Fig. 4). Both of the rHuPON1$_{K192}$-injected mice survived the high-level DZO exposure, which would have killed uninjected *PON1*$^{-/-}$ mice. Toxicity associated with the high-level DZO exposure, as measured by ChE inhibition, was similar to that seen in control mice receiving doses as low as 1–1.5 mg/kg (not shown).

Discussion

There is a need for therapeutic quantities of rHuPON1 to treat ongoing toxic OP pesticide exposures, as well as possible nerve agent exposures. The possibility also exists for using therapeutic rHuPON1 to treat carotid artery disease (18). A microbial expression system that will facilitate the rapid screening of PON1 variants with increased catalytic efficiency of agent hydrolysis, a requisite for therapeutically efficacious rHuPON1, has not been available until now (19).

The experiments described here demonstrate the first successful expression and purification of active, untagged native and engineered PON1 from *E. coli*. The *E. coli*-produced unglycosylated, untagged PON1 is best suited for such needs because of the ease of scale-up production and the low likelihood of immunogenic response. The naturally occurring PON1$_{R192}$ alloform and an engineered variant with increased efficacy for OP hydrolysis, K192, were expressed, purified, and characterized. The injected rHuPON1 had a half-life comparable to that of the injected native PON1 purified from plasma (14, 15). It was nontoxic and protected *PON1*$^{-/-}$ mice from lethal OP exposures when injected before or after exposure. The *PON1*$^{-/-}$ mice were chosen for these studies for two reasons: (*i*) they have no detectable DZOase activity in their plasma, making it easy to accurately determine the persistence of the injected rHuPON1 over time through a simple enzymatic assay of small plasma samples, and (*ii*) these mice are very sensitive to DZO exposure. Protecting the *PON1*$^{-/-}$ mice from DZO exposures that would be lethal in wild-type mice provided an excellent test of the therapeutic efficacy of the engineered rHuPON1$_{K192}$. In previous work, we demonstrated that increasing the levels of plasma PON1 in wild-type mice through injection of purified rabbit PON1 significantly increased their resistance to OP exposure (14, 15).

Other laboratories have not found it possible to express and purify active rHuPON1 from bacterial cells (19). The successful expression of active rHuPON1 depends on the media used and on a reduced temperature for growth. The *E. coli* system did not produce active rHuPON1 when grown at temperatures of 37°C or above.

The purification of rHuPON1 was achieved through several column chromatography steps. HIC and DEAE chromatography have previously proven useful for purifying PON1 from plasma (20, 21). The final specific activity of rHuPON1 was comparable to that of purified PON1 from human serum (22). Although the PON1$_{K192}$ variant has not been observed in human populations, it was chosen for expression and purification because of the high rate of OP hydrolysis by rabbit PON1 (which has lysine at position 192) observed in our earlier studies (15). As expected, the rHuPON1$_{K192}$ variant demonstrated higher catalytic efficiency for OP pesticide targets than the natural human rHuPON1$_{R192}$ variant and much higher catalytic efficiency than human PON1$_{Q192}$. Interestingly, the rHuPON1$_{K192}$ had higher K_m values for all three OPs tested; however, its maximal velocities also were significantly higher, resulting in overall higher catalytic efficiency for the rHuPON1$_{K192}$ variant.

To examine the therapeutic potential of the *E. coli*-produced rHuPON1, we tested the purified rHuPON1$_{K192}$ in the *PON1*$^{-/-}$ mouse model system used to demonstrate the importance of PON1 in OP detoxification. The rHuPON1$_{K192}$ injected into the *PON1*$^{-/-}$ mice was nontoxic and had a long half-life. We have demonstrated previously that for purified PON1 injected into *PON1*$^{-/-}$ mice, a catalytic efficiency of ≈77 was adequate to provide protection against OP exposure (15). rHuPON1$_{K192}$ had a catalytic efficiency of 118 for the hydrolysis of DZO (Table 2), compared with 73–75 for native human PON1. Of note, the *PON1*$^{-/-}$ mice not only tolerated injection with rHuPON1, but also exhibited no adverse effects.

To determine the protective capacity against OP exposure, we exposed *PON1*$^{-/-}$ mice to high doses of DZO 10 min before injection of rHuPON1$_{K192}$. Whereas *PON1*$^{-/-}$ mice exposed to 3 or 7 mg/kg DZO would have died (15), the mice injected with rHuPON1$_{K192}$ survived and exhibited milder OP toxicity compared with the control mice exposed to sublethal levels of DZO.

Table 2. Kinetic analysis of substrate hydrolysis by rHuPON1 variants

Substrate	rHuPON1 variant	K_m, mM	V_{max}, units/mg	V_{max}/K_m
Paraoxon	K192	0.925 ± 0.029	11.66 ± 0.06	12.61 ± 0.32
	R192	0.868 ± 0.016	6.61 ± 0.18	7.62 ± 0.23
DZO	K192	2.57 ± 0.62	301 ± 51	118 ± 10
	R192	1.33 ± 0.08	119 ± 5	89.8 ± 2.3
Chlorpyrifos-oxon	K192	0.317 ± 0.02	245 ± 3	777 ± 66
	R192	0.131 ± 0.005	34.7 ± 0.3	266 ± 12
Phenyl acetate	K192	3.22 ± 0.79	3,020 ± 300	966 ± 166
	R192	0.957 ± 0.02	680 ± 0	711 ± 16

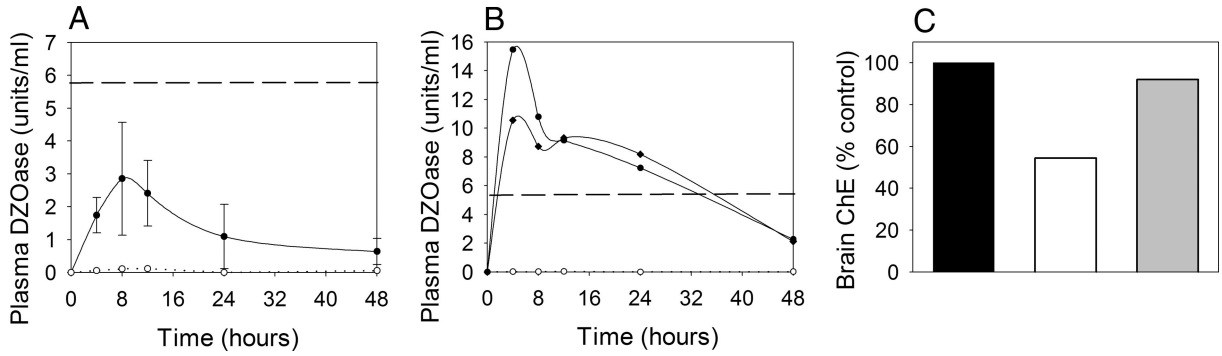

Fig. 3. PON1 levels and protection after injection of rHuPON1$_{K192}$ into *PON1*$^{-/-}$ mice. (*A*) Plasma PON1 DZOase levels (●) after i.p. injection of 1.12 units of rHuPON1$_{K192}$ into *PON1*$^{-/-}$ mice (average of three mice). Maximal PON1 levels were approximately half those observed in wild-type mice, represented by the dashed line. Note the lack of DZOase in plasma of the saline-injected *PON1*$^{-/-}$ mice (○). (*B*) Plasma PON1 DZOase levels after i.p. and i.m. injection of a total of 192 μg of rHuPON1$_{K192}$ (3.91 units) into each of two *PON1*$^{-/-}$ mice. The dashed line represents DZOase activity in plasma of wild-type mice. Female and male mice injected with saline had no DZOase activity (○). DZOase of female (♦) and male (●) mice injected with rHuPON1 are shown separately. At 48 h after rHuPON1 injection, the mice were challenged with 1 mg/kg of DZO. (*C*) Injection of rHuPON1$_{K192}$ protected brain ChE from inhibition, measured 6 h after DZO exposure. Brain ChE activity was expressed as a percentage of untreated *PON1*$^{-/-}$ mice (black bar). Inhibition in the rHuPON1$_{K192}$-injected mice is represented by the gray bar; inhibition in the saline-injected mice, by the white bar (average of two mice for each group).

These experiments clearly demonstrate the therapeutic potential 2of *E. coli*-expressed engineered rHuPON1. Variants with even higher catalytic efficiencies should be attainable and should prove useful for treating OP pesticide and nerve agent exposures.

Materials and Methods

Engineering and Expression of rHuPON1. The plasmid pStaby1.2 (Eurogentec) was modified to generate pStaby-NcoI by replacing an NdeI restriction endonuclease (RE) site downstream of the T7 promoter with an NcoI RE site using the polymerase chain reaction primers StabyNcoI-F (5′-CATGCTTGCCATGGCTAGCATGACTGGTGGACAG-3′) and StabyNcoI-R (5′-CTAGCTTGCCATGGGTATATCTCCTTCTTAAAGTTAAAC-3′), followed by NcoI RE (New England Biolabs) digestion, 0.8% agarose gel purification, ligation using T4 DNA ligase (New England Biolabs), and clonal isolation in XL1-blue (Stratagene) *E. coli*. Human PON1$_{Q192}$ gene coding sequence (23), with an NcoI RE site at the translational start codon and a SacI RE site at the translation stop codon, was cloned into NcoI-SacI-digested pET32a (Novagen). Site-directed mutagenesis (24) was used to change the Q192 position to encode either R192 or K192. The resulting plasmids contained NcoI-NotI fragments encoding rHuPON1$_{R192}$ or rHuPON1$_{K192}$ that were introduced into the pStaby-NcoI plasmid by ligating 0.8% agarose gel-purified NcoI to NotI DNA fragments of PON1 to a NcoI-NotI-digested pStaby-NcoI vector using T4 DNA ligase, followed by clonal isolation in XL1-blue *E. coli*. Expression of rHuPON1 in bacteria was carried out using the Staby system expression strain SE1 (Eurogentec). SE1 cells were transformed with either pStaby-*PON1*$_{R192}$ or pStaby-*PON1*$_{K192}$, as well as with the plasmid pRARE (encoding genes for tRNA rare in *E. coli*; Invitrogen) and selected on L-broth (Teknova) agar plates containing 50 μg/ml of carbenicillin and 34 μg/ml of chloramphenicol. A 14-l Microferm MF-114 fermentor (New Brunswick Scientific), with an air flow of 6 liters/min and stirring at 500 rpm, was used for production of rHuPON1 expressing *E. coli*. A culture of 200 ml of SE1 cells containing pRARE and pStaby-PON1$_{R192}$ growing exponentially at room temperature in L-broth supplemented with antibiotics was added to 12 liters of L-broth containing antibiotics, 10 mM $CaCl_2$, 2% glycerol, and trace elements (25) at 23°C. When the A_{600} reached 0.8, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.25 mM. After 16 h, 167 g of cells were harvested by centrifugation from the rHuPON1$_{R192}$-expressing cultures and stored at −80°C. Identical methods were used for expression of rHuPON1$_{K192}$, yielding 347 g of cells. We are currently using the Eurogentec plasmid pSCodon1.2, which encodes the tRNAs for the rare codons and eliminates the need for antibiotic in the media. We used the two-plasmid system before pSCodon1.2 became available.

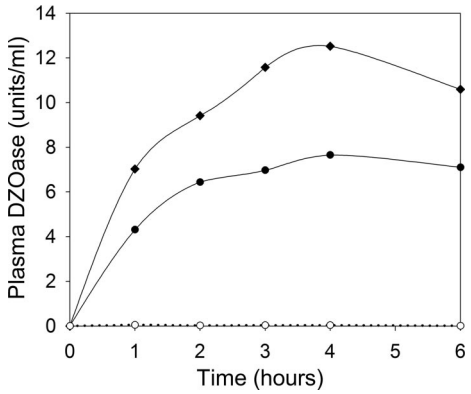

Fig. 4. Plasma PON1 levels over time after i.p./i.m. injection of 192 μg (3.91 units) of rHuPON1$_{K192}$ into each of two male *PON1*$^{-/-}$ mice that had received DZO exposures of either 3 or 7 mg/kg 10 min before the rHuPON1 injection. None of the control mice displayed any plasma DZOase activity (○). One male mouse was challenged with 3 mg/kg of DZO, then injected with rHuPON1$_{K192}$ (♦); the other was treated with 7 mg/kg, then injected with the protein (●). The mice were euthanized at 6 h after DZO exposure for determination of brain ChE activity.

Purification of rHuPON1. The AREase activity of PON1 was measured by following the production of phenol from phenyl acetate hydrolysis, as described previously (26, 27), because the expression strain lacks endogenous AREase activity. *E. coli* cells (200 g of rHuPON1$_{K192}$ or 167 g of rHuPON1$_{R192}$) were combined with 100 ml of 0.1-mm glass beads (Biospec Products), 100 μl of a protease inhibitor mixture (539134; Calbiochem), 125 units of Benzonase (Novagen), and lysis buffer (20 mM Tris, pH 8.0, 1 mM $CaCl_2$, 5 mM dithiothreitol) to a final volume of 350 ml. Cells containing soluble active rHuPON1 were disrupted by using a BeadBeater (Biospec Products) with ten 1-min pulses, alternating with 1 min of cooling. Extracts were centrifuged for 20 min at 17,000 × *g*, and the supernatant was decanted. Cell pellet fractions were pooled and resuspended in lysis buffer containing 1% Tergitol (Sigma) to a final volume of 350 ml, then reextracted with the BeadBeater as described above. After centrifugation at 17,000 × *g*, the second supernatant was combined with the initial lysis supernatant before purification.

The rHuPON1$_{K192}$ was purified through seven column chromatography steps (Fig. 1). Cell extracts were loaded onto a 70-ml resin bed volume DEAE Sepharose Fast Flow column (1.5 × 39 cm) (GE Healthcare Biosciences AB). The column was equilibrated and washed with DEAE buffer I (20 mM Tris [pH 8.0], 1 mM $CaCl_2$, 1 mM dithiothreitol, and 0.1% Tween-20 detergent) before the bound proteins were eluted with a 40-column volume gradient of 0–0.5 M NaCl in DEAE buffer I (Fig. 1*A*). Fractions containing AREase activity were pooled and desalted on a 900-ml Sephadex G-25 desalting column (7.6 × 20 cm) (Amersham Pharmacia) equilibrated and run with 20 mM Tris [pH 8.0] and 1 mM $CaCl_2$ buffer. The resulting pooled, active fractions were loaded onto a 40-ml resin bed volume DEAE Sepharose Fast Flow column (1.5 × 23 cm) equilibrated with DEAE buffer I and eluted with a 40-column volume NaCl gradient in DEAE buffer I as described above (Fig. 1*B*). Arylesterase-containing fractions were pooled and loaded onto a 30-ml ceramic HA (type II, 40μ; BioRad) column (1.5 × 10 cm). The HA column was washed with HA buffer [2

mM potassium phosphate (pH 6.8)], 0.1 mM $CaCl_2$, 0.1% Tween-20), then eluted with a 40-column volume HA buffer gradient from 2 mM to 100 mM potassium phosphate (pH 6.8) (Fig. 1*C*). AREase-containing fractions were pooled and loaded onto an 11-ml resin bed volume DEAE Sepharose Fast Flow column (0.7 × 29 cm), washed with DEAE buffer II (20 mM Tris [pH 7.1], 1 mM $CaCl_2$), and eluted with a 40-column volume 0–0.75 M NaCl gradient (Fig. 1*D*). AREase-containing fractions were pooled, and NaCl was added to a final concentration of 3 M. The 3 M NaCl pool was loaded onto a 6-ml t-butyl Sepharose Fast Flow HIC column (0.7 × 15 cm) (GE Healthcare), washed with HIC buffer (10 mM Tris [pH 8.0], 1 mM $CaCl_2$) containing 3 M NaCl, and eluted with a reverse salt gradient of 40 column volumes, 3–0 M NaCl in HIC buffer. The t-butyl HIC column was then washed with HIC buffer containing 0.1% Tween-20 to elute the AREase activity (Fig. 1*E*). AREase-containing fractions were pooled, concentrated, and flowed through a 400-ml gel filtration Superdex 200 column (2.6 × 64 cm) (GE Healthcare) equilibrated with Superdex buffer (150 mM NaCl, 20 mM Tris [pH 8.0], 1 mM $CaCl_2$) (Fig. 1*F*). The rHuPON1 was further purified by pooling the AREase-containing fractions and loading them onto a 1-ml DEAE column (0.5 × 5 cm) equilibrated with DEAE buffer III (20 mM Tris [pH 7.7], 1 mM $CaCl_2$). The column was eluted with 50 column volumes of a 0–0.75 M NaCl gradient (Fig. 1*G*). The rHuPON1$_{R192}$ variant was purified using a similar method, but replacing the HA column with a second Sephadex G-25 column (data not shown).

Kinetic Analysis of rHuPON1 Variants. Measurements of PON1 hydrolysis of paraoxon, DZO, and chlorpyrifos-oxon were carried out as described previously (26). In brief, PON1 activity was measured in 0.1 M Tris·HCl (pH 8.5), 2 M NaCl, and 2 mM $CaCl_2$ with varying concentrations of paraoxon, DZO, or chlorpyrifos-oxon. The reaction was initiated by mixing 10 μl of purified rHuPON1 with 200 μl of substrate in an assay buffer. The absorbance was monitored continuously for 4 min. The reactions were carried out in a Molecular Devices SPECTRAmax PLUS Microplate spectrophotometer. The amount of hydrolysis product was calculated from the initial linear rates of hydrolysis using extinction coefficients of 18 $mM^{-1}cm^{-1}$ at A_{405} for *p*-nitrophenol (paraoxon hydrolysis), 3 $mM^{-1}cm^{-1}$ at A_{270} for 2-isopropyl-4-methyl-6-hydroxy pyrimidine (DZO hydrolysis), 5.56 $mM^{-1}cm^{-1}$ at A_{310} for 3,5,6-trichloro-2-pyridinol (chlorpyrifos-oxon hydrolysis), or 1.310 $mM^{-1}cm^{-1}$ at A_{270} for phenol (phenyl acetate hydrolysis) (28). All kinetic analyses were determined with at least three substrate concentrations below and three substrate concentrations above the K_m values, with catalytic constants determined by plotting substrate concentration/velocity versus substrate concentration (29). Units of activity are expressed as μmol of hydrolysis product formed per minute.

PON1 Knockout (*PON1*$^{-/-}$) Mice. The generation of the *PON1*$^{-/-}$ mice has been described previously (30, 31). The mice were housed in either barrier or modified specific pathogen-free facilities with 12-h dark–light cycles and free access to food and water. All 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. Animal use protocols were approved by the University of Washington's Institutional Animal Care and Use Committee.

Exposure and Protection of Mice. Recombinant PON1 protein was administered to mice by i.p. injection or a combination of i.m. and i.p. injections, as indicated. For i.p. injections, a 23G needle was used to inject 200 μl of rHuPON1$_{K192}$ or sterile normal saline into the peritoneal cavity. For i.m. injections, a 30G needle was used to inject 100 μl of rHuPON1$_{K192}$ or sterile saline into the lateral quadriceps (50 μl into each quadricep). Blood (<50 μl; no greater than 1% of total body weight) was drawn from the saphenous vein at the indicated times after the injections. DZO (Chem Service) was dissolved in acetone and applied on a shaved area (4 cm^2) of the back of 12-wk-old mice at 5 μl/g body weight, either 48 h after or 10 min before injection of rHuPON1$_{K192}$. Control animals received acetone application only. After the mice were euthanized by cervical dislocation, the organs were dissected, placed on dry ice, and stored at −80°C until assayed. Some of the mice injected with rHuPON1$_{K192}$ were monitored for up to 4 months to detect any long-lasting negative side effects. DZOase assays were performed as described above but with 150 mM NaCl in the assay buffer. Brain ChE assays were performed as described previously (31).

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