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Recombinant paraoxonase 1 protects against sarin and soman toxicity following microinstillation inhalation exposure in guinea pigs☆

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ABSTRACT

To explore the efficacy of paraoxonase 1 (PON1) as a catalytic bioscavenger, we evaluated human recombinant PON1 (rePON1) expressed in *Trichoplusia ni* larvae against sarin and soman toxicity using microinstillation inhalation exposure in guinea pigs. Animals were pretreated intravenously with catalytically active rePON1, followed by exposure to 1.2 X LCt_{50} sarin or soman. Administration of 5 units of rePON1 showed mild increase in the blood activity of the enzyme after 30 min, but protected the animals with a significant increase in survival rate along with minimal signs of nerve agent toxicity. Recombinant PON1 pretreated animals exposed to sarin or soman prevented the reduction of blood O_2 saturation and pulse rate observed after nerve agent exposure. In addition, rePON1 pretreated animals showed significantly higher blood PON1, acetylcholinesterase (AChE), and butyrylcholinesterase activity after nerve agent exposure compared to the respective controls without treatments. AChE activity in different brain regions of rePON1 pretreated animals exposed to sarin or soman were also significantly higher than respective controls. The remaining activity of blood PON1, cholinesterases and brain AChE in PON1 pretreated animals after nerve agent exposure correlated with the survival rate. In summary, these data suggest that human rePON1 protects against sarin and soman exposure in guinea pigs.
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1. Introduction

Human serum paraoxonase 1 (PON1, EC 3.1.8.1) is a polymorphic enzyme that is expressed mainly in the liver, secreted to blood, and associated with high density lipoproteins (HDL) (Costa et al., 2003; Humbert et al., 1993; Primo-Parmo et al., 1996). PON1 associated with HDL is a 45 kDa arylesterase which has been reported to protect low density lipoproteins from oxidative modifications and thus exerts the anti-atherogenic property of PON1 in atherosclerosis and cardiovascular diseases (Costa et al., 2005a; Harel et al., 2004; Josse and Masson, 2001; Mackness et al., 2002). PON1 has also been described as a potential catalytic bioscavenger by a number of groups due to its capacity to hydrolyze organophosphates (OPs) and chemical warfare nerve agents (CWNAs) (Costa et al., 2005b; Furlong et al., 2005; Otto et al., 2009; Otto et al., 2010; Stevens et al., 2008). It can hydrolyze large amounts of OPs and CWNAs compared to the stoichiometric bioscavenger human butyrylcholinesterase (BChE), which acts in a 1:1 ratio, and would thus require smaller doses and would reduce the cost of treatment (Doctor et al., 1991; Doctor and Saxena, 2005; Lenz et al., 2005; Lenz et al., 2007; Rochu et al., 2007).

The mechanism of action of OPs and CWNAs has been well described as the irreversible inhibition of acetylcholinesterase (AChE) in the central and peripheral nervous system leading to abnormal accumulation of acetylcholine resulting in overstimulation of cholinergic transmission (Aldridge and Davison, 1953; Bajgar, 2004; Flynn and Wecker, 1986). An effective countermeasure strategy for OP and CWNA toxicity has been centered on the development of catalytic bioscavenger enzymes as pretreatments which can rapidly hydrolyze large amounts of OPs and CWNAs in the blood before they reach their targets. Recent research and development identified human PON1 as a suitable candidate catalytic bioscavenger against OP and CWNA mediated toxicity (Costa

Abbreviations: PON1, paraoxonase 1; HDL, high density lipoprotein; rePON1, recombinant PON1; OP, organophosphate; CWNA, chemical warfare nerve agent; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; p-NPA, p-nitrophenylacetate; BCA, bicinchoninic acid; Sarin, 2-(fluoro-methylphosphoryl)oxypropane; Soman, 2-(fluoromethylphosphoryl)oxy-3,3-dimethylbutane.

☆ *Disclosure*: The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army, the Navy, or the Department of Defense, USA.

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et al., 2005b; Furlong et al., 2005; Li et al., 1995; Otto et al., 2009; Otto et al., 2010; Rochu et al., 2007; Stevens et al., 2008). Most of these studies were either performed under *in vitro* conditions, or in rodent models of OP toxicity (Duysen et al., 2010; Fu et al., 2005; Li et al., 1995; Wang et al., 2010). We also reported efficient hydrolysis of the CWNA tabun in comparison to sarin and soman by PON1 using *in vitro* analysis (Valiyaveettil et al., 2010). Efficacy of PON1 against CWNA toxicity in animal models needs to be investigated.

Recently, we have reported the protective efficacy of human and rabbit serum purified PON1 against sarin and soman inhalation toxicity in guinea pigs using microinstillation technology (Valiyaveettil et al., 2011). Although serum purified enzymes can be used for treatment, recombinant enzymes are preferred for the ease of large scale production and elimination of polymorphism and heterogeneity. Pooling of serum from multiple donors may lead to purification of multiple PON1 mutants including one which has lower catalytic activity. Another major concern with the use of human derived protein therapeutics is the inherent potential for infectious agents like hepatitis and human immunodeficiency virus (Grillberger et al., 2009). Nevertheless, recombinant enzymes may have difference in stability, pharmacokinetics and efficacy, which need to be investigated. Significant hydrolysis of sarin and soman by human recombinant PON1 (rePON1) expressed in *Trichoplusia ni* larvae by *in vitro* analysis prompted us to further investigate the protective efficacy against CWNA toxicity (Valiyaveettil et al., 2010). In this paper, we evaluated the efficacy of human rePON1 against sarin and soman toxicity in guinea pigs using microinstillation inhalation exposure technology (Nambiar et al., 2007; Nambiar et al., 2006).

2. Materials and methods

2.1. Materials

P-nitrophenylacetate (p-NPA), phenylacetate, acetylthiocholine, butyrylthiocholine, tetra monoisopropyl pyrophosphortetramide, huperzine A, 5,5′-dithiobis (2-nitrobenzoic acid), and 4,4′-dipyridyl disulfide, 4,4′-dithiodipyridine were purchased from Sigma (St. Louis, MO). Tissue protein extraction reagent and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce (Rockford, IL). CWNAs, sarin (99.8% pure) and soman (99.6% pure) were obtained from Edgewood Chemical and Biological Center and used at US Army Medical Research Institute of Chemical Defense (USAMRICD), Aberdeen Proving Ground, MD.

2.2. Animals

All animal procedures were performed at USAMRICD in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals (NRC Publication 1996 edition) with an approved Institutional Animal Care and Use Committee protocol. Male Hartley guinea pigs (250–300 g, Charles River Laboratories, Wilmington, MA) were used for the studies.

2.3. Expression and purification of recombinant human PON1

Recombinant PON1 was expressed in *T. ni* larvae and was purified as described earlier in collaboration with Chesapeake PERL (Savage, MD) (Otto et al., 2010; Valiyaveettil et al., 2010). Briefly, baculovirus expression vector containing human PON1 cDNA (encoding wild type human PON1 with Q192 polymorphism) was orally infected to larvae followed by incubation at controlled conditions for 96 h. The harvested larvae were stored under frozen conditions until the recovery of PON1 enzyme. The extraction and purification of rePON1 was performed by using multiple chromatography procedures as described earlier (Otto et al., 2010; Valiyaveettil et al., 2010). The purity of rePON1 was analyzed by SDS–PAGE and Western blotting. The catalytic activity of purified rePON1 (>99% pure) was analyzed using p-NPA substrate and expressed as units/mg protein or units/ml (Valiyaveettil et al., 2010). Enzyme activity was calculated with the molar extinction coefficient ($18\ mM^{-1}\ cm^{-1}$) of p-nitrophenol. The purified rePON1 showed a specific activity of 8.5 units/mg protein.

2.4. Sarin and soman microinstillation inhalation exposure

Control and rePON1 pretreated guinea pigs were exposed to sarin and soman using the microinstillation inhalation exposure methodology as described earlier (Nambiar et al., 2007; Nambiar et al., 2006). Briefly, the animals were anesthetized (telazol, 40 mg/kg, i.m. and medetomidine, 0.125 mg/kg, s.c.) and saline or rePON1 (5 units) were administered through saphenous vein using a 28.5 gauge needle. Ear blood was collected from animals before and after the administration of rePON1. Animals were intubated and a microcatheter was inserted and placed 2 cm above the bifurcation of the trachea for aerosolization of the CWNA. Thirty minutes after administration of rePON1, the animals were exposed to sarin ($846\ mg/m^3$) or soman ($841\ mg/m^3$) using the microinstillation equipment (Trudell Medical, Ontario, Canada) with a pulse rate of 40 pulses/min for 2–4 min (Che et al., 2008; Perkins et al., 2010). Blood and tissue samples were collected from terminally ill animals. The surviving animals were allowed to recover for 24 h, euthanized by exsanguination and blood and tissues were collected for biochemical analysis.

2.5. Pulse rate and blood O_2 saturation measurement

The blood O_2 saturation (measured as % saturation of peripheral oxygen, SpO_2) and pulse rate (measured as beats per min, bpm) of the animals were recorded by using a pulse oximeter (Nonin Medical Instruments, Minneapolis, MN). The data were recorded at pre-intubation, post-intubation and 30 s intervals during and after exposure to CWNA for 15 min. Mean ± SEM values were plotted against time using GraphPad Prism software.

2.6. Analysis of blood and brain enzyme activity

Blood samples collected at various time points were diluted with de-ionized water. Brain tissue was dissected into different regions (frontal cortex, hind cortex, mid brain, cerebellum and hippocampus) and homogenized with 1:7 (wt/vol) tissue protein extraction reagent. The activity of PON1, AChE and BChE in the blood and AChE in the brain was determined as described earlier (Ellman et al., 1961; Valiyaveettil et al., 2011). Protein content in the brain samples was estimated by BCA protein assay kit. The activity of various enzymes in the blood and brain was expressed as μmol/min/ml or μmol/min/mg protein, respectively.

2.7. Data analysis

The animal survival data was plotted using Kaplan–Meier method and analyzed by Logrank test. The pulse rate and blood O_2 saturation data at multiple time points were analyzed by two-way ANOVA with Bonferroni post-test. Blood and brain enzyme activity data were analyzed by using Mann–Whitney test.

3. Results

3.1. Recombinant human PON1 protects against sarin and soman toxicity

The survival data of guinea pigs pretreated with 5 units of rePON1 (~600 μg of protein) followed by exposure to $846\ mg/m^3$ of sarin or $841\ mg/m^3$ of soman are shown in the Kaplan–Meier curve (Fig. 1). Most of the control animals exposed to sarin or soman without any treatment died within 10–15 min, while majority of rePON1 pretreated animals exposed to sarin or soman survived for 24 h. The non-surviving animals in rePON1 pretreated group died 2–4 h after CWNA exposure. Guinea pigs pretreated with 5 units of rePON1 and then exposed to $846\ mg/m^3$ sarin showed ~80% survival rate that is significantly higher than that seen in control animals exposed to sarin ($p = 0.009$). In animals exposed to $841\ mg/m^3$ of soman, rePON1 pretreatment showed a ~71% survival rate that was significant compared to untreated controls ($p = 0.015$). The animals that survived for 24 h in both sarin and soman groups were healthy and active.

3.2. Recovery of pulse rate and blood O_2 saturation in rePON1 pretreated guinea pigs exposed to CWNAs

Guinea pigs exposed to sarin ($846\ mg/m^3$) showed a gradual drop in the heart rate during the entire 15 min of recording until the death of the animal, as reported previously (Valiyaveettil et al., 2011) (Fig. 2A). Recombinant PON1 pretreated animals exposed to sarin showed a similar decrease in the pulse rate up to 5 min,

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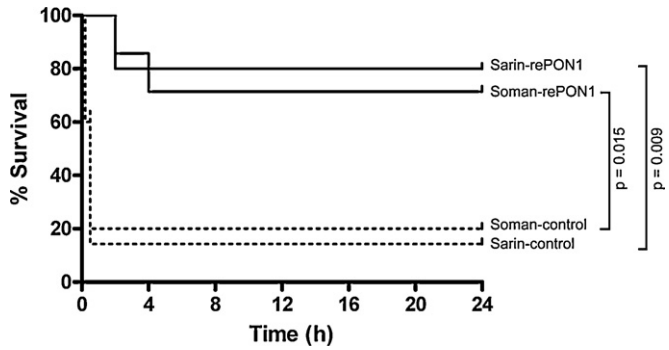

Fig. 1. Kaplan–Meier survival curve of guinea pigs pretreated with rePON1 and exposed to sarin and soman. Male Hartley guinea pigs were intravenously administered with saline or human recombinant PON1 (5 units) and 30 min later exposed to 846 mg/m^3 of sarin or 841 mg/m^3 of soman using microinstillation method as described in "Section 2". The surviving animals were allowed to recover for 24 h. The percentage of survival of guinea pigs was plotted against time in GraphPad Prism using Kaplan–Meier survival curve and the statistical significance was analyzed by Logrank test. $n = 14$ for saline controls exposed to sarin; $n = 10$ for saline controls exposed to soman; $n = 5$ for human recombinant PON1 (rePON1) exposed to sarin, $n = 7$ for rePON1 exposed to soman.

which returned back to baseline by 8 min (Fig. 2A). The pulse rate in rePON1 pretreated animals exposed to sarin showed a statistically significant increase 10 min after the start of sarin exposure compared to the respective sarin controls (Fig. 2A). In the case of soman (841 mg/m^3), the control guinea pigs showed a sharp drop in pulse rate for the first 5 min. Pulse rate tend to return back, but remained below baseline levels up to 10 min and then decreased further until the death of the animal (Fig. 2B). Pulse rates in animals pretreated with rePON1 and exposed to soman showed a relatively lesser decrease and were quickly returned and maintained at baseline levels and that was statistically significant compared to untreated soman controls from 10 min after exposure (Fig. 2B).

The blood O_2 saturation curve of sarin exposed animals showed a decrease till the end of 15 min, although there was a drift toward normal levels at 7.5 min (Fig. 2C). Recombinant PON1 pretreated animals exposed to sarin showed a drop in the blood O_2 saturation that peaked around 6 min and then returned back to baseline at 10 min and maintained until 15 min of recording. The blood O_2 saturation at 10, 11, 14 and 15 min after sarin exposure in rePON1 pretreated animals was significantly higher than the respective sarin controls (Fig. 2C). In soman exposed animals, the blood O_2 saturation curve showed a pattern resembling pulse rate with a decrease at 4–5 min and a trend of returning back to baseline at 8–9 min (Fig. 2D). Recombinant PON1 pretreated animals exposed to soman also showed a pattern similar to pulse rate that returned back to baseline at 8 min and that was significantly higher than the respective soman controls at 11 and 15 min (Fig. 2D). The restoration of blood O_2 saturation and pulse rate in rePON1 pretreated guinea pigs exposed to sarin or soman correlated with the increase in survival rate.

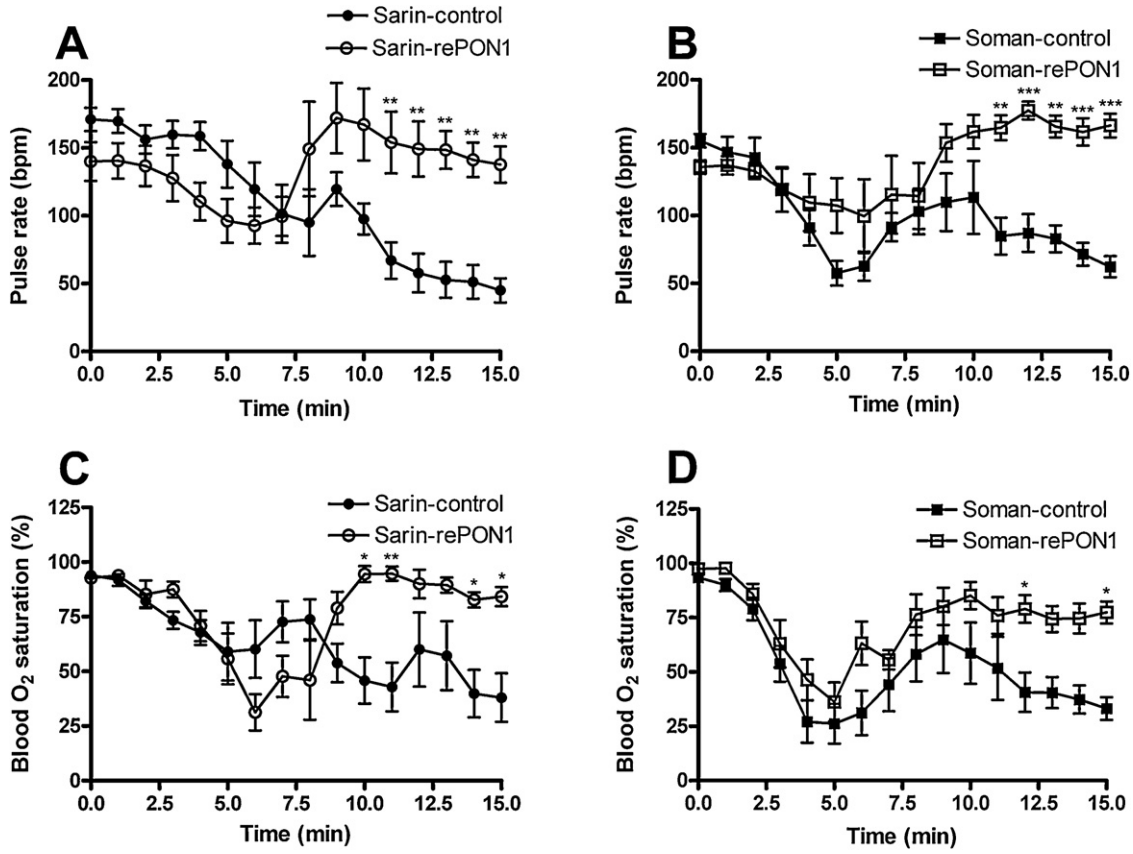

Fig. 2. Pulse rate and blood O_2 saturation curves of animals exposed to sarin and soman. Guinea pigs were administered with saline or rePON1 and exposed to sarin (A and C) or soman (B and D) as described in Fig. 1 and "Section 2". The pulse rate (A and B) in beats per min (bpm) and blood O_2 saturation (C and D) were recorded using a pulse oximeter every 30 s for 15 min and mean ± SEM values were plotted against time (min). The data was analyzed by using two-way ANOVA with Bonferroni post-test. Saline controls exposed to sarin or soman, $n = 8$; human recombinant PON1 (rePON1) exposed to sarin, $n = 4$; and rePON1 exposed to soman, $n = 5$ ((*) $0.05 < p < 0.01$; (**) $0.01 < p < 0.001$; (***) $p < 0.001$).

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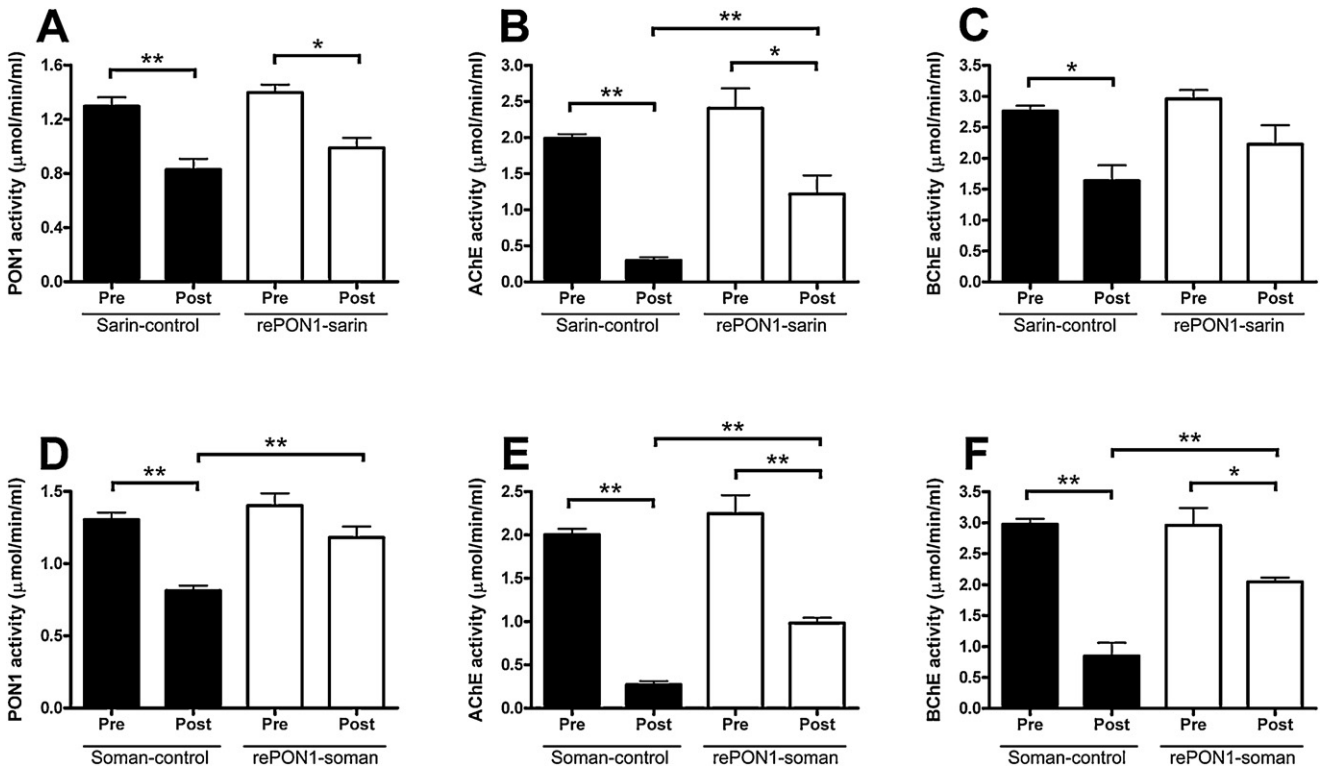

Fig. 3. Blood activity of PON1, AChE and BChE in guinea pigs exposed to sarin and soman. As described in Fig. 1, the animals were pretreated with rePON1 followed by exposure to sarin (A–C) or soman (D–F). The activity of PON1 (A and D), AChE (B and E), and BChE (C and F) in the pre- and post-exposed blood samples were analyzed as described in "Section 2". The results were expressed as μmol/min/ml of blood ($n = 6$ for control, and 4–5 for rePON1; (*) $0.05 < p < 0.01$; (**) $0.01 < p < 0.001$).

3.3. Activity of PON1, AChE and BChE in the blood of guinea pigs treated with rePON1and exposed to sarin and soman

As reported earlier, the activity of PON1, AChE and BChE in the blood of guinea pigs exposed to sarin (846 mg/m^3) or soman (841 mg/m^3) showed a significant reduction compared to the respective controls (Valiyaveettil et al., 2011) (Fig. 3). Administration of rePON1 showed a marginal insignificant increase in the activity of PON1 and AChE in the blood of guinea pigs (Figs. 3A, B, D, and E), while blood BChE activity was unaltered (Figs. 3C and F). Recombinant PON1 pretreated animals exposed to sarin or soman also showed significant decreases in blood PON1, AChE and BChE activities, but this decrease was considerably lower than that of respective nerve agent exposed controls (Fig. 3). The residual blood PON1, AChE and BChE activities in rePON1 pretreated animals exposed to sarin or soman correlated with the increased survival rate after CWNA exposure.

3.4. Brain AChE activity in rePON1 pretreated sarin and soman exposed guinea pigs

Guinea pigs exposed to sarin or soman showed a significant reduction in the activity of AChE in different regions of the brain as observed earlier (Valiyaveettil et al., 2011) (Fig. 4). AChE activity in most of the brain regions of rePON1 pretreated animals exposed to sarin was significantly higher than that of nerve agent controls (Figs. 4A–E). The frontal cortex of PON1 pretreated animals exposed to sarin showed AChE activity similar to that of unexposed controls (Fig. 4A). In the case of soman, all the brain regions of rePON1 pretreated animals showed significantly higher AChE activity compared to nerve agent controls (Figs. 4A–E). Recombinant PON1 pretreated animals exposed to sarin or soman retained ~40–70% of AChE activity, possibly contributing to the survival of animals after CWNA exposure.

4. Discussion

Taken together, the results described here demonstrate that human rePON1 produced in *T. ni* larvae and administered intravenously to guinea pigs protected against sarin and soman inhalation toxicities. These data support our previous observation that purified human and rabbit serum PON1 protects against CWNA toxicity (Valiyaveettil et al., 2011). The observed protective efficacy of rePON1 was reflected by the increase in survival rate, lack of behavioral symptoms, restoration of pulse rate and blood O_2 saturation, significantly higher levels of blood bioscavenger enzymes and blood and brain AChE levels in pretreated animals compared to the respective nerve agent exposed controls, indicating that PON1 pretreatment decreases the overall toxicity of sarin and soman in guinea pigs. Significant protection was observed with 5 units of rePON1, which is approximately 600 μg of protein per animal (~2.5 mg/kg). Higher doses of rePON1, in the range of 10–20 units (~5–10 mg/kg), may be adequate to efficiently protect against 2–5 X LCt_{50} of nerve agent exposure, which has to be confirmed by performing more detailed experiments.

As we reported earlier with human and rabbit serum PON1, the decrease in pulse rate and blood O_2 saturation curves due to CWNA exposure was returned to the baseline values in rePON1 pretreated animals exposed to nerve agents, indicating that PON1 pretreatment leads to immediate restoration of normal respiration in the surviving animals (Valiyaveettil et al., 2011). Animals pretreated with rePON1 and then exposed to sarin showed a lower blood O_2 saturation at 5–8 min compared to untreated animals exposed to sarin. Although this drop in blood O_2 saturation was not significant, such a drop was also observed in animals pretreated with human serum purified PON1 previously (Valiyaveettil et al., 2011). In contrast, such a drop in blood O_2 saturation was not observed with rabbit serum purified PON1 (Valiyaveettil et al., 2011). This might be due to the higher catalytic efficiency of rabbit serum PON1

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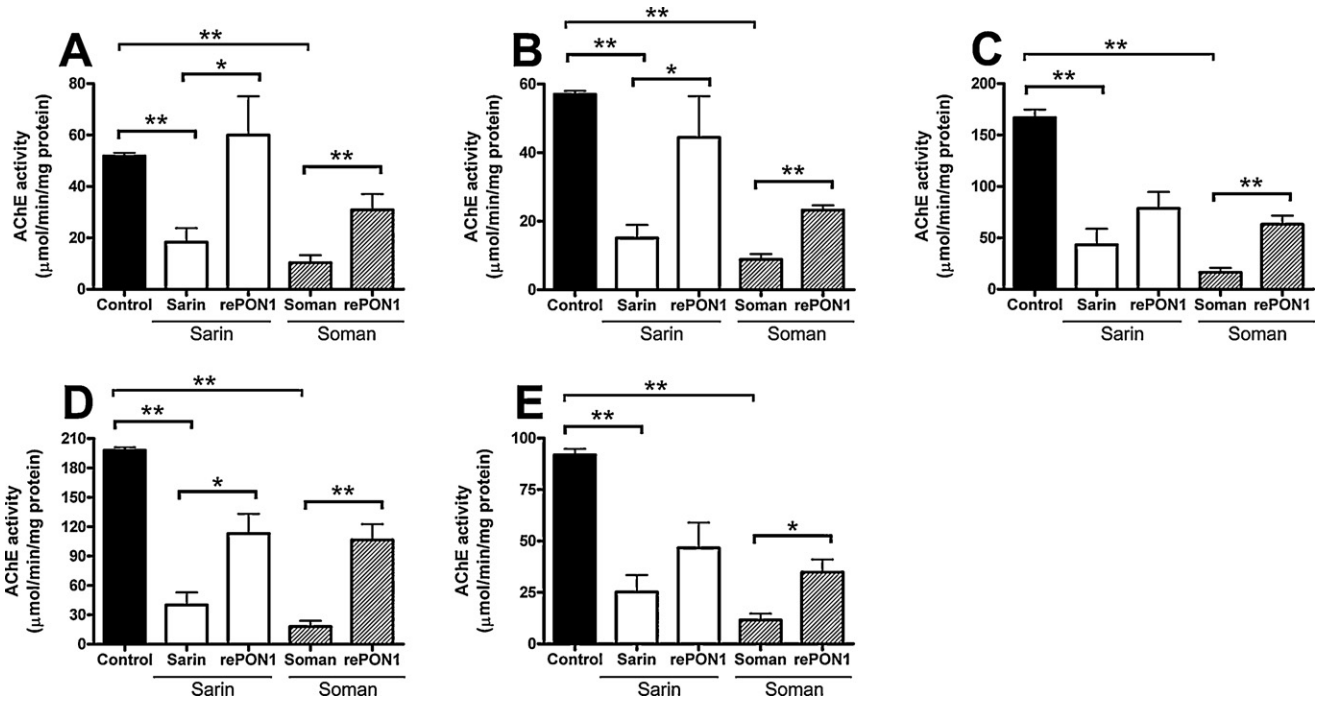

Fig. 4. Brain AChE activity in sarin and soman exposed guinea pigs. The animals were pretreated with rePON1 and exposed to sarin or soman as described in Fig. 1, and different regions of the brain were dissected and extracted with tissue protein extraction reagent. The activity of AChE in the brain extracts were analyzed as described in "Section 2". The results were expressed as μmol/min/mg protein. Shown are the mean ± SEM values for AChE activity from frontal cortex (A), hind cortex (B), mid brain (C) cerebellum (D), and hippocampus (E) (control unexposed, $n = 6$; sarin or soman exposed, $n = 6$; and rePON1, $n = 4–5$; (*) $0.05 < p < 0.01$; (**) $0.01 < p < 0.001$).

compared to recombinant or human serum PON1 against sarin and soman as observed earlier (Valiyaveettil et al., 2010).

Administration of rePON1 showed mild increase in the activity of PON1 in the blood of guinea pigs after 30 min. Upon nerve agent exposure, the activities of PON1, BChE and AChE were significantly decreased in controls and rePON1 pretreated animals. However, the rePON1 pretreated animals exposed to CWNA showed significantly higher activity of blood enzymes compared to the respective agent exposed controls, indicating the *in vivo* hydrolysis of CWNAs by rePON1. The increased enzyme activity in the blood of rePON1 pretreated animals exposed to CWNAs can further protect the guinea pigs against repeated exposure of CWNAs. Similarly, AChE activity from different parts of the brain of rePON1 pretreated animals exposed to nerve agents was higher compared to the respective controls indicating that rePON1 pretreatment protects the central nervous system. The preservation of the activity of AChE and bioscavenger enzymes in the brain and blood of PON1 pretreated animals may be a key reason for the increased survival rate of animals.

As observed earlier with administration of purified serum PON1, intravenous injection of rePON1 showed only a minimum increase in the blood activity of the enzyme in guinea pigs after 30 min (Valiyaveettil et al., 2011). This might be possibly due to the rapid degradation/clearance of the exogenous PON1 in guinea pigs (Boado et al., 2011). The rapid clearance of exogenous PON1 raises the need for pharmacokinetic analysis of the rePON1 and the development of PON1 mutants with better pharmacokinetics (Boado et al., 2011). Reduced half-life of PON1 in circulation can possibly be attributed to poor association of exogenously administered PON1 with HDL, which is the natural *in vivo* carrier of the enzyme (Gaidukov et al., 2009; Harel et al., 2004; Sorenson et al., 1999). Modulators of PON1 which can increase the endogenous expression of the enzyme may overcome the drawback of reduced half-life associated with exogenous PON1 (Costa et al., 2011; Costa et al., 2005c). Natural association of endogenously induced PON1 with HDL may increase the circulatory half-life of PON1. The marginal increase in the activity of PON1 with exogenous administration of recombinant or human and rabbit serum purified PON1 was sufficient to protect against 1.2 X LCt_{50} of nerve agents (Valiyaveettil et al., 2011). Dietary polyphenolic compounds such as quercetin and resveratrol are reported to increase the endogenous expression of PON1 by 2–3 fold which possibly could be adequate to protect against lethal doses of CWNA exposure (Boesch-Saadatmandi et al., 2010; Gouedard et al., 2004).

5. Conclusion

In conclusion, PON1 from multiple sources (recombinant and human and rabbit serum purified) protected against 1.2 X LCt_{50} sarin and soman in guinea pigs using microinstillation inhalation exposure (Valiyaveettil et al., 2011). The protective efficacy was observed in conjunction with mild increase in the blood levels of the enzyme, possibly owing to rapid clearance of exogenous PON1. Recombinant PON1 pretreated animals retain higher levels of cholinesterase and PON1activities that might be responsible for the increased protection and survival. Research efforts to generate rePON1 with higher catalytic efficiency and enhanced pharmacokinetics can drive the future development of PON1 as catalytic bioscavenger suitable to combat against CWNA exposure.

Conflict of interest statement

The authors declare that there are no financial or personal conflicts of interest.

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