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Resveratrol Induces Catalytic Bioscavenger Paraoxonase 1 Expression and Protects Against Chemical Warfare Nerve Agent Toxicity in Human Cell Lines

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Abstract Current advances in enzyme bioscavenger prophylactic therapy against chemical warfare nerve agent (CWNA) exposure are moving towards the identification of catalytic bioscavengers that can degrade large doses of organophosphate (OP) nerve agents without self destruction. This is a preferred method compared to therapy with the purified stoichiometric bioscavenger, butyrylcholinesterase, which binds OPs 1:1 and would thus require larger doses for treatment. Paraoxonase-1 (PON-1) is one such catalytic bioscavenger that has been shown to hydrolyze OP insecticides and contribute to detoxification in animals and humans. Here we investigated the effects of a common red wine ingredient, Resveratrol (RSV), to induce the expression of PON-1 in the human hepatic cell line HC04 and evaluated the protection against CWNA simulants. Dose-response curves showed that a concentration of 20 μM RSV was optimal in inducing PON-1 expression in HC04 cells. RSV at 20 μM increased the extracellular PON-1 activity approximately 150% without significantly affecting the cells. Higher doses of RSV were cytotoxic to the cells. Resveratrol also induced PON-1 in the human lung cell line A549. RSV pre-treatment significantly ($P = 0.05$) protected the hepatic cells against exposure to $2\times$ LD_{50} of soman and sarin simulants. However, lung cells were protected against soman simulant exposure but not against sarin simulant exposure following RSV treatment. In conclusion, these studies indicate that dietary inducers, such as RSV, can up-regulate PON-1, a catalytic bioscavenger, which can then hydrolyze and protect against CWNA-induced toxicity, providing a prospective new method to protect against CWNA exposure. J. Cell. Biochem. 103: 1524–1535, 2008.

Key words: nerve agents; organophosphates; transcriptional inducers; paraoxonase gene expression; neuroprotection

Organophosphorous (OP) chemical warfare nerve agents (CWNAs) inhibit post-synaptic acetylcholinesterase (AChE), which prevents the hydrolysis of the neurotransmitter acetylcholine (ACh) leading to hypercholinergy, seizures/*status epilepticus*, and potentially death. In addition to the central nervous system (CNS), CWNAs also inhibit AChE in the circulatory and respiratory systems, as well as variety of other bodily systems. Blood and plasma cholinesterase, such as human butyrylcholinesterase (BChE) levels play an important role in OP-induced toxicity and have been previously used to determine exposure to CWNAs [Magnotti et al., 1987a,b,c; Kropp and Richardson, 2003a,b; Bajgar et al., 2004a,b; Gordon et al., 2005]. In general, more than 60% inhibition of blood AChE is required to produce CNS seizures [Maxwell et al., 1987; Ehrich et al., 1995]. Therefore, a key strategy

Abbreviations used: CWNA, chemical warfare nerve agent; OP, organophosphate; AChE: acetylcholinesterase; ACh, acetylcholine; BChE, butyrylcholinesterase; RSV, resveratrol; PON-1, paraoxonase-1; HDL, high density lipoprotein; FBS, fetal bovine serum; DMSO, dimethylsulfoxide; *p*-NPA, *para*-nitrophenylacetate; T-PER, tissue protein extraction reagent; CNS, central nervous system; LD_{50}, median lethal dose; GD, soman; GB, sarin

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to protect against CWNA exposure is prophylactic treatment with purified human plasma cholinesterase.

Current investigations with purified enzyme show that use of cholinesterases (AChE and BChE) as bioscavengers is the most prominent method for protection against organophosphate (OP) intoxication [Doctor et al., 1991]. At present, human plasma butyrylcholinesterase is being developed as a prophylactic therapy for nerve agent exposure [Doctor and Saxena, 2005]. It is known for most CWNAs that 1–2 mol of OP inhibits 1 mol of cholinesterase [Raveh et al., 1989; Doctor et al., 1991; Doctor and Saxena, 2005]. As a result, a large dose of the bioscavenger is required to protect against median lethal dose (LD_{50}) exposure to nerve agents. A more effective strategy is therapy with enzymes that can rapidly hydrolyze large amounts of CWNAs in the blood before they reach the brain and other vital organs. Continuous efforts are underway to obtain a catalytic bioscavenger that can more effectively protect against lethal CWNA exposure.

Human serum paraoxonase (PON-1) (EC 3.1.8.1) is a polymorphic enzyme which hydrolyzes OP insecticides and contributes significantly to the detoxification of several OPs in animals and humans [Mackness et al., 1998]. Interspecies differences in PON-1 activity correlate well with observed LD_{50} values for OPs [Davies et al., 1996b]. Administration of external PON-1 protects against OP poisoning in rodents [Li et al., 1995a]. PON-1 has also been shown to hydrolyze nerve agents soman and sarin, and is being considered as a catalytic bioscavenger [Geldmacher-von Mallinckrodt et al., 1984]. Veterans with Gulf War Syndrome and neurological problems had very low levels of PON-1 activity [Haley, 2003]. Recently, it was shown that intravenous administration of naked PON-1 DNA expresses the enzyme and protects mice against the nerve agent challenge [Fu et al., 2005a]. Neurological symptoms were reduced in the animals, indicating that PON-1 hydrolyzed soman in the blood and decreased the amount that reached the brain. Thus, PON-1 therapy could be used to increase the threshold for CWNA toxicity, extend the so-called 'golden hour' (first 1 h after lethal attack), and protect military and civilian personnel at risk of exposure.

Paraoxonase-1 is mainly synthesized in the liver and secreted to the blood, where it associates exclusively with high density lipoproteins (HDLs) [Mackness and Walker, 1988b; Hassett et al., 1991b]. Although PON-1 protein is not readily detectable in other tissues, PCR techniques show that PON-1 mRNA is present in many other tissues, including the lungs [Primo-Parmo et al., 1996c]. The secreted blood PON-1 protein is 355 amino acids long and retains its hydrophobic leader sequence with the exception of the methionine, which is a structural requirement for PON-1's association with HDL. PON-1 knockout mice with no plasma or liver paraoxonase activity showed a dramatic increase in the sensitivity to chloropyriform oxon [Shih et al., 1998]. PON-1 administered to knockout mice to restore plasma enzyme showed protection against chloropyriform oxon [Costa et al., 2003d]. These results indicate that PON-1 can be used to catalytically bioscavenge nerve agents and protect against their cytotoxicity.

Dietary polyphenolic compounds such as quercetin increase the levels of PON-1 mRNA and activity [Gouedard et al., 2004a]. Secreted PON-1 activity and mRNA levels are also increased by fenofibric acid [Gouedard et al., 2003]. PON-1 activity was increased nearly threefold by the red wine ingredient resveratrol in animals [Gouedard et al., 2004b]. These dietary compounds may be useful to protect against low-dose exposure to CWNAs, or to reduce the prophylactic therapeutic doses of PON-1 for protection against lethal CWNAs. Here, we investigated the effect of resveratrol on PON-1 expression in human liver and lung cell lines, and studied the protective effect against analogs of the chemical warfare agents soman and sarin.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The mouse liver cell line HC04 was obtained by a Cooperative Research and Development Agreement from AFIRMS, Bangkok, Thailand. The human lung cell line A549 was obtained from American Type Culture Collection (Manassas, VA). Both cell lines were cultured in RPMI-1640 Medium (Quality Biological, Inc., Gaithersburg, MD) with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium

pyruvate, and 10% fetal bovine serum (FBS, Quality Biological, Inc., Gaithersburg, MD). The cells were cultured at 37°C in an incubator with humidified air and 5% CO_2.

Depletion of Paraoxonases From Fetal Bovine Serum

To minimize the interference during transcriptional induction and the enzyme assay, endogenous serum PON-1 in the fetal bovine serum was depleted using heat inactivation [Simeon and Pavkovic, 1993]. In brief, 50 ml of FBS was thawed to room temperature from frozen storage. The thawed FBS was then incubated at 55°C overnight. The incubated FBS was then filter-sterilized by passing it through 0.22 μm filters. PON-1 depletion was verified by comparing the results of the enzyme assay. This method of heat-inactivation removed nearly 97.3% PON-1 activity (control FBS PON-1 activity 219.2 ± 4.3 ΔOD/min, Heat inactivated FBS, 5.9 ± 0.4, $n = 10$) of the FBS without altering the overall protein amount.

Analysis of the Effect of Dietary Inducers on PON-1 Activity

Cells were plated (2×10^4 cells/well/ml) in 48-well tissue-culture plates (Costar) using PON-1 depleted FBS medium. Resveratrol (RSV, Sigma Chemical Co., St Louis, MO) was diluted in dimethylsulfoxide (DMSO) and various doses of RSV were added to the cells and incubated for different time periods as indicated in each experiment. DMSO diluted similarly was added as a vehicle to the control wells. In order to rule out whether the effect of RSV was due to its anti-oxidant properties, ascorbic acid (10 mM), a general anti-oxidant, was also tested for its effects on PON-1 induction.

Paraoxonase-1 (PON-1) Microassay

Paraoxonase-1 activity and expression was determined using methods adopted from [Furlong et al., 1989] and [Mueller et al., 1983]. For the PON-1 assay, an increase in optical density was monitored at 405 nm for 5 min in a reaction mixture containing 20 μl enzyme, 10 μl of 50 mM *para*-nitrophenylacetate (*p*-NPA), 160 μL of 0.1 M Trizma (TRIS) buffer, and 10 μl calcium chloride ($CaCl_2$) for a final volume of 200 μl. This reaction mixture produces a color change directly related to the amount of PON-1 present in the tested sample. Culture supernatant (20 μl) at the end of the incubation was used as the source of extracellular enzyme.

Protein Determination

Cells were lysed using Tissue Protein Extraction Reagent (T-PER) (Sigma Chemical Co.). Three hundred microliters of the reagent was directly added to the cells and pipetted up and down. Total protein content of the cell lysate was determined by Bradford protein assay (Sigma) using bovine serum albumin as a standard. Ten microliters of the lysate was added to 300 μl of reaction mixture and the optical density was measured at 595 nm in a microplate reader.

Organophosphate Treatment and MTT Cytotoxicity Assay

The organophosphate nerve agent analog simulants; isopropyl *p*-nitrophenyl methylphosphonate (for GB) and pinacolyl *p*-nitrophenyl methylphosphonate (for GD) has been tested for toxicity in H. attenuata [Lum et al., 2003]. We synthesized these analogs in our lab for this study. The structure of the simulants is shown in Figure 1 and has been verified by NMR Spectroscopy. For soman and sarin simulants (soman-NO_2 and sarin-NO_2), the fluorine group is replaced with a *p*-nitrophenyl group. These simulants were diluted in PON-1 depleted medium, added to the cells, and incubated for 2 days. Cell survival was quantified by incubating the cells for 5 h with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml), which yields a blue formazan product in living cells but not in dead cells or their lytic debris [Mosmann, 1983]. The resulting colored product was solubilized in DMSO and the absorbance measured at 550 nm using a microplate reader.

Cell Morphology Analysis by Microscopy

Cell morphology following RSV treatment or organophosphate-induced toxicity was analyzed by phase contrast microscopy using an Olympus microscope, model IX51.

Determination of Protection Against Nerve Agent Simulants

To determine the protective effects of RSV, cells were cultured as described above with various doses of RSV. After 24 h, $2XLD_{50}$ of each

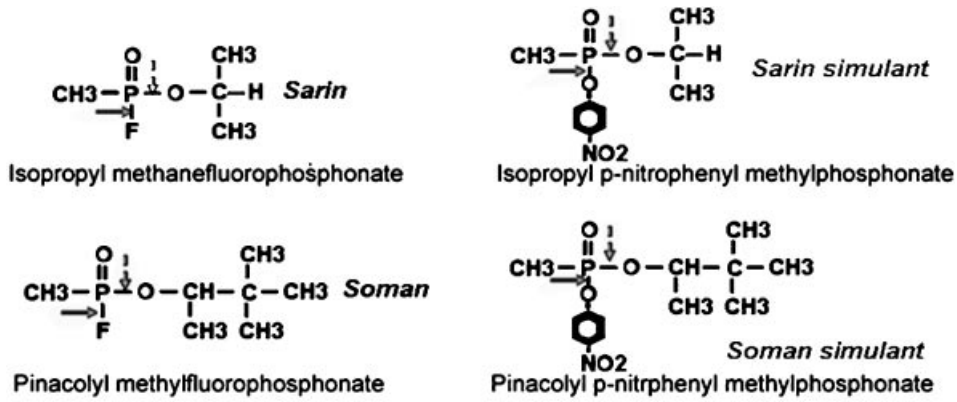

Fig. 1. The structures of soman and sarin simulants synthesized for PON-1 protection studies. Simulants of soman and sarin were synthesized in our department. The structures of the simulants have been verified by NMR Spectroscopy. For soman and sarin simulants (soman-NO_2 and sarin-NO_2), the fluorine group is replaced with a *p*-nitrophenyl group.

soman and sarin OP surrogate was added to the cells. After 48 h of OP incubation, the cells were subjected to a MTT cytotoxicity assay. Fifty microliters of 5 mg/ml MTT were added and the cells were incubated for 2–4 h at 37°C. The cells were washed and solubilized in DMSO. The resulting absorbance at 550 nm compared to that of control cells indicated survivability and protective effects.

Statistical Analysis

The statistical analysis of triplicates was performed by Graphpad Prism 4.0 (San Diego, CA). *P*-values less than 0.05 were considered significant.

RESULTS

Depletion of Serum PON-1 by Heat Inactivation

To reduce the background interference of PON-1 found in the FBS supplement and to determine accurate expression of PON-1 following induction with dietary inducers, the serum PON-1 was depleted using overnight heat inactivation. PON-1 already present in the serum could increase the background and mask the results or exhibit feedback inhibition of enzyme expression. This method of heat-inactivation removed nearly 97.3% PON-1 activity (control FBS PON-1 activity 219.2 ± 4.3 ΔOD/min, heat inactivated FBS, 5.9 ± 0.4 ΔOD/min, $n = 10$) of the FBS without altering the overall protein amount, thus reducing the background activity in our assays.

Resveratrol Upregulates PON-1 Expression in Human Hepatic Cell Line HC04

HC04 cells incubated with 2, 5, 10, 20, 50, 100, and 200 μM resveratrol that was added once at the beginning of the incubation for 24 h. At 24 h, microscopy of the culture plate showed that cell morphology was not altered in RSV-treated cells up to 20 μM compared to control vehicle treated cells. However, RSV doses higher than 20 μM were cytotoxic to the cells (Fig. 2A). Many of the cells were rounded and dead at doses around 50 μM and higher at 24 h. A similar result of cellular toxicity was obtained by MTT cytotoxicity assay (Fig. 2E).

The extracellular PON-1 activity in the culture supernatant was measured as described in the Materials and Methods section. In RSV-treated cells, the extracellular PON-1 level gradually increased with the dose up to 200 μM at 24 h. Without cell death, the optimum induction of PON-1 was approximately 1.5-fold at 20 μM RSV treatment (Fig. 2B). Although higher doses of RSV expressed more PON-1 activity, it was cytotoxic to HC04 cells. The cellular protein level was slightly decreased in cells incubated with 50–200 μM RSV (Fig. 2C). Further incubation of the cells without changing the medium for 48 h resulted in no further increase in the extracellular PON-1 activity. Intracellular PON-1 activity determined after lysis of the cells incubated with

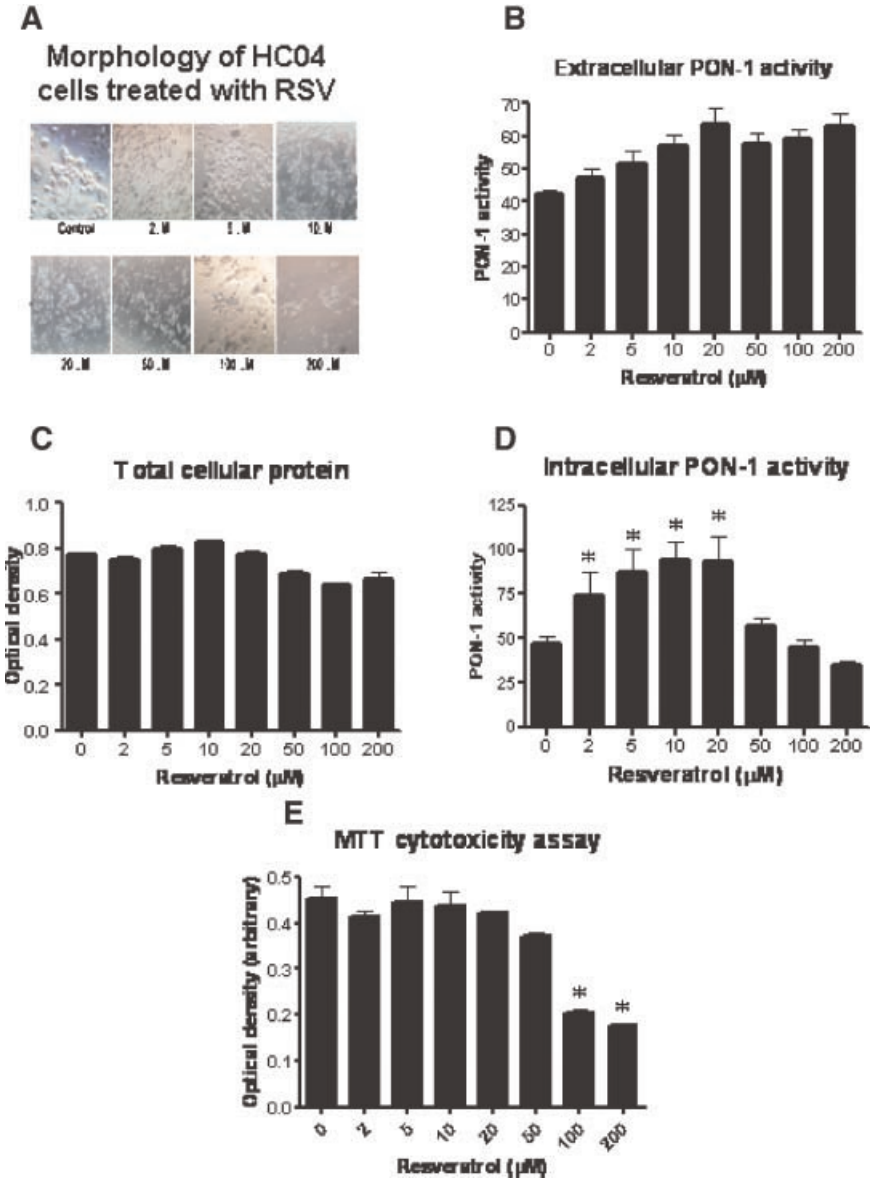

Fig. 2. Effect of resveratrol on the human liver cell line HC04. **A**: Photomicrographs (20×) of HC04 control cells or cells treated with various doses of RSV for 24 h. **B:** Extracellular PON-1 activity was determined by the microassay of 20 μl of the culture supernatant. The effect of RSV on the PON-1 level was determined to be significant ($P < 0.05$). **C**: Cells were lysed with T-PER, and the total cellular protein was measured by Bradford's dye binding assay. A slight decrease was noted, but this difference was not found to be significant. **D**: After lysing the cells, intracellular PON-1 activity was determined by the microassay. All the assays were performed in atleast triplicates and the data expressed as mean ± SEM. Significant effect was observed at the 2–20 μM doses (marked by asterisks, $P < 0.05$). **E**: MTT cytotoxicity assay of cells treated with RSV showing a significant decrease in metabolic activity with increasing concentrations of RSV (marked by asterisks, $P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2–20 μM was increased very similar to the extracellular PON-1 induction up to 20 μM (Fig. 2D). MTT cytotoxicity assay revealed that at higher concentrations RSV is cytotoxic to cells and decreased the metabolic activity (Fig. 2E). These data suggest that a concentration of 20 μM PON-1 is suitable for protection against nerve agent exposure.

To determine if resveratrol induction of PON-1 activity is due to its anti-oxidant effects, the cells were treated similarly with ascorbic acid (10 mM), a general anti-oxidant. Ascorbic acid did not show any significant effect on PON-1 induction (data not shown), indicating that the effects of RSV were not due to its anti-oxidant properties.

Effect of Resveratrol on PON-1 Expression in the Human Lung Cell Line A549

In vivo, PON-1 is expressed in liver cells and secreted to the serum. Although PON-1 is not expressed in other cells, it has been shown that PON-1 mRNA is present in other tissues such as lung cells. To determine whether resveratrol can induce PON-1 in tissue culture cells other than liver, human lung cells (A549) cells were incubated with 2–200 μM resveratrol for 24 h and assayed for PON-1 activity. The basal level of PON-1 in A-549 cells was less than HC04 liver cells. Extracellular PON-1 level increased in RSV treated A549 cells at 24 h. The optimum induction of PON-1 was approximately 13–15% at 2–20 μM RSV treatment (Fig. 3B) without any cell death. Although higher doses of RSV induced PON-1, it was cytotoxic and the cellular protein level slightly decreased in cells incubated with 50–200 μM RSV (Fig. 3C). These results suggest that RSV induced PON-1 in human lung cells to a lesser extent compared to liver cell lines.

Effect of the Nerve Agent Simulants on HC04 and A549 Cells

To evaluate the protective effect of RSV treatment against the cytotoxicity of chemical warfare nerve agent exposure, we first determined the toxicity of simulants of soman and sarin in HC04 cells. Starting from concentrations of 2.164 mg/ml for soman-NO_2 and 2.044 mg/ml for sarin-NO_2 the cells were incubated with serial two-fold dilutions of sarin-NO_2 and soman-NO_2 for 2 days. Significant cytotoxicity was evident with soman-NO_2 and sarin-NO_2 even at low concentrations (0.01–0.05 mg/ml) by microscopy. Nerve agent simulant-induced cytotoxicity in HC04 cells was visible by floating dead cells and a reduction in the number of cells adhered to the cell culture plate. The MTT cytotoxicity assay that

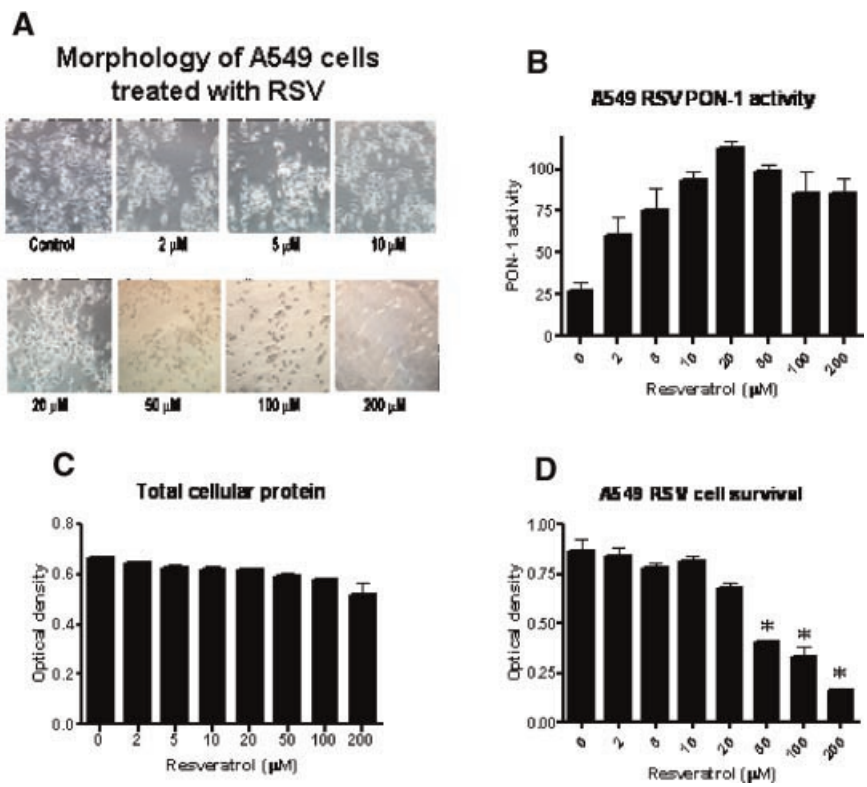

Fig. 3. Effect of resveratrol on human lung cell line A549. **A**: Photomicrographs (20×) showing A549 cells that were treated with various doses of RSV for 24 h. **B**: Extracellular PON-1 activity was determined by the microassay with 20 μl of the culture supernatant. Significant increases were observed at all doses (asterisks, $P < 0.05$). **C**: Cells were lysed with 1% Nonidet-P40 and the total cellular protein was determined using Bradford's dye binding assay. All assays were performed in at least triplicates and the data expressed as mean ± SEM. No significant change in Optical Density was observed. **D**: MTT cytotoxicity assay of A549 cells treated with RSV showed a decrease in metabolic activity with increasing concentrations of RSV, significantly at 50–200 μM RSV (marked by asterisks, $P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

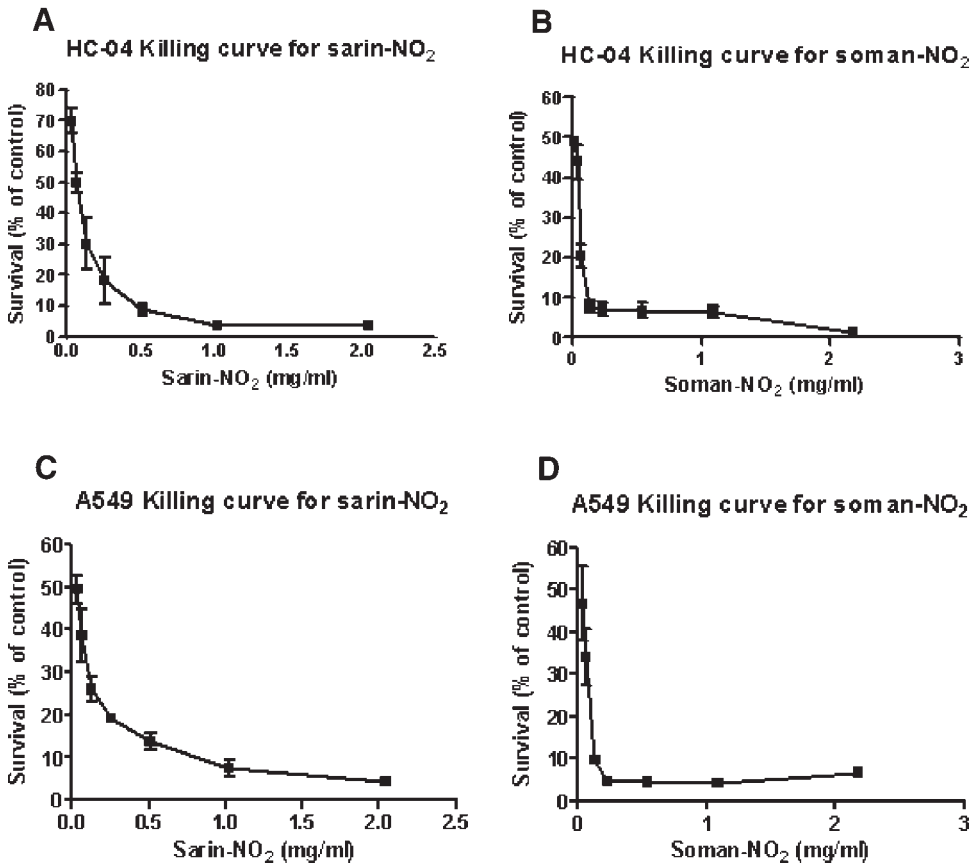

Fig. 4. The cytotoxicity of soman and sarin simulants in HC04 and A549 cells. The cytotoxicity of nerve agent simulants were measured by the MTT assay as described in the Materials and Methods section and represented as the percent of control. A,B: Human liver cell line HC04; C,D: Human lung cell line A549. A,C: Soman stimulant. B,D: Sarin stimulant. All the assays were performed in triplicates and the data is expressed as mean ± SEM.

determines metabolic survival yielded an LD_{50} of 0.068 mg/ml for soman-NO_2 0.064 mg/ml for sarin-NO_2 in HC04 cells (Fig. 4A,B). Sarin-NO_2 was albeit less toxic than soman-NO_2. A similar cytotoxicty result was also observed in the human lung cell line (Fig. 4C,D).

Resveratrol Treatment Protects the Cells From Soman-NO_2 and Sarin-NO_2 Induced Cytotoxicity

To determine whether the PON-1 induction following RSV-treatment would protect the cells against OP exposure, HC04 cells were incubated with 2, 5, 10, and 20 μM resveratrol for the optimal induction period of 24 h. Next, 2× LD_{50} of soman-NO_2 and sarin-NO_2 were added to each of the wells containing RSV and control cells and incubated for 2 days. Microscopy results showed that the cells treated first with 2–20 μM RSV followed by soman-NO2 or sarin-NO2 survived and retained their original cellular morphology (Cell 5A and 5B). Survivability was greatest at 20 μM RSV treated cells. The protection of cells by RSV against nerve agent simulant cytotoxicity was measured by the MTT cytotoxicity assay and represented as a percentage of control samples treated with RSV alone. Consistent with the cellular morphology, the cytotoxicity data shows that 2–20 μM RSV significantly ($P < 0.0001$) protected the HC04 cells against nerve agent simulant exposure (Fig. 5C). The protection was nearly 100% in samples treated with 2, 5, 10, and 20 μM RSV indicating that a small expression of PON-1 can induce significant protection.

The protection of RSV-induced PON-1 was also evaluated in the A549 human lung cell line. As shown in Figure 6 cells treated with RSV protected against the toxicity of soman-NO_2 simulant. However, sarin-NO_2 toxicity was not protected by RSV treatment in A549 cells

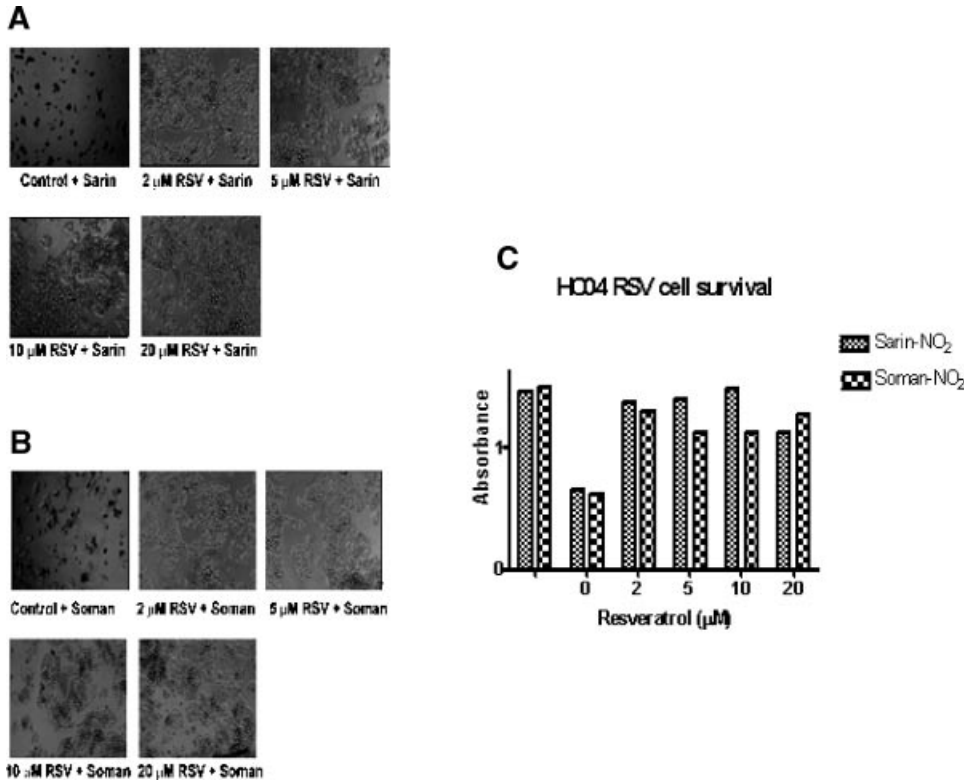

Fig. 5. RSV protects HC04 cells from the cytotoxicity of nerve agent simulants. **A**: Photomicrographs (20×) showing cells treated with various RSV and 2× LD_{50} of sarin for 24 h. **B**: Photomicrographs (20×) showing cells treated with various doses of RSV and 2× LD_{50} of soman for 24 h. **C**: The protective effect of RSV was measured against 2× LD_{50} of nerve agent simulant by the MTT cytotoxicity assay as described in the Materials and Methods section. The protection was expressed as a percentage of control cells treated with RSV but not treated simulants. All the assays were performed in triplicates and the data expressed as mean.

(Fig. 6B). The level of protection against soman-NO_2 protection correlated with the level of expression of PON-1 (Figs. 6 and 3B). The protection was optimum at 20 μM RSV treated cells (Fig. 6B). The cell morphology was completely preserved in RSV treated A549 cells exposed to soman-NO_2 (Fig. 6A). Thus, induction of cellular PON-1 by RSV can bioscavenge the organophosphate soman-NO_2 and protect against its toxicity in A549 lung cell line.

DISCUSSION

The major finding in this study is that the red wine ingredient resveratrol induces paraoxonase-1 expression in a human liver cell line, HC04, and to a lesser extent in human lung cell line A549, thus protecting against the cytotoxicity induced by chemical warfare agent soman and sarin simulants. As expected, the induction of the enzyme was much higher in the liver cell line compared to human lung cell line. Human PON-1 is mainly synthesized in the liver and secreted to the blood, where it associates exclusively with high density lipoproteins (HDLs) [Mallinckrodt et al., 1979; Mackness and Walker, 1988a; Hassett et al., 1991a; Adkins et al., 1993; Primo-Parmo et al., 1996b; Boright et al., 1998; Cole et al., 2003; Costa et al., 2003c, 2005b; Furlong et al., 2005]. Although PON-1 protein is not readily detectable in other tissues, PCR techniques show that PON-1 mRNA is present in many tissues in the body [Primo-Parmo et al., 1996a]. This observation could be the reason why RSV induces PON-1 to a lower extent in the lung cell line.

Nearly 100% protection at the lowest dose of RSV in the hepatic cell line indicates that a small amount of PON-1 is sufficient for protection against lethal exposure to nerve agents, an inherent property of a catalytic bioscavenger. Thus, dietary inducers of PON-1 might be sufficient to protect against low-dose exposure to CWNAs. Since PON-1 also has anti-oxidant

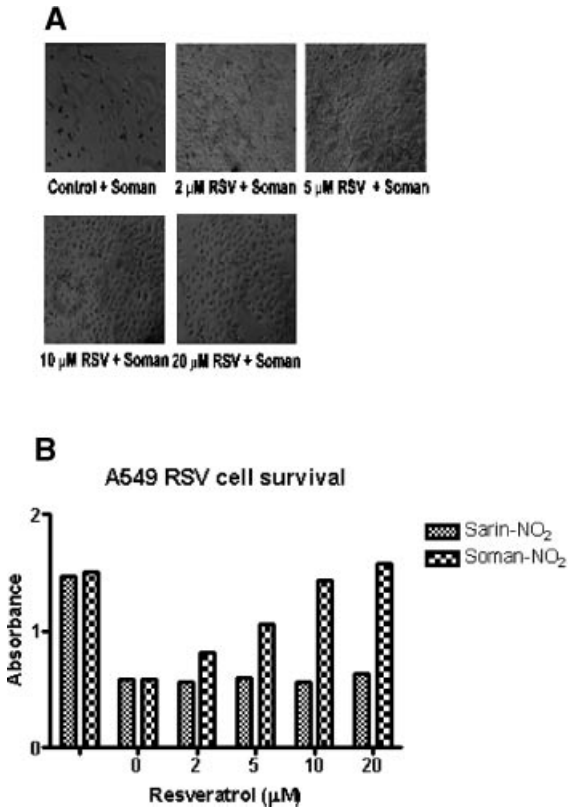

Fig. 6. RSV protects human lung A549 cells from the cytotoxicity of soman simulants. **A**: Photomicrograph (20×) of showing cells treated with various doses of RSV and 2× LD_{50} soman simulant. **B**: The protective effect of RSV was measured in the nerve agent stimulant by the MTT cytotoxicity assay as described in Materials and Methods section. The protection was expressed as a percentage of control cells treated with RSV but not treated simulants. All the assays were performed in triplicates and the data expressed as mean.

properties, it should provide additional protection against stress and deployment-related sickness under hostile conditions of war [Aviram, 1999; Boemi et al., 2004].

It is not clear why resveratrol treatment of lung cells did not protect against the sarin simulant but protected against the soman simulant. One possibility is that sarin is hydrolyzed to a lesser extent by PON-1 compared to soman [La Du, 1996; Davies et al., 1996a; Yamasaki et al., 1997; Costa et al., 1999c; Furlong et al., 2000]. So the lower expression of PON-1 and low catalytic activity against sarin may not be adequate for protection. RSV induced PON-1 at a lower concentration, however, it showed less protection against sarin simulant in the lung cell line. On the other hand, liver produces other bioscavengers such as butyrylcholinesterase, carboxylesterase and albumin. A marginal induction of these bioscvaengers may provide additive effect and protect against sarin-NO_2 toxicity. Preliminary experiments with resveratrol did not reveal any induction of BChE, AChE or carboxylesterase in liver cells (Nambiar et al., unpublished work). Further studies are required to understand the molecular mechanism of this discrepancy. It is interesting to note that the sarin simulant was also not hydrolyzed by the induction of PON-1 in the human lung cell line by fenofibrate, another inducer of PON-1.

Protection against nerve agent by PON-1 in human lung cells is important in lieu of the fact that inhalation is predicted to be the major route of exposure to chemical warfare agents during war or terrorism. Removal of residual agent in the airway is important in reducing systemic absorption.

Transcriptional induction of bioscavengers is an innovative new strategy for combating organophosphorous chemical warfare agent exposure. We have previously shown that Trichostatin A, a histone deactylase inhibitor, has been shown to transcriptionally induce endogenous AChE expression and protect neuronal cells from OP poisoning [Curtin et al., 2005]. Induction of endogenous enzymes will minimize the production of antibodies that would otherwise be formed in response to the complex structure and post-translational modification of the administered purified enzymes from pooled human plasma. Also, the cost of drug, labor, and treatment will be much lower by using transcriptional inducers compared to purified enzyme treatments. Another advantage of using dietary transcriptional inducers is that the elevated enzyme level can be maintained as long as the inducer is present in the body and has a non-invasive administration. Moreover, induction of intracellular PON-1 can protect against the intracellular toxicity of OPs unlike purified enzymes that may not be endocytosed into the cells. While induction of cholinesterases is a powerful tool to combat the loss of the enzyme caused by CWNAs, it is only a 1:1 stoichiometric effect, demanding a large dose of enzyme treatment for protection. PON-1 acts catalytically and is not self destructive, thus necessitating only a small amount of induction for a significant protective effect against CWNA exposure [Li et al., 1995b; Costa et al., 1999b].

Resveratrol has been shown to induce PON-1 gene expression in mouse hepatocytes, and has

been credited for the cardio-protective effects of red wine [Gouedard et al., 2004c]. While resveratrol has been expressed, its protective ability against chemical warfare nerve agents has not been tested. Our results indicate that RSV, which induces only a modest increase in the amount of PON-1, can significantly protect cells from CWNA-induced toxicity. Previous studies indicate that RSV specifically induced PON-1 at the transcriptional level [Gouedard et al., 2004d]. Also, there is no significant increase in the protein level in cells treated with resveratrol, indicating that the protective effect is not due to the possible expression of other factors.

In order to rule out whether the effect of RSV was due to its anti-oxidant properties, ascorbic acid (10 mM), a general anti-oxidant was also evaluated for PON-1 induction. Ascorbic acid (10 mM), was shown to have no significant effect on PON-1 induction (data not shown), indicating that the effects of RSV on PON-1 induction were not due to its anti-oxidant properties.

The fact that RSV induces PON-1 by transcriptional induction and protects against CWNA exposure is supported by the observation that it needs more than a 6 h exposure for detectable induction and 24 h for optimal induction and protection against CWNA exposure. Continuous addition of RSV at 6–12 h intervals may further induce the PON-1 expression and protect against higher doses of nerve agents.

Recently it has been shown that administration of naked PON-1 plasmid DNA to the tail mice induced PON-1 expression [Fu et al., 2005b]. These mice also survived soman exposure indicating that PON-1 expression can protect against nerve agents in animals. It has also been reported that endogenous levels of PON-1 or the presence of mutations in its gene determine the threshold of OP toxicity [Li et al., 1993; Costa et al., 1999a, 2003a,b, 2005a; Worth, 2002]. Currently, we are in the process of testing dietary PON-1 inducers for protection against nerve agent exposure in a rat seizure model using radiotelemetry instrumentation.

In summary, the activity of the chemical warfare nerve agent catalytic bioscavenger PON-1 can be induced by an ingredient of red wine, resveratrol. The induced enzyme can bioscavenge the nerve agent simulant and protect the human hepatic cells from organophosphate-induced cytotoxicity. Resveratrol also induced PON-1 in the human lung cell line A549 and protected against soman simulant. Thus, dietary inducers of PON-1 such as resveratrol could be used as novel pretreatment for chemical warfare nerve agent exposure.

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REFERENCES

Adkins S, Gan KN, Mody M, La Du BN. 1993. Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: Glutamine or arginine at position 191, for the respective A or B allozymes. Am J Hum Genet 52:598–608.

Aviram M. 1999. Cholesterol oxidation, macrophage foam cells and atherosclerosis: Importance of antioxidants and paraoxonase. Harefuah 136:620–626.

Bajgar J, Sevelova L, Krejcova G, Fusek J, Vachek J, Kassa J, Herink J, De Jong LP, Benschop HP. 2004a. Biochemical and behavioral effects of soman vapors in low concentrations. Inhal Toxicol 16:497–507.

Bajgar J, Sevelova L, Krejcova G, Fusek J, Vachek J, Kassa J, Herink J, De Jong LP, Benschop HP. 2004b. Biochemical and behavioral effects of soman vapors in low concentrations. Inhal Toxicol 16:497–507.

Boemi M, Sirolla C, Testa R, Cenerelli S, Fumelli P, James RW. 2004. Smoking is associated with reduced serum levels of the antioxidant enzyme, paraoxonase, in Type 2 diabetic patients. Diabet Med 21:423–427.

Boright AP, Connelly PW, Brunt JH, Scherer SW, Tsui LC, Hegele RA. 1998. Genetic variation in paraoxonase-1 and paraoxonase-2 is associated with variation in plasma lipoproteins in Alberta Hutterites. Atherosclerosis 139:131–136.

Cole TB, Jampsa RL, Walter BJ, Arndt TL, Richter RJ, Shih DM, Tward A, Lusis AJ, Jack RM, Costa LG, Furlong CE. 2003. Expression of human paraoxonase (PON1) during development. Pharmacogenetics 13:357–364.

Costa LG, Li WF, Richter RJ, Shih DM, Lusis A, Furlong CE. 1999a. The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism. Chem Biol Interact 119–120:429–438.

Costa LG, Li WF, Richter RJ, Shih DM, Lusis A, Furlong CE. 1999b. The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism. Chem Biol Interact 119–120:429–438.

Costa LG, Li WF, Richter RJ, Shih DM, Lusis A, Furlong CE. 1999c. The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism. Chem Biol Interact 119–120:429–438.

Costa LG, Cole TB, Furlong CE. 2003a. Polymorphisms of paraoxonase (PON1) and their significance in clinical toxicology of organophosphates. J Toxicol Clin Toxicol 41:37–45.

Costa LG, Richter RJ, Li WF, Cole T, Guizzetti M, Furlong CE. 2003b. Paraoxonase (PON 1) as a biomarker of susceptibility for organophosphate toxicity. Biomarkers 8:1–12.

Costa LG, Richter RJ, Li WF, Cole T, Guizzetti M, Furlong CE. 2003c. Paraoxonase (PON 1) as a biomarker of susceptibility for organophosphate toxicity. Biomarkers 8:1–12.

Costa LG, Richter RJ, Li WF, Cole T, Guizzetti M, Furlong CE. 2003d. Paraoxonase (PON 1) as a biomarker of susceptibility for organophosphate toxicity. Biomarkers 8:1–12.

Costa LG, Cole TB, Vitalone A, Furlong CE. 2005a. Measurement of paraoxonase (PON1) status as a potential biomarker of susceptibility to organophosphate toxicity. Clin Chim Acta 352:37–47.

Costa LG, Vitalone A, Cole TB, Furlong CE. 2005b. Modulation of paraoxonase (PON1) activity. Biochem Pharmacol 69:541–550.

Curtin BF, Tetz LM, Compton JR, Doctor BP, Gordon RK, Nambiar MP. 2005. Histone acetylase inhibitor trichostatin A induces acetylcholinesterase expression and protects against organophosphate exposure. J Cell Biochem 96:839–849.

Davies HG, Richter RJ, Keifer M, Broomfield CA, Sowalla J, Furlong CE. 1996a. The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. Nat Genet 14:334–336.

Davies HG, Richter RJ, Keifer M, Broomfield CA, Sowalla J, Furlong CE. 1996b. The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. Nat Genet 14:334–336.

Doctor BP, Saxena A. 2005. Bioscavengers for the protection of humans against organophosphate toxicity. Chem Biol Interact 157–158:167–171.

Doctor BP, Raveh L, Wolfe AD, Maxwell DM, Ashani Y. 1991. Enzymes as pretreatment drugs for organophosphate toxicity. Neurosci Biobehav Rev 15:123–128.

Ehrich M, Jortner BS, Padilla S. 1995. Comparison of the relative inhibition of acetylcholinesterase and neuropathy target esterase in rats and hens given cholinesterase inhibitors. Fundam Appl Toxicol 24:94–101.

Fu AL, Wang YX, Sun MJ. 2005a. Naked DNA prevents soman intoxication. Biochem Biophys Res Commun 328: 901–905.

Fu AL, Wang YX, Sun MJ. 2005b. Naked DNA prevents soman intoxication. Biochem Biophys Res Commun 328: 901–905.

Furlong CE, Richter RJ, Seidel SL, Costa LG, Motulsky AG. 1989. Spectrophotometric assays for the enzymatic hydrolysis of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase. Anal Biochem 180:242–247.

Furlong CE, Li WF, Richter RJ, Shih DM, Lusis AJ, Alleva E, Costa LG. 2000. Genetic and temporal determinants of pesticide sensitivity: Role of paraoxonase (PON1). Neurotoxicology 21:91–100.

Furlong CE, Cole TB, Walter BJ, Shih DM, Tward A, Lusis AJ, Timchalk C, Richter RJ, Costa LG. 2005. Paraoxonase 1 (PON1) status and risk of insecticide exposure. J Biochem Mol Toxicol 19:182–183.

Geldmacher-von Mallinckrodt M, Diepgen TL, Enders PW. 1984. Interethnic differences of human serum paraoxonase activity-relevance for the detoxification of organophosphorous compounds. Arch Belg (Suppl):243–251.

Gordon RK, Haigh JR, Garcia GE, Feaster SR, Riel MA, Lenz DE, Aisen PS, Doctor BP. 2005. Oral administration of pyridostigmine bromide and huperzine A protects human whole blood cholinesterases from ex vivo exposure to soman. Chem Biol Interact

Gouedard C, Koum-Besson N, Barouki R, Morel Y. 2003. Opposite regulation of the human paraoxonase-1 gene PON-1 by fenofibrate and statins. Mol Pharmacol 63: 945–956.

Gouedard C, Barouki R, Morel Y. 2004a. Dietary polyphenols increase paraoxonase 1 gene expression by an aryl hydrocarbon receptor-dependent mechanism. Mol Cell Biol 24:5209–5222.

Gouedard C, Barouki R, Morel Y. 2004b. Induction of the paraoxonase-1 gene expression by resveratrol. Arterioscler Thromb Vasc Biol 24:2378–2383.

Gouedard C, Barouki R, Morel Y. 2004c. Induction of the paraoxonase-1 gene expression by resveratrol. Arterioscler Thromb Vasc Biol 24:2378–2383.

Gouedard C, Barouki R, Morel Y. 2004d. Induction of the paraoxonase-1 gene expression by resveratrol. Arterioscler Thromb Vasc Biol 24:2378–2383.

Haley RW. 2003. Gulf war syndrome: Narrowing the possibilities. Lancet Neurol 2:272–273.

Hassett C, Richter RJ, Humbert R, Chapline C, Crabb JW, Omiecinski CJ, Furlong CE. 1991a. Characterization of cDNA clones encoding rabbit and human serum paraoxonase: The mature protein retains its signal sequence. Biochemistry 30:10141–10149.

Hassett C, Richter RJ, Humbert R, Chapline C, Crabb JW, Omiecinski CJ, Furlong CE. 1991b. Characterization of cDNA clones encoding rabbit and human serum paraoxonase: The mature protein retains its signal sequence. Biochemistry 30:10141–10149.

Kropp T, Richardson R. 2003a. Relative inhibitory potencies of chlorpyrifos oxon, chlorpyrifos methyl oxon, and mipafox for acetylcholinesterase versus neuropathy target esterase. J Toxicol Environ Health A 66:1145–1157.

Kropp T, Richardson R. 2003b. Relative inhibitory potencies of chlorpyrifos oxon, chlorpyrifos methyl oxon, and mipafox for acetylcholinesterase versus neuropathy target esterase. J Toxicol Environ Health A 66:1145–1157.

La Du BN. 1996. Structural and functional diversity of paraoxonases. Nat. Med 2:1186–1187.

Li WF, Costa LG, Furlong CE. 1993. Serum paraoxonase status: A major factor in determining resistance to organophosphates. J Toxicol Environ Health 40:337–346.

Li WF, Furlong CE, Costa LG. 1995a. Paraoxonase protects against chlorpyrifos toxicity in mice. Toxicol Lett 76:219–226.

Li WF, Furlong CE, Costa LG. 1995b. Paraoxonase protects against chlorpyrifos toxicity in mice. Toxicol Lett 76:219–226.

Lum KT, Huebner HJ, Li Y, Phillips TD, Raushel FM. 2003. Organophosphate nerve agent toxicity in Hydra attenuata. Chem Res Toxicol 16:953–957.

Mackness MI, Walker CH. 1988a. Multiple forms of sheep serum A-esterase activity associated with the high-density lipoprotein. Biochem J 250:539–545.

Mackness MI, Walker CH. 1988b. Multiple forms of sheep serum A-esterase activity associated with the high-density lipoprotein. Biochem J 250:539–545.

Mackness B, Durrington PN, Mackness MI. 1998. Human serum paraoxonase. Gen Pharmacol 31:329–336.

Magnotti RA Jr, Eberly JP, Quarm DE, McConnell RS. 1987a. Measurement of acetylcholinesterase in erythrocytes in the field. Clin Chem 33:1731–1735.

Magnotti RA, Jr., Eberly JP, Quarm DE, McConnell RS. 1987b. Measurement of acetylcholinesterase in erythrocytes in the field. Clin Chem 33:1731–1735.

Magnotti RA, Jr., Eberly JP, Quarm DE, McConnell RS. 1987c. Measurement of acetylcholinesterase in erythrocytes in the field. Clin Chem 33:1731–1735.

Mallinckrodt M, Hommel G, Dumbach J. 1979. On the genetics of the human serum paraoxonase (EC 3.1.1.2). Hum Genet 50:313–326.

Maxwell DM, Brecht KM, O'Neill BL. 1987. The effect of carboxylesterase inhibition on interspecies differences in soman toxicity. Toxicol Lett 39:35–42.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63.

Mueller RF, Hornung S, Furlong CE, Anderson J, Giblett ER, Motulsky AG. 1983. Plasma paraoxonase polymorphism: A new enzyme assay, population, family, biochemical, and linkage studies. Am J Hum Genet 35: 393–408.

Primo-Parmo SL, Sorenson RC, Teiber J, La Du BN. 1996a. The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. Genomics 33:498–507.

Primo-Parmo SL, Sorenson RC, Teiber J, La Du BN. 1996b. The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. Genomics 33:498–507.

Primo-Parmo SL, Sorenson RC, Teiber J, La Du BN. 1996c. The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. Genomics 33:498–507.

Raveh L, Ashani Y, Levy D, De La HD, Wolfe AD, Doctor BP. 1989. Acetylcholinesterase prophylaxis against organophosphate poisoning. Quantitative correlation between protection and blood-enzyme level in mice. Biochem Pharmacol 38:529–534.

Shih DM, Gu L, Xia YR, Navab M, Li WF, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM, Lusis AJ. 1998. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. Nature 394:284–287.

Simeon V, Pavkovic E. 1993. Heat inactivation of paraoxonase and arylesterase activities in human and rabbit serum. Chem Biol Interact 87:103–107.

Worth J. 2002. Paraoxonase polymorphisms and organophosphates. Lancet 360:802–803.

Yamasaki Y, Sakamoto K, Watada H, Kajimoto Y, Hori M. 1997. The Arg192 isoform of paraoxonase with low sarin-hydrolyzing activity is dominant in the Japanese. Hum Genet 101:67–68.