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Novel approaches to mitigating parathion toxicity: targeting cytochrome P450–mediated metabolism with menadione

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Abstract

Accidental or intentional exposures to parathion, an organophosphorus (OP) pesticide, can cause severe poisoning in humans. Parathion toxicity is dependent on its metabolism by the cytochrome P450 (CYP) system to paraoxon (diethyl 4-nitrophenyl phosphate), a highly poisonous nerve agent and potent inhibitor of acetylcholinesterase (AChE). We have been investigating inhibitors of CYP-mediated bioactivation of OPs as a method of preventing or reversing progressive parathion toxicity. It is well recognized that NADPH–cytochrome P450 reductase, an enzyme required for the transfer of electrons to CYPs, mediates chemical redox cycling. In this process, the enzyme diverts electrons from CYPs to support chemical redox cycling, which results in inhibition of CYP-mediated biotransformation. Using menadione as the redox-cycling chemical, we discovered that this enzymatic reaction blocks metabolic activation of parathion in rat and human liver microsomes and in recombinant CYPs important to parathion metabolism, including CYP1A2, CYP2B6, and CYP3A4. Administration of menadione to rats reduces metabolism of parathion, as well as parathion-induced inhibition of brain cholinesterase activity. This resulted in inhibition of parathion neurotoxicity. Menadione has relatively low toxicity and is approved by the FDA for other indications. Its ability to block parathion metabolism makes it an attractive therapeutic candidate to mitigate parathion-induced neurotoxicity.

Keywords

redox cycling; parathion; paraoxon; menadione; organophosphorus pesticides

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Conflicts of interest

The authors declare no conflicts of interest.

Introduction

Organophosphates (OPs) are widely used as pesticides, as they are inhibitors of many serine esterases, notably including acetylcholinesterase (AChE).[1,2] Exposure of humans to OPs, either as a result of accidental or intentional exposures, can result in severe neurotoxicity.[1–3] The most common organophosphates are modified esters of phosphoric acid. Phosphotriesters, which contain a phosphoryl (P=O bond) group, are direct inhibitors of AChE, while phosphorothioate pesticides, which contain a thiophosphoryl (P=S bond) group, are indirect inhibitors of AChE and require metabolic activation to became biologically active oxons.[3] This latter class of cholinesterase inhibitors includes a variety of OP pesticides, such as parathion, chlorpyrifos, malathion, and disulfoton, each of which becomes more toxic than the parent compound following metabolic activation by the cytochrome P450 (CYP) system (see Fig. 1 for structures). The former class of OPs includes traditional nerve warfare agents, such as tabun, sarin, soman, VX, and oxono metabolites of phosphorothioate pesticides. As a result of inhibiting AChE, these agents cause an accumulation of acetylcholine in synapses, a process that can cause overstimulation of muscarinic and nicotinic cholinergic receptors.[1,3] This can lead to a “cholinergic crisis,” leading to tremors, convulsions, and increased secretions.[1,3]

Because of the threat of toxicity resulting from exposure to OPs, there remains a considerable interest in the development and use of effective antidotes. Two drugs that are currently approved for OP poisoning are atropine and pralidoxime (2-PAM).[4,5] Atropine is a competitive AChE antagonist but is limited by the fact that it only targets muscarinic AChE.[4] 2-PAM binds to OP-inactivated AChE and regenerates the enzyme; however, it is a charged molecule and does not efficiently cross the blood–brain barrier.[6] New strategies to generate more effective drugs to treat OP intoxication have included oxime reactivators that can cross the blood–brain barrier,[7,8] chemical scavengers such as cyclodextrins,[9] and bioscavengers, which include enzymes that neutralize and/or detoxify OPs, such as AChE, butyrylcholinesterases, carboxylesterases, paraoxonases, and phosphotriesterases.[10–13] Many of these drug candidates do not possess a broad spectrum for many OP substrates; enzymes scavengers are also difficult to produce, and many lack an efficient delivery system to target organs.[14–16]

An alternative approach to protect against the toxicity of indirect OP AChE inhibitors is to limit their metabolism to active compounds; this can be accomplished by increasing enzymes that detoxify OPs and inhibiting CYPs critical for their metabolism. Earlier studies using pretreatments with agents that either increased detoxification enzymes or inhibited CYP activity met with only limited success. For example, compounds that increase detoxification of OPs, such as chlorcyclizine, phenobarbital, beta-napthoflavone, and polychlorinated biphenyls reduce the toxicity of the OP and parathion in animal models; however, several days of pretreatments are required.[17,18] Compounds that directly inhibit CYPs, such as cimetidine (1-cyano-2-methyl-3-[2-[(5-methyl-1H-imidazol-4-yl)methylsulfanyl]ethyl]guanidine), a H2 histamine receptor blocker, unexpectedly enhanced oxon formation from parathion, as well as parathion toxicity.[19,20] Piperonyl butoxide, a pesticide that also inhibits CYP activity, was found to reduce the lethality of parathion in mice.[21] However, in rats, it had no effect on parathion-induced inhibition of brain

cholinesterase.[22] Whereas nonselective CYP antagonists, including SKF525A (2-diethylaminoethyl 2,2-diphenylpentanoate) and ketoconazole, were found to inhibit OP metabolism in rodents, their ability to suppress OP intoxication was limited, possibly due to the fact that they also inhibit degradation of the biologically active OP oxons.[23,24] Because of deficits in the ability of different inhibitors of OP metabolism to prevent or reverse toxicity, none have been approved by the Food and Drug Administration for use in humans suffering from OP intoxication.

The cytochrome P450 system and chemical redox cycling

CYPs play essential roles in the biotransformation of a wide variety of endogenous and exogenous compounds, including OPs.[25,26] Biotransformation is dependent on the transfer of reducing equivalents from NADPH to the CYPs, a process mediated by the flavin adenine dinucleotide (FAD) and the flavin mononucleotide (FMN)–containing enzyme NADPH–cytochrome P450 reductase.[26] This reaction involves an interflavin electron transfer between stable semiquinone FAD/FMN intermediates in NADPH–cytochrome P450 reductase followed by a sequential two-electron transfer to CYP heme. Of interest is the fact that NADPH–cytochrome P450 reductase also mediates a process known as *chemical redox cycling*.[27] In this NADPH-dependent process, redox-active compounds are enzymatically reduced to radical anions, which are unstable. Readily reducing molecular oxygen, these radicals generate superoxide anion and, in the process, regenerate the parent compound, which is then free, once again, to be reduced by NADPH–cytochrome P450 reductase. As this process continually recurs, utilizes, a disproportionate amount of reducing equivalents and oxygen, generates reactive oxygen species, and does not appear to be metabolically relevant for cell function, it has been referred to as “futile metabolism” or “futile redox cycling.”[2728] Superoxide anions formed through this cycle can be converted to hydrogen peroxide via superoxide dismutase.[28] While hydrogen peroxide can be detoxified by enzymes, such as catalase, glutathione peroxidase, and the peroxiredoxins, in the presence of transition metals, hydrogen peroxide can also form highly toxic hydroxyl radicals, which can cause cell damage.[29] Superoxide anions can also react with nitric oxide, forming peroxynitrite, a reactive nitrogen intermediate that can induce nitrosative stress.[30]

In addition to generating reactive oxygen species, which can lead to oxidative and nitrosative stress, chemical redox cycling has the potential to deplete cells of NADPH and oxygen, compromising metabolic reactions requiring these co-substrates.[31] Low oxygen tension can also stabilize radical anions; their reactivity with cellular macromolecules, including DNA, lipids, and proteins, can also lead to toxicity. Potential damage will depend on localized concentrations of the redox-cycling chemical, length of time of exposure to these agents, activity of redox-cycling enzymes, and the ability of cells and tissue to compensate by increasing metabolic activity under low oxygen tension and regenerating NADPH. Under normal homeostatic conditions, it would be expected that levels of NADPH would not be compromised and that diffusion of oxygen into cells and tissues would be sufficient to overcome losses during redox cycling. There are a number of bioreductive anticancer drugs that generate radical species during redox cycling.[32] It is thought that the long half-life of these radicals at low oxygen tensions in tumors increases their reactivity and results in increased antitumor activity.[32] Conversely, redox cycling under aerobic conditions and the

generation of reactive oxygen species are thought to be important in the antitumor and/or antimicrobial action of a number of chemotherapeutic agents, including nitrofurantoin, mitomycin C, and the anthracycline antibiotics.[33–35]

Chemical redox cycling by NADPH–cytochrome P450 reductase inhibits CYP activity and OP metabolism

Our laboratory has been characterizing the redox-cycling activity of the *p*-quinone menadione (methyl-1,4-naphthoquinone), also known as vitamin K3.[36] In the presence of NADPH–cytochrome P450 reductase, menadione readily redox cycles, generating superoxide anion and hydrogen peroxide. Due to its relatively low activation energy, this reaction preferentially diverts electrons from their supply to CYPs, resulting in inhibition of CYP-mediated biotransformation reactions (see Fig. 2 for a model of the effects of menadione on CYP-mediated metabolism). It is not specific for an individual CYP. Thus, using recombinant enzymes, we found that menadione inhibits multiple human P450 isoforms from different subfamilies, including CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP 3A7 (see Fig. 3 for an example of chemical redox cycling and the inhibition of CYP activity).[36] These data are consistent with earlier studies showing that menadione can inhibit CYP-mediated aniline-*p*-hydroxylation and aminopyrine-*N*-demethylation in rat liver microsomes.[37]

As CYP activity is required for OP metabolism, we tested the ability of menadione to inhibit metabolic activation of an OP to its oxon. For these studies we used parathion (O,O-diethyl O-(4-nitrophenyl) phosphorothioate), an important agricultural OP pesticide that is considered a high-priority chemical threat. The toxicity of parathion is dependent on CYP-mediated conversion to paraoxon (diethyl 4-nitrophenyl phosphate), a highly active inhibitor of cholinesterases.[36] Enzymes important in metabolizing parathion include CYP1A2, CYP2B6, and CYP3A4;[38] menadione was found to inhibit metabolism of parathion to paraoxon by each of these enzymes. Moreover, menadione was also found to inhibit parathion metabolism in human and rat liver microsomes (see Fig. 4 showing that menadione inhibits parathion metabolism).[36] In these studies, we also confirmed that menadione inhibited CYP-mediated metabolism of parathion to paraoxon by demonstrating that it prevented inhibition of AChE. Thus, human liver microsome–mediated metabolism of parathion to paraoxon led to the inhibition of AChE, which was blocked by menadione (see Fig. 4, showing that menadione inhibits parathion metabolism and AChE inhibition induced by parathion and its metabolites).

Menadione inhibits parathion intoxication in rats

Since it blocks parathion metabolism in rodents, menadione would be expected to inhibit signs of parathion intoxication, and indeed this was found to be the case. Treatment of rats with parathion (8 mg/kg, oral gavage) was found to induce neurotoxicity, which included a significant startle response, tremulous jaw movements, tremors, and convulsions.[36] Each of these signs of toxicity was significantly diminished in rats treated with menadione (40 mg/kg, intraperitoneal) 30 min after parathion exposure. As expected, metabolism of

parathion to paraoxon in the rats was also inhibited, as menadione caused a significant increase in levels of brain and serum parathion. Moreover, inhibition of cholinesterase activity in the forebrain and hindbrain, an indication of paraoxon formation in rats treated with parathion, was blocked by menadione (Table 1).[36] These data demonstrate that menadione is effective in inhibiting parathion metabolism *in vivo*.

Summary

CYP-mediated metabolism of parathion to paraoxon is critical for its ability to interfere with cholinergic signaling and cause toxicity. By redox cycling with NADPH–cytochrome P450 reductase in an energetically favorable reaction, menadione inhibits CYP activity, blocks the formation of paraoxon from parathion, and reduces or prevents a cholinergic crisis. These data suggest that menadione may be an effective pharmacologic approach to mitigating parathion poisoning in humans, as well as poisoning by other phosphorothioate pesticides. Since menadione can inhibit other metabolic enzymes using NADPH–cytochrome P450 reductase as an electron donor, its use may be limited to short-term treatments for acute OP exposure to prevent toxicity. It should also be noted that menadione will only be effective in preventing OP metabolism and subsequent neurotoxicity, as it is not expected to reverse OP-inactivated AChE. Menadione is an FDA-approved drug for use in humans with a known acceptable safety profile. This should facilitate our ability to further develop the drug for clinical use as a countermeasure against parathion toxicity.

Acknowledgments

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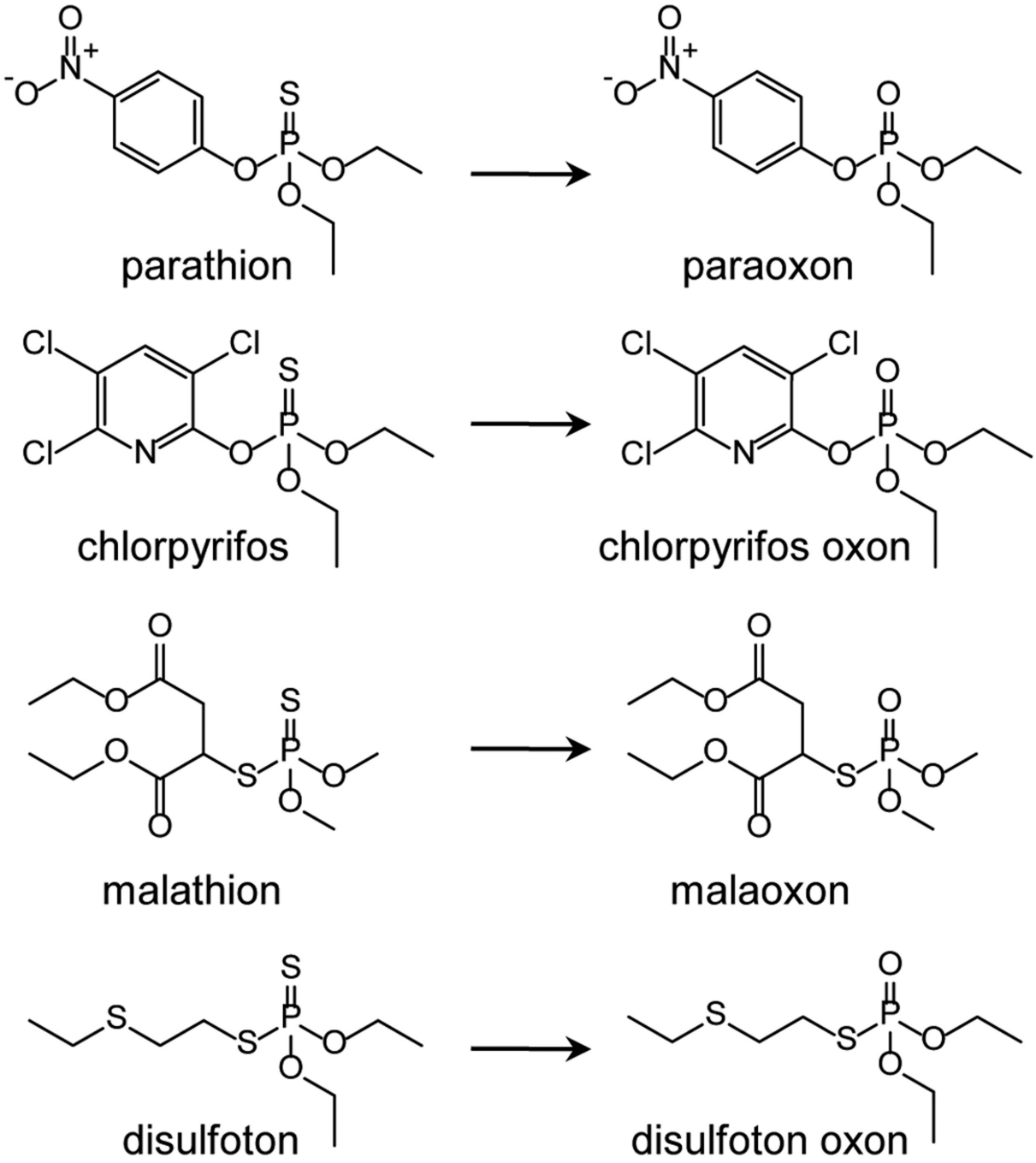

Figure 1.
Examples of phosphorothioate pesticides and corresponding oxon metabolites generated following CYP-mediated metabolism.

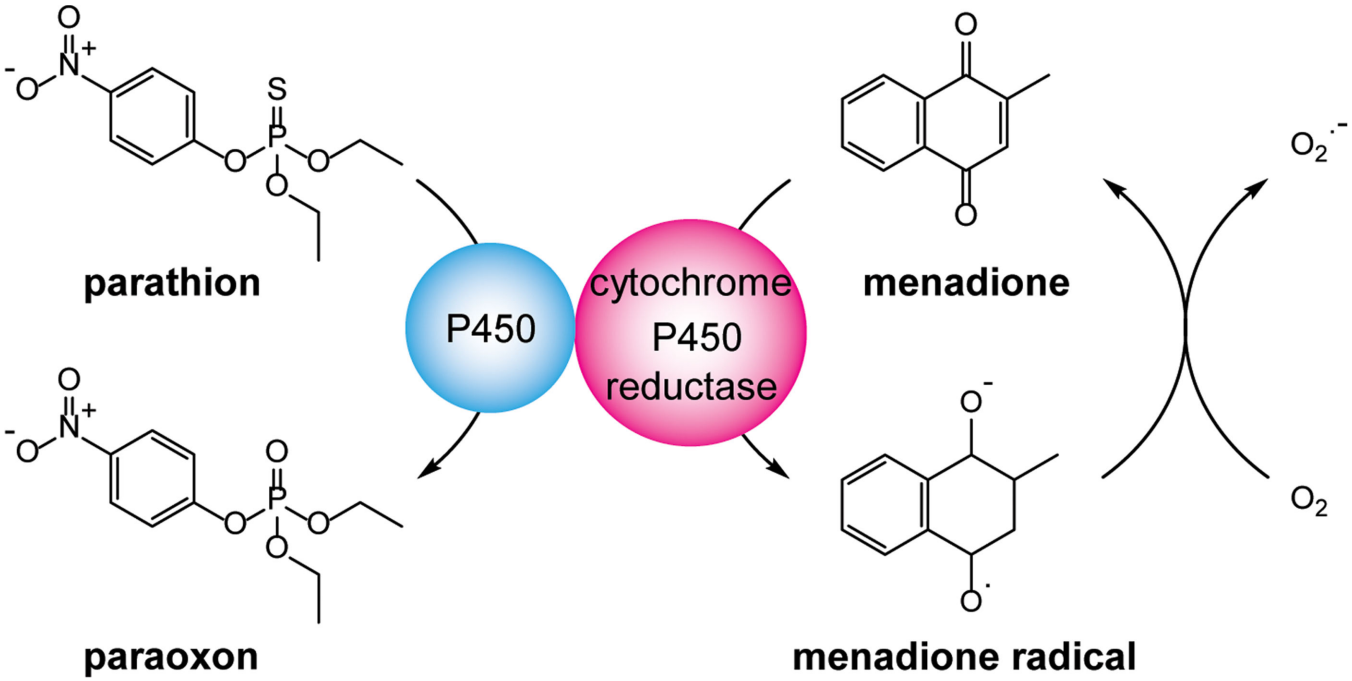

Figure 2.

Redox cycling of menadione suppresses parathion metabolism by the P450 system. NADPH–cytochrome P450 reductase catalyzes the one-electron reduction of menadione, generating a semiquinone radical. Under aerobic conditions, these radicals react rapidly with molecular oxygen to form superoxide anion, regenerating menadione in the process. Redox cycling diverts electrons from CYP-mediated parathion metabolism. This inhibits formation of paraoxon and suppresses parathion toxicity.

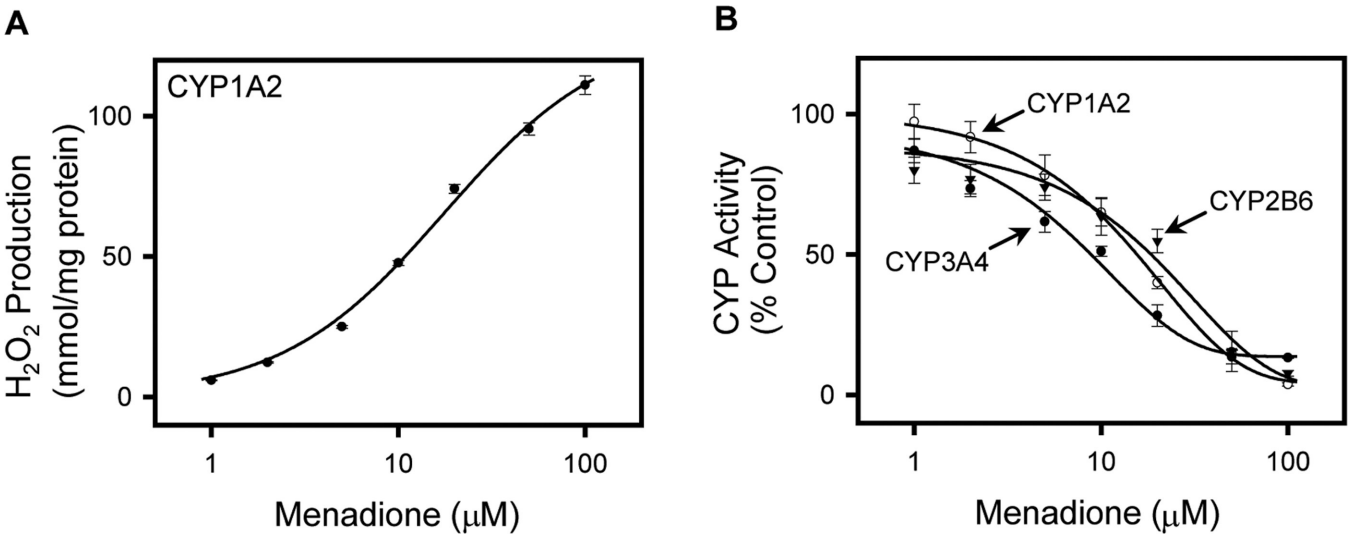

Figure 3.

Menadione redox cycling inhibits CYP enzyme activities. (A) CYP1A2 mediates menadione redox cycling as measured by the formation of hydrogen peroxide (H_2O_2). H_2O_2 formation was assayed using the Amplex Red assay and is presented as mmol/min/mg protein. (B) Menadione causes a concentration-dependent inhibition of the activities of the major CYPs that mediate parathion metabolism. CYP1A2, CYP2B6, and CYP3A4 are recombinant human CYP enzyme preparations prepared from insect cells coexpressing human NADPH–cytochrome P450 reductase. Substrates used to measure CYP1A2, CYP2B6, and CYP3A4 activity were 7-ethoxyresorufin, 7-ethoxymethyloxy-3-cyanocoumarin, and dibenzylfluorescein, respectively. The data points shown the mean ± SE, $n = 3$. Summary of data from Ref. 36.

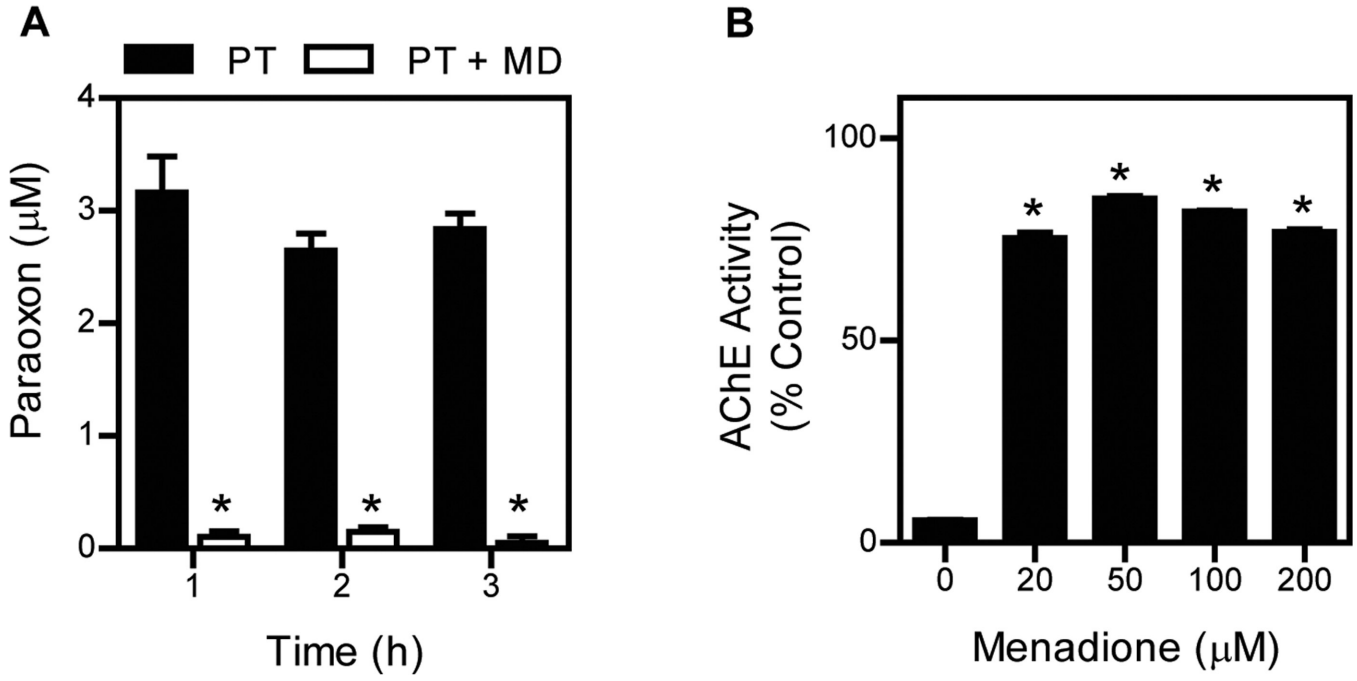

Figure 4.

Menadione redox cycling inhibits parathion metabolism. (A) Human liver microsomes were treated with 20 μM parathion (PT) in the absence and presence of 50 μM menadione. At the indicated times, reactions were assayed for paraoxon. Data are mean ± SE (n= 3). Asterisks show that the formation of paraoxon in the presence of menadione was significantly different ($P < 0.05$) from that in the absence of menadione. (B) The ability of menadione to reverse the inhibitory effects of parathion on AChE activity in reactions with human liver microsomes. Reactions containing human liver microsomes were supplemented with AChE. Incubations with parathion inhibited AChE activity, and this was reversed by menadione. The figures show that menadione redox cycling readily inhibited paraoxon formation. Each point is the mean ± SE (n = 3). Asterisks show that the inhibition of AChE in the presence of menadione was significantly different ($p < 0.05$) from that in the absence of menadione. Summary of data from Ref. 36.

Table 1

Effects of menadione on parathion levels and esterase activities in rat tissues[36]

	Treatment	
	Parathion	**Parathion + menadione**
Parathion levels in tissue		
Liver (μg/g tissue)	1.9 ± 0.3	1.9 ± 0.5
Brain (μg/g tissue)	0.1 ± 0.05	0.9 ± 0.3
Serum (μg/mL)	0.4 ± 0.1	2.4 ± 0.3
CES activity (% control) in tissue		
Liver	6.4 ± 0.5	15.3 ± 1.5
Serum	7.0 ± 1.8	18.0 ± 2.1
ChE activity (% control) in brain		
Forebrain	7.3 ± 1.1	90.0 ± 3.6
Hindbrain	15.5 ± 1.3	86.8 ± 2.3

Rats were administered parathion (8 mg/kg, oral gavage) followed 20 min later by menadione (40 mg/kg, IP). Sixty minutes after parathion treatment, rats were sacrificed. Carboxylesterase (CES) and cholinesterase (ChE) activities were determined using p-nitrophenylvalerate and acetylthiocholine as the substrates, respectively.