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Lysophospholipase inhibition by organophosphorus toxicants

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Abstract

Lysophospholipases (LysoPLAs) are a large family of enzymes for removing lysophospholipids from cell membranes. Potent inhibitors are needed to define the importance of LysoPLAs as targets for toxicants and potential therapeutics. This study considers organophosphorus (OP) inhibitors with emphasis on mouse brain total LysoPLA activity relative to the mipafox-sensitive neuropathy target esterase (NTE)-LysoPLA recently established as 17% of the total activity and important in the action of OP delayed toxicants. The most potent inhibitors of total LysoPLA in mouse brain are isopropyl dodecylphosphonofluoridate (also for LysoPLA of *Vibrio* bacteria), ethyl octylphosphonofluoridate (EOPF), and two alkyl-benzodioxaphosphorin 2-oxides (BDPOs)[(*S*)-octyl and dodecyl] (IC50 2–8 nM). OP inhibitors acting in vitro and in vivo differentiate a more sensitive portion but not a distinct NTE-LysoPLA compared with total LysoPLA activity. For 10 active inhibitors, NTE-LysoPLA is 17-fold more sensitive than total LysoPLA, but structure–activity comparisons give a good correlation (r^2 = 0.94) of IC50 values, suggesting active site structural similarity or identity. In mice 4 h after intraperitoneal treatment with discriminating doses, EOPF, tribufos (a plant defoliant), and dodecanesulfonyl fluoride inhibit 41–57% of the total brain LysoPLA and 85–99% of the NTE-LysoPLA activity. Total LysoPLA as well as NTE-LysoPLA is decreased in activity in $Nte^{+/-}$-haploinsufficient mice compared to their $Nte^{+/+}$ littermates. The lysolecithin level of spinal cord but not brain is elevated significantly following EOPF treatment (3 mg/kg), thereby focusing attention on localized rather than general alterations in lysophospholipid metabolism in OP-induced hyperactivity and toxicity.

Keywords: Ethyl octylphosphonofluoridate; Lysolecithin; Lysophospholipase; Neuropathy target esterase; Organophosphorus inhibitor; *Vibrio*

Introduction

Lysophospholipases (LysoPLAs) are a large family of enzymes regulating lysophospholipid and lysolecithin levels in cell membranes (Wang and Dennis, 1999). LysoPLAs are present in many tissues and vary in molecular mass from approximately 25 to at least 155 kDa, but the relative role of the multiple isoforms is largely unknown (Quistad et al., 2003; Wang and Dennis, 1999). Lysophospholipids are critical to cell survival and function and are associated with several diseases such as hyperlipidemia, inflammation, and myocardial ischemia (Wang and Dennis, 1999). Lysolecithin acts directly on G protein-coupled receptors and induces receptor-mediated signal transduction (Kabarowski et al., 2001; Xu, 2002). It also causes demyelination of neuronal sheaths often accompanied by axonal lesions (Hall, 1972; Prineas et al., 2002; Quarles et al., 1999).

Organophosphorus (OP) toxicants have played a major role in characterizing one of the brain LysoPLAs called neuropathy target esterase (NTE)-LysoPLA (Quistad et al., 2003). In mouse brain, NTE-LysoPLA is the mipafox-sensitive (50 μM) portion (17%) of total LysoPLA activity (Quistad et al., 2003). It is similar or identical in genomic, proteomic, and OP-sensitivity profiles to NTE of human and hen brain (Johnson and Glynn, 2001; van Tienhoven et al., 2002; Winrow et al., 2003) associated with OP-induced polyneuropathy and demyelination (Ehrich and Jortner, 2001; Johnson and Glynn, 2001).

Abbreviations: BDPO, benzodioxaphosphorin 2-oxide or 2-substituted-4*H*-1,3,2-benzodioxaphosphorin 2-oxide; DFP, diisopropyl fluorophosphate; EOPF, ethyl octylphosphonofluoridate; GC, gas chromatography; LC, liquid chromatography; LysoPLA, lysophospholipase; Me, CH_3; MeCN, acetonitrile; MS, mass spectroscopy; NTE, neuropathy target esterase; OP, organophosphorus; Ph, phenyl; $PyrCl_3$, 3,5,6-Cl_3-2-pyridyl as in chlorpyrifos oxon; TLC, thin layer chromatography.

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Potent inhibitors of mouse brain NTE-LysoPLA are ethyl octylphosphonofluoridate (EOPF), isopropyl dodecylphosphonofluoridate, and (*S*)-octyl-benzodioxaphosphorin 2-oxide (BDPO) (IC50 <1 nM) (Quistad et al., 2003; Segall et al., 2003a). The OP sensitivity of the remaining 83% of mouse brain LysoPLA activity has not been reported. Other LysoPLAs appear from two reports to be less sensitive, that is, IC50 600 nM for methyl arachidonylfluorophosphonate with expressed human brain LysoPLA (Wang et al., 1999) and 5 mM for diisopropyl fluorophosphate (DFP) with murine LysoPLA I from macrophage-like cells (Wang et al., 1997). The OP sensitivity and pharmacological profile, although not previously determined, are also of interest for the bacterial LysoPLA of *Vibrio cholerae* because removal of the gene *lypA* encoding LysoPLA L_2 reduces cholera toxin production (Whayeb et al., 1996a).

Lysophospholipid levels are controlled by the relative activities of several enzymes involved in their synthesis and breakdown (Wang and Dennis, 1999). Low LysoPLA activity in brain, genetically or OP-induced, might be expected to elevate lysolecithin levels. However, the effect of an OP on brain lysolecithin level would ultimately depend on its specificity for inhibition of LysoPLA compared with other enzymes in the biosynthesis and degradation pathways. Localized effects might also be involved in lysolecithin accumulation as already known for NTE and NTE-LysoPLA inhibition (Ehrich and Jortner, 2001; Kamijima and Casida, 1999).

There are three goals of this study. The first is to discover potent OP inhibitors for LysoPLAs and define if the sensitivity and specificity profiles vary with the enzyme source and type, that is, mouse brain total LysoPLA and NTE-LysoPLA compared with mouse and human liver LysoPLAs and *Vibrio* LysoPLA. The second goal is to determine for mouse brain if the mipafox-sensitive 17% of total LysoPLA activity designated NTE-LysoPLA is distinctly different than the remaining portion based on OP inhibitor sensitivity and specificity both in vitro and in vivo. The third is to define the relationship, if any, between total mouse brain LysoPLA activity and nerve lysolecithin level based on both genetic and OP inhibitor criteria.

Materials and methods

Chemicals. Caution: some of the test compounds have high toxicity in mice (Wu and Casida, 1996). Most candidate inhibitors were available from previous investigations in this laboratory (Quistad et al., 2002, 2003; Segall et al., 2003a, 2003b; Wu and Casida, 1992, 1995, 1996; Zhang and Casida, 2001). 6-*N*-Biotinylaminohexyl isopropyl fluorophosphate was obtained from Toronto Research Chemicals (North York, ON, Canada) and phenyl phenylacetate from Aldrich (Milwaukee, WI).

Mouse experiments. Male albino Swiss–Webster mice (ca. 25 g) were from Harlan Laboratories (Indianapolis, IN). They were maintained under standard conditions with access to water and food ad libitum. The mice generally were treated intraperitoneally with candidate inhibitors in dimethyl sulfoxide (30 μl). Some also received lysolecithin intraperitoneally in water (50 μl), alone or 4 h after EOPF (10 mg/kg). The mice were euthanized by cervical dislocation at indicated times with removal of blood by cardiac puncture. Blood was added to tubes containing 0.1 M citrate (200 μl, Becton Dickinson, Franklin Lakes, NJ) and centrifuged (700 × *g*, 10 min) to recover plasma. Brain was removed and frozen before analysis. The studies were carried out in accordance with the *Guiding Principles in the Use of Animals in Toxicology* as adopted by the Society of Toxicology in 1989.

Mice genetically deficient in NTE ($Nte^{+/-}$-haploinsufficient) were compared with the corresponding wild-type ($Nte^{+/+}$) mice (129S6/SvEvTac, male, ca. 4 months old) (Winrow et al., 2003) using frozen brains provided by Carrolee Barlow, Christopher Winrow, and Rob Helton of the Salk Institute for Biological Studies (La Jolla, CA).

General procedure for enzyme-coupled assays for total LysoPLA and NTE-LysoPLA activities and lysolecithin level. An enzyme-coupled microplate assay is described by Kishimoto et al. (2002) for determination of lysolecithin levels in plasma. The sequential action of four added enzymes (LysoPLA, *sn*-glycero-3-phosphocholine phosphodiesterase, choline oxidase, and peroxidase) converts lysolecithin to *sn*-glycero-3-phosphocholine, choline, hydrogen peroxide, and a purple derivative (in the presence of two chromogenic agents). This procedure is modified in the present investigation to determine total LysoPLA and NTE-LysoPLA activities (Quistad et al., 2003) and lysolecithin level. Two reagents (A and B) are used. Reagent A contains *sn*-glycero-3-phosphocholine phosphodiesterase (0.2 units/ml), choline oxidase (10 units/ml), peroxidase (20 units/ml), and 3-(*N*-ethyl-3-methylanilino)-2-hydroxysulfonate (3 mM). Reagent B is 5 mM 4-aminoantipyrine. The fourth enzyme (*Vibrio* sp. LysoPLA; also known as phospholipase B) is added separately and only in assays for lysolecithin levels. All enzymes and reactants are obtained from Sigma (St. Louis, MO) and added in Tris buffer [100 mM (pH 8.0) containing 1 mM calcium chloride and 0.01% Triton X-100]. One additional step is required to determine NTE-LysoPLA activity because, by definition, it is the mipafox (50 μM)-sensitive portion of the total LysoPLA activity (Quistad et al., 2003). In contrast to NTE assays with phenyl valerate as the substrate, where the portion of the activity resistant to paraoxon (40 μM) is part of the definition (Johnson, 1977), with lysolecithin as the substrate paraoxon is omitted because there is no inhibition of either NTE-LysoPLA or total LysoPLA. This overall procedure is suitable for assaying OP inhibitors because only LysoPLA of the four added enzymes is inhibited by the OP concen-

trations (μM) used, for example, *n*-$C_{12}H_{25}P(O)(OC_3H_7$-*i*)F (10), EOPF (0.1), dodecyl-BDPO (1), and mipafox (50). The IC50 values were derived usually from 3-fold concentration differentials giving 15–85% inhibition. Mean IC50 and SE values reported represent at least three experiments.

Mouse brain and Vibrio total LysoPLA activity assays. Mouse brain was homogenized at 20% (w/v) in 50 mM Tris buffer (pH 8.0) containing 0.2 mM EDTA. The supernatant fraction (700 × *g*, 10 min) was used to assay total LysoPLA activity. Reagents A (120 μl) and B (80 μl) were added to individual chambers containing Tris buffer as above (45 μl). Brain homogenate supernatant fraction (15 μl) was then added followed by the candidate inhibitor (0.1–100 000 nM) in dimethyl sulfoxide (5 μl). After 15-min incubation at 25 °C, lysolecithin (250 μM final concentration) was added in Tris buffer (50 μl) and total LysoPLA activity was measured by kinetic assay of absorbance at 570 nm for 10 min at 25 °C using a microplate reader (Molecular Devices, Sunnyvale, CA). The activity was linear with regard to protein level and time and measured as mAU/min.

LysoPLA from *Vibrio* was diluted in Tris buffer before assay as above, replacing the brain homogenate with 0.05 units *Vibrio* LysoPLA per replicate.

Mouse brain NTE-LysoPLA activity assays. The above procedure for total LysoPLA activity was modified in only one step to determine NTE-LysoPLA activity. After incubating the brain preparation with the candidate inhibitor for 15 min, mipafox was added in 50 mM Tris-citrate (5 μl) to give 0 or 50 μM final concentration with an additional 20-min incubation before adding lysolecithin. Each brain preparation from mice treated with inhibitors was used to determine both the total LysoPLA and NTE-LysoPLA activities (Quistad et al., 2003).

Liver microsomal total LysoPLA and NTE-LysoPLA activity assays. Mouse liver microsomes were prepared by homogenization of liver (20% w/v) in 50 mM Tris–0.2 mM EDTA (pH 8.0, 4 °C). After centrifugation at 10 000 × *g* for 30 min, the supernatant was recentrifuged at 100 000 × *g* for 60 min and the microsomal pellet resuspended in the same buffer to the original volume. Pooled human liver microsomes (20 mg protein/ml) were obtained from BD Biosciences (Bedford, MA). Microsomes [10–15 μl containing 58 (mouse) or 100 (human) μg protein] were added to the enzyme-coupled system (same volumes of reagents and total volume as above) for analysis of total LysoPLA and NTE-LysoPLA activities.

Phospholipid levels of pons/medulla oblongata. Pons/medulla oblongata samples (ca. 50 mg) were provided to Lipomics Technologies (West Sacramento, CA) for analysis of phospholipids resolved into lipid classes by thin layer chromatography (TLC) followed by transmethylation to fatty acid esters quantified by capillary gas chromatography (GC) (Watkins et al., 2002).

Lysolecithin levels of whole brain and plasma analyzed by enzyme-coupled assay. Lysolecithin assayed colorimetrically with coupled enzymes was extracted from individual brains (ca. 0.4 g each) by two methods: (1) homogenize at 20% w/v in 50% aqueous acetonitrile (MeCN) and (2) homogenize with 4 ml $CHCl_3$/MeOH (1:2) followed by dilution with $CHCl_3$ (1 ml) and water (1 ml) to recover the lower $CHCl_3$ phase containing lipids (Bligh and Dyer, 1959), including lysolecithin. In each case, homogenates were centrifuged (700 × *g*, 10 min). An aliquot (30 μl) of the 50% MeCN extract was assayed directly, whereas an aliquot (50 μl) from extraction with $CHCl_3$/MeOH was evaporated and lipids were suspended in Tris buffer (150 μl) from which replicates (50 μl) were assayed. Reagents A (120 μl) and B (80 μl) containing three of the four enzymes were added to individual chambers containing Tris buffer (total volume 315 μl including aliquot of brain extract added last). Finally, *Vibrio* LysoPLA (0.05 units in 5 μl Tris buffer per replicate) was introduced and the absorbance recorded at 570 nm after 30 min at 25 °C. Likewise, a 10-μl aliquot of citrate-diluted blood plasma was analyzed.

Lysolecithin levels of nerve tissues. Total lipid was extracted from 70 to 110 mg of nerve tissue (spinal cord, pons/medulla oblongata, frontal lobe) by homogenizing at 25 °C for 3 min with $CHCl_3$/MeOH (2:1). Lipid (3 mg) was fractionated by Alltech silica column (0.5 × 1 cm) eluted with 1 ml each of ethyl acetate, ethyl acetate/MeOH (1:1), MeOH, and finally 95% MeOH/5% water to recover combined lecithin and lysolecithin. The major lysolecithin ($C_{16:0}$) was quantified by electrospray liquid chromatography/mass spectroscopy (LC/MS), monitoring *m*/*z* 478 [M^+-H_2O] after flow injection of 3 μl.

Results

In vitro sensitivity of mouse brain LysoPLA to inhibitors

The in vitro sensitivity to inhibitors provides one means of differentiating one esterase from another, for example, NTE-LysoPLA from total LysoPLA. The same sensitivity ratio of IC50 values over a large range of inhibitor types suggests that the active sites are similar. Fifteen OP and two organosulfur compounds were evaluated by IC50 comparisons (Table 1). Inhibition of total LysoPLA activity was determined relative to the NTE-LysoPLA component (17% of total, inhibited by 50 μM mipafox). Two fluorophosphonates [EOPF and *n*-$C_{12}H_{25}P(O)(OC_3H_7$-*i*)F] are particularly potent (IC50 2 nM) (Fig. 1). A biotinylated fluorophosphate and diisopropyl fluorophosphate (DFP) are 100- and 6000-fold less active, respectively. Paraoxon is

Table 1
In vitro sensitivity of mouse brain and *Vibrio* lysophospholipases to organophosphorus and organosulfur inhibitors

Compound	IC50 (nM)[a]	
	Mouse brain total LysoPLA	*Vibrio* sp.
Fluoro OP		
n-$C_8H_{17}P(O)(OC_2H_5)F$ (EOPF) (**1**)	1.8 ± 0.1	90 ± 15
n-$C_{12}H_{25}P(O)(OC_3H_7$-*i*$)F$ (**2**)	2.2 ± 0.5	6.7 ± 1.3
$RP(O)(OC_3H_7$-*i*$)F$ R = biotin-NH$(CH_2)_6$O-	200 ± 53	>100000 (17 ± 1)[b]
(*i*-PrO)$_2$P(O)F (DFP) (**3**)	12000 ± 200	>100000 (0 ± 1)[b]
(*i*-PrNH)$_2$P(O)F (mipafox)	>50000 (17 ± 2)[b]	>50000 (0 ± 0)[b]
$(C_2H_5O)_2P(O)OPh$-4-NO_2 (paraoxon)	>40000 (0 ± 0)[b]	>100000 (2 ± 1)[b]
BDPO		
(*S*)-*n*-C_8H_{17}- (*S*-*n*-octyl) (**4**)	5.0 ± 1.9	1300 ± 200
n-$C_{12}H_{25}$- (*n*-dodecyl)	8.3 ± 2.6	34 ± 6
(*R*)-*n*-C_8H_{17}- (*R*-*n*-octyl) (**5**)	260 ± 42	>100000 (45 ± 1)[b]
n-$C_{18}H_{37}$- (*n*-octadecyl)	330 ± 50	67 ± 9
o-CH_3-PhO- (*o*-tolyloxy) (**6**)	3800 ± 1700	>100000 (24 ± 13)[b]
OP insecticide or analog		
(*n*-$C_5H_{11}O)_2P(O)OCH = CCl_2$ (**7**)	28 ± 5	>100000 (6 ± 4)[b]
(*n*-$C_5H_{11}O)_2P(O)OPyrCl_3$ (chlorpyrifos pentyl oxon)	76 ± 16	360 ± 15
$(C_2H_5O)_2P(O)OPyrCl_3$ (**8**) (chlorpyrifos oxon)	1300 ± 240	18000 ± 3000
$(CH_3O)_2P(O)OCH = CCl_2$ (dichlorvos)	>100000 (24 ± 6)[b]	>100000 (12 ± 4)[b]
Sulfonyl fluoride		
n-$C_{12}H_{25}$- (*n*-dodecyl) (**9**)	5100 ± 1600	680 ± 130
n-C_8H_{17}- (*n*-octyl) (**10**)	10000 ± 2000	>100000 (31 ± 2)[b]

[a] Mean ± SE (n = 3–7).
[b] Inhibition (%) at indicated dose, mean ± SE (n = 3).

inactive. (*S*)-*n*-Octyl- and *n*-dodecyl-BDPOs also are potent inhibitors (IC50 5–8 nM). (*R*)-Octyl- and octadecyl-BDPO have intermediate activity (IC50 260–330 nM) and *o*-tolyloxy-BDPO is much less active. Two pentyl derivatives of insecticides, (*n*-$C_5H_{11}O)_2P(O)OCH = CCl_2$ from dichlorvos and (*n*-$C_5H_{11}O)_2P(O)OPyrCl_3$ from chlorpyrifos oxon, are moderately potent inhibitors of total LysoPLA (IC50 28–76 nM) although their methyl or ethyl analogs are much less active. Dodecane- and octanesulfonyl fluorides are generally less active (IC50 5100–10000 nM) than the OPs examined. Octyl and dodecyl side chains provide optimal inhibition and are generally equivalent with the following order of potency: fluoro OP > BDPO > alkanesulfonyl fluoride.

NTE-LysoPLA on average is 17-fold more sensitive than total LysoPLA for 10 compounds and a log–log plot of IC50 values gives an excellent structure–activity correlation (r^2 = 0.94), suggesting strong structural similarities for the inhibitor binding sites of NTE-LysoPLA and total LysoPLA (Fig. 2).

Five OP, carbamate, and carboxylate inhibitors were tested at discriminating concentrations (Table 2). NTE-LysoPLA generally is inhibited more than total LysoPLA. At 2000 μM, the phosphinate $Ph_2P(O)F$ inhibits about half of the total LysoPLA, whereas the same level of inhibition occurs at 200 μM for two carbamates (the sulfoxide of EPTC herbicide and phenyl *N*-benzylcarbamate). Phenyl valerate and phenyl phenylacetate, substrates of NTE (Johnson, 1977), are weak inhibitors at 200 μM.

In vitro sensitivity of mouse and human liver LysoPLAs to inhibitors

The level of total LysoPLA activity in liver microsomes (mouse, human) is similar to that of mouse brain (absorbance change of 8–12 mAU/min for 100 μg protein) (Table

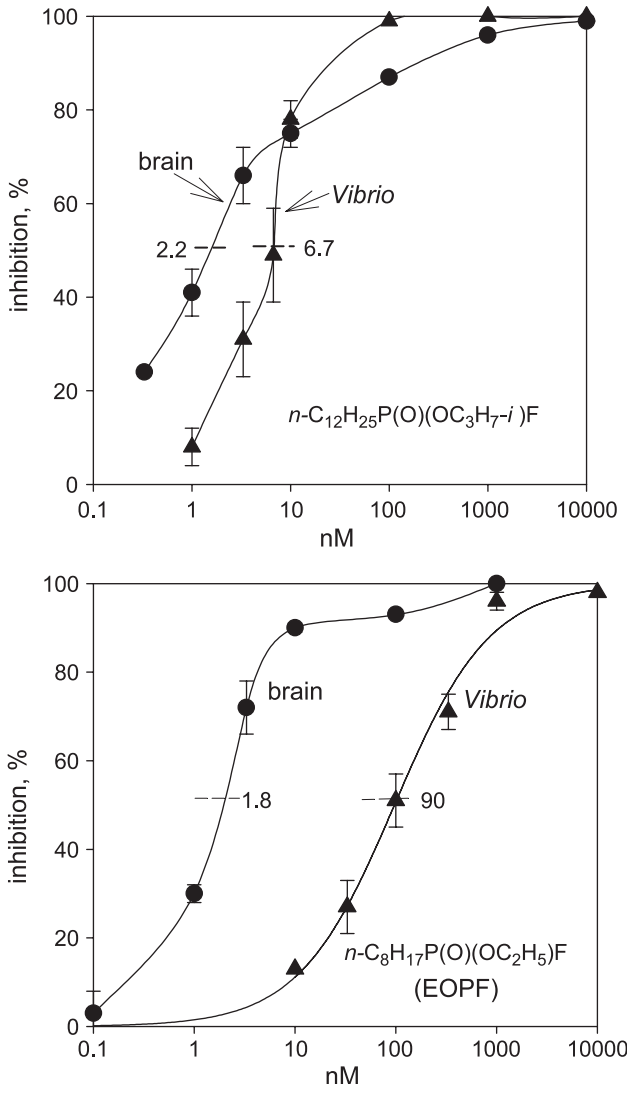

Fig. 1. Two potent organophosphorus inhibitors of total LysoPLA from mouse brain and *Vibrio* sp.

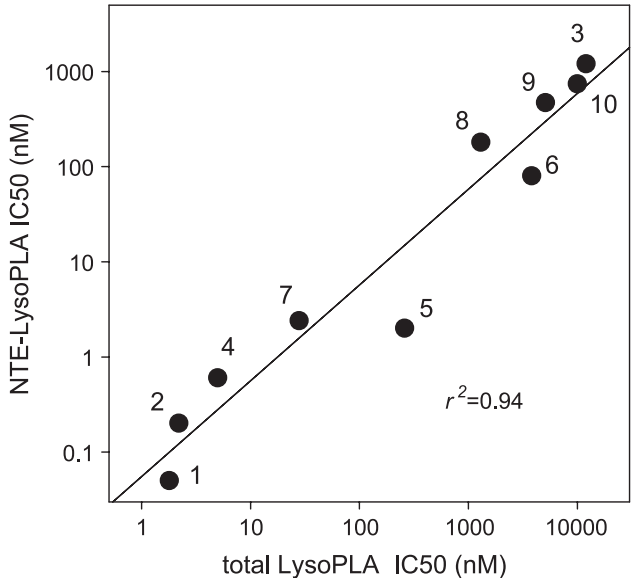

Fig. 2. Relationship between in vitro sensitivities of mouse brain total LysoPLA and NTE-LysoPLA activities assayed with mipafox at 0 and 50 μM, respectively. Numbers for the points plotted refer to compound designations in Table 1, which gives the total LysoPLA data. IC values for NTE-LysoPLA are from Quistad et al. (2002, 2003) and Segall et al. (2003a), except for chlorpyrifos oxon (180 ± 70 nM, $n = 3$) determined in this study.

3). Total LysoPLA in liver is moderately sensitive to EOPF IC50 (150–270 nM), but mouse brain total LysoPLA (Table 1) is >100-fold more sensitive than the corresponding liver enzyme(s). The NTE-LysoPLA component of total LysoPLA (sensitive to 50 μM mipafox, insensitive to 40 μM paraoxon) is greatest in human liver microsomes followed by mouse liver and brain (50 ± 3%, 16 ± 2%, and 17 ± 2% of total, respectively). Inhibition of NTE-LysoPLA by mipafox is similar for mouse brain (IC50 13 μM) compared to liver microsomes (IC50 24 μM), but mouse brain NTE-LysoPLA (Table 1) is about 400-fold more sensitive to EOPF than the liver enzymes (this study and Quistad et al., 2003).

Table 2
In vitro sensitivity of mouse brain lysophospholipases (total and NTE) to organophosphorus, carbamate, and carboxylate inhibitors

Compound	μM	Inhibition (%)[a]	
		Total LysoPLA	NTE-LysoPLA
OP			
$Ph_2P(O)F$ (diphenyl fluorophosphinate)	2000	52 ± 2	74 ± 12
Carbamate			
$(n\text{-}C_3H_7)_2NC(O)S(O)C_2H_5$ (EPTC sulfoxide)	200	51 ± 1	85 ± 8
$PhOC(O)NHCH_2Ph$ (phenyl *N*-benzylcarbamate)	200	47 ± 3	67 ± 3
Carboxylate			
$PhOC(O)C_4H_9$-*n* (phenyl valerate)	200	21 ± 1	16 ± 4
$PhOC(O)CH_2Ph$ (phenyl phenylacetate)	200	6 ± 6	52 ± 20

[a] Mean ± SE ($n = 3$).

Table 3
In vitro sensitivity of mouse and human liver lysophospholipases (total and NTE) to discriminating inhibitors

Compound	IC50 (nM)[a]			
	Total LysoPLA		NTE-LysoPLA	
	Mouse	Human	Mouse	Human
EOPF	270 ± 50	150 ± 13	ca. 20	37 ± 3
Mipafox	>100000 (19,21)[b]	50000 ± 3000	24000 ± 200	24000 ± 900
Paraoxon	>40000	>40000	>40000	>40000

[a] Mean ± SE ($n = 3–4$).
[b] Inhibition (%) at 100,000 nM for individual replicates.

In vitro sensitivity of Vibrio LysoPLA to inhibitors

$n\text{-}C_{12}H_{25}P(O)(OC_3H_7\text{-}i)F$ is the most potent inhibitor of *Vibrio* LysoPLA (IC50 7 nM). EOPF is the only other highly potent fluoro OP (13-fold less active) (Table 1, Fig. 1). Dodecyl- and octadecyl-BDPOs are moderately potent (IC50 34–67 nM), whereas other BDPOs are much less active. Chlorpyrifos pentyl oxon and dodecanesulfonyl fluoride are somewhat less active (IC50 360–680 nM). The general level of potency for active compounds in each series is: fluoro OP > BDPO > alkanesulfonyl fluoride. In contrast to LysoPLA in mouse brain, the corresponding *Vibrio* enzyme is inhibited much more by analogs with dodecyl rather than octyl side chains (Fig. 1). Although the enantiomeric preference for (*S*)-*n*-octyl-BDPO is the same for LysoPLA from *Vibrio* and mouse brain, both (*S*)- and (*R*)-isomers are considerably poorer inhibitors with *Vibrio*.

In vivo sensitivity of mouse brain LysoPLA to inhibitors

In vivo inhibitor sensitivity provides another way of comparing enzyme active site specificities. Six OP and organosulfur compounds were administered intraperitoneally to mice with assay of total LysoPLA and NTE-LysoPLA at 4 h post-treatment. The compounds selected are known delayed toxicants inhibitory to NTE-LysoPLA (Quistad et al., 2003; Segall et al., 2003b; Wu and Casida, 1996). Generally, NTE-LysoPLA is inhibited more effectively than total LysoPLA, but EOPF, $n\text{-}C_{12}H_{25}SO_2F$, and tribufos (a plant defoliant) inhibit 41–57% of the total LysoPLA at 10, 100, and 100 mg/kg, respectively. The *n*-octyl-BDPO enantiomers and *o*-tolyloxy-BDPO are less effective on total LysoPLA (Table 4).

Table 4
In vivo sensitivity of mouse brain lysophospholipases (total and NTE) to organophosphorus and organosulfur inhibitors

Compound (mg/kg)	Inhibition (%)[a]	
	Total LysoPLA	NTE-LysoPLA[b]
EOPF		
1		18 ± 15
2	23 ± 8	89 ± 12
3	33 ± 3	100 ± 0
10	45 ± 7	99 ± 1
n-$C_{12}H_{25}SO_2F$		
100	57 ± 6	92 ± 9
Tribufos		
30	9 ± 3	7 ± 8
100	41 ± 11	85 ± 16
o-Tolyloxy-BDPO		
10	16 ± 10	70 ± 4
30	40 ± 9	89 ± 7
100	34 ± 3	87 ± 16
(S)-n-Octyl-BDPO		
5	3 ± 2	71 ± 8
(R)-n-Octyl-BDPO		
5	0 ± 0	7 ± 8

[a] Mean ± SE (n = 5–6); 4 h post-treatment.
[b] Total and NTE-LysoPLA determined using the same mice. Data for NTE-LysoPLA from Quistad et al. (2003).

Brain LysoPLA activities and lysolecithin levels in $Nte^{+/+}$ and $Nte^{+/-}$-haploinsufficient mice

Total LysoPLA and NTE-LysoPLA activities were determined for wild-type ($Nte^{+/+}$) and $Nte^{+/-}$-haploinsufficient mice (Table 5). In this strain, NTE-LysoPLA constitutes 29% of the total LysoPLA in $Nte^{+/+}$ mice and NTE-LysoPLA is reduced 41% in the $Nte^{+/-}$ mice (Winrow et al., 2003). The decrease in total LysoPLA activity in $Nte^{+/-}$ mice is 2.5-fold greater than predicted from the reduction of NTE-LysoPLA alone (4.3 versus 1.7 mAU/min), indicating that *nte* confers activity for lysolecithin hydrolysis beyond that determined in the mipafox-sensitive assay.

Lysolecithin levels in the 50% MeCN extract of whole brain analyzed by enzyme-coupled assay are not significantly different for $Nte^{+/+}$ mice (control, normalized, 100 ± 10) and $Nte^{+/-}$ mice (77 ± 15 relative to control), possibly related to the small number of replicates (n = 3).

Table 5
Brain lysophospholipase activities (total and NTE) in $Nte^{+/+}$- and $Nte^{+/-}$-haploinsufficient mice

Activity	mAU/min		
	$Nte^{+/+}$	$Nte^{+/-}$	Change
Total LysoPLA	14.3 ± 0.5[a]	10.0 ± 1.0	−4.3
NTE-LysoPLA[b]	4.1 ± 0.2	2.4 ± 0.2	−1.7

[a] Mean ± SE (n = 7 for $Nte^{+/+}$ and 4 for $Nte^{+/-}$).
[b] Winrow et al. (2003).

Table 6
Phospholipid levels in pons/medulla oblongata of mice following EOPF treatment at 3 mg/kg analyzed by TLC/GC

Phospholipid	Tissue level (μmol/g)[a]		
	Control	EOPF	Change (%)[b]
Lysolecithin[c]	1.4	1.7	+21
Lecithin	23.3	25.0	+7
Phosphatidyl ethanolamine	20.7	22.0	+6
Cardiolipin	4.4	4.4	0
Phosphatidyl serine	32.5	31.5	−3
Sphingomyelin	5.7	4.8	−16

[a] Mean, SD averaged 37% of mean (n = 3); 16–24 h post-treatment; changes not statistically significant.
[b] Fatty acid ratios not significantly changed within each phospholipid class (data not shown).
[c] The major components ($C_{16:0}$ and $C_{18:0}$) increased 25 and 27%, respectively.

Lysolecithin levels in nerve tissue and plasma of mice treated with EOPF

EOPF is a delayed neurotoxicant for mice at 3 mg/kg ip with poisoning signs generally occurring 16–24 h post-treatment. Typically mice are asymptomatic for about 16 h, then over the next 1–2 h exhibit piloerection, sluggishness, and finally stop moving until death. Lysolecithin is moderately toxic to mice (intraperitoneal LD50 400–800 mg/kg) with organs, including brain, having a bloody appearance. However, this toxicity is synergized less than 2-fold by 4-h prior treatment with EOPF (10 mg/kg).

A possible relationship of EOPF toxicity to nerve phospholipid content was examined in three ways. First, phospholipids in pons/medulla oblongata were quantitated by class and fatty acid components. This analysis established that EOPF increases the level of lysolecithin more than that of other phospholipid classes without altering the fatty acid ratios, but with the small sample size (n = 3), the observed increase was not statistically significant (Table 6). Second, lysolecithin levels in the $CHCl_3$/MeOH (1:2) extracts of whole brain were analyzed by enzyme-coupled assay. This approach suggests that relative to controls (100 ± 20, n = 9), EOPF increases the lysolecithin level to 150 ± 26 (n = 12) (3 mg/kg, 16–24 h post-treatment) and 139 ± 58 (n = 3) (10 mg/kg, 4 h post-treatment), but the results are not statistically significant. Third, a very specific LC/MS meth-

Table 7
Lysolecithin levels in nerve tissue of mice following EOPF treatment at 3 mg/kg analyzed by LC/MS

Nerve tissue	Tissue level (%)[a]		
	Control	EOPF	Change (%)
Spinal cord	100 ± 19	130 ± 7	+30**
Frontal lobe	100 ± 5	107 ± 10	+7
Pons/medulla oblongata	100 ± 6	102 ± 7	+2

[a] Mean ± SD (n = 6); 16–24 post-treatment; lipid fractionated by silica column and the major lysolecithin ($C_{16:0}$) quantified by integration of area for m/z 478; area relative to control normalized to 100.
** $P < 0.01$ (unpaired Student's t test).

od was used to quantitate the major lysolecithin component ($C_{16:0}$) in different nerve regions, indicating no significant change for frontal lobe or pons/medulla oblongata but a highly significant increase for spinal cord (Table 7).

Plasma lysolecithin levels are not significantly changed by EOPF (3 mg/kg; 16–24 h post-treatment), that is, control 420 ± 160 μM and treated 340 ± 150 μM (mean ± SD; n = 4–10).

Discussion

OP inhibitors of LysoPLAs: potency and specificity profiles

Three OPs (compounds **1**, **2**, and **4**) are the most active inhibitors yet reported for mouse brain NTE-LysoPLA (IC50 0.05–0.6 nM) (Quistad et al., 2003) and total LysoPLAs (IC50 2–5 nM) (this report), far exceeding the potencies of OPs examined earlier on other LysoPLAs (Wang et al., 1997, 1999).

The similar activity levels of NTE-LysoPLA in brain and liver are somewhat surprising because earlier studies of NTE distribution showed no activity in liver or kidney (Johnson, 1982). However, recent investigations with genetically modified mice show NTE-LysoPLA in kidney and testes (Winrow et al., 2003). Inhibition of liver NTE-LysoPLA probably is not significant toxicologically because the delayed neurotoxicant EOPF is much more potent with NTE-LysoPLA of neural origin, making this a more likely target for toxicity.

The structure–activity requirements for inhibition of *Vibrio* LysoPLA differ from those for the corresponding mouse brain enzyme(s). n-$C_{12}H_{25}P(O)(OC_3H_7$-$i)F$ retained the highest potency for *Vibrio* LysoPLA (IC50 7 nM). Low sequence homology is expected comparing LysoPLA from mammalian brain with that from *Vibrio* because the deduced amino acid sequence for LysoPLA L_2 from *Vibrio cholerae* has only 38% identity to the same enzyme from the more closely related *Escherichia coli* (Whayeb et al., 1996b).

Mouse brain LysoPLAs: comparison of NTE-LysoPLA and total LysoPLA

Brain NTE-LysoPLA is the proposed target for OP-induced delayed toxicity in mice (Quistad et al., 2003). The present study shows that not only brain NTE-LysoPLA but also total LysoPLA activity is inhibited by several OP and organosulfur compounds in vitro and in vivo. Although NTE-LysoPLA is more sensitive than total LysoPLA, the structure–activity relationships are similar, suggesting common structural features for the active sites of these LysoPLAs. In the $Nte^{+/-}$-haploinsufficient mice, the decrease in total LysoPLA activity is greater than predicted from the reduction of NTE-LysoPLA alone. These findings do not indicate that NTE-LysoPLA is a unique and distinct enzyme from the other mouse brain LysoPLA(s).

Lysolecithin level relative to LysoPLA activity

LysoPLAs are the principal enzymes for removing lysolecithin from cell membranes, including in human brain (Ross and Kish, 1994; Sun et al., 1987; Wang and Dennis, 1999), so their inhibition could lead to higher levels of lysolecithin. However, the in vivo lysolecithin level is a balance of biosynthesis and degradation. The relatively high lysolecithin level in mouse plasma (340–420 μM) is comparable to that in humans (234 μM) (Kishimoto et al., 2002). A lipidomics approach to brain analysis reveals no dramatic EOPF-induced changes in the abundance of various phospholipid classes or their constituent fatty acids. More specifically, lysolecithin levels in brain and blood are similar for control and EOPF-poisoned mice. However, there is a small (30%), but statistically significant, increase for lysolecithin in spinal cord. On this basis, delayed toxicity may result from localized rather than general alterations in lysophospholipid metabolism or signal transduction pathways in nerve tissues.

Conclusion

The acute lethality of most OP toxicants, including insecticides and chemical warfare agents, is attributable to selective inhibition of acetylcholinesterase as the primary target. However, some of these compounds are also potent NTE-LysoPLA inhibitors and therefore serve as probes to understand the role of lysophospholipids in human diseases.

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