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Cellular function of neuropathy target esterase in lysophosphatidylcholine action

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ABSTRACT

Neuropathy target esterase (NTE) plays critical roles in embryonic development and maintenance of peripheral axons. It is a secondary target of some organophosphorus toxicants including analogs of insecticides and chemical warfare agents. Although the mechanistic role of NTE *in vivo* is poorly defined, it is known to hydrolyze lysophosphatidylcholine (LPC) *in vitro* and may protect cell membranes from cytotoxic accumulation of LPC. To determine the cellular function of NTE, Neuro-2a and COS-7 cells were transfected with a full-length human NTE-containing plasmid yielding recombinant NTE (rNTE). We find the same inhibitor sensitivity and specificity profiles for rNTE assayed with LPC or phenyl valerate (a standard NTE substrate) and that this correlation extends to the LPC hydrolases of human brain, lymphocytes and erythrocytes. All of these LPC hydrolases are therefore very similar to each other in respect to a conserved inhibitor binding site conformation. NTE is expressed in brain and lymphocytes and contributes to LPC hydrolase activities in these tissues. The enzyme or enzymes responsible for erythrocyte LPC hydrolase activity remain to be identified. We also show that rNTE protects Neuro-2a and COS-7 cells from exogenous LPC cytotoxicity. Expression of rNTE in Neuro-2a cells alters their phospholipid balance (analyzed by liquid chromatography–mass spectrometry with single ion monitoring) by lowering LPC-16:0 and LPC-18:0 and elevating glycerophosphocholine without a change in phosphatidylcholine-16:0/18:1 or 16:0/18:2. NTE therefore serves an important function in LPC homeostasis and action.

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Introduction

Neuropathy target esterase (NTE) is a member of the serine hydrolase patatin-like phospholipase family (PNPLA) designated PNPLA6 (Wilson et al., 2006). Members of this family act as triglycerol lipases, retinylester lipases and phospholipases (PLA) (Kienesberger et al., 2008). NTE (a 1327-amino acid enzyme) and NEST (a recombinant enzyme comprised of human NTE residues 727–1216) hydrolyze lysophosphatidylcholine (LPC) much faster than phosphatidylcholine

(PC) (van Tienhoven et al., 2002; Quistad et al., 2003) and may act as phospholipase B (PLB) enzymes (Glynn, 2006). NTE is expressed in brain, testis, muscle, and other tissues (Wilson et al., 2006). Human NTE-related esterase (PNPLA7), with 65% homology to NTE at the amino acid level (Winrow et al., 2003), hydrolyzes LPC and lysophosphatidic acid (LPA) but not PC or triglycerides and may play a role in energy metabolism (Kienesberger et al., 2008). Mouse NTE, *Drosophila* Swiss Cheese protein, and yeast YML059c are 97, 61 and 51% homologous to human NTE. The endogenous substrate of yeast NTE is proposed to be PC or LPC since YML059c degrades PC to glycerophosphocholine (GPC) in living cells (Zaccheo et al., 2004).

Metabolic reactions related to NTE and LPC cellular function are shown in Fig. 1. LPC levels of cellular membranes are tightly regulated by several LPC hydrolases (LPCHs) (Ross and Kish, 1994; Wang and Dennis, 1999). NTE hydrolyzes membrane-associated lipids, suggesting a cellular role in their metabolism (van Tienhoven et al., 2002). NTE, lysophospholipases (LysoPLAs) and other LPCHs hydrolyze LPC to GPC (Casida and Quistad, 2004), an important osmolyte in kidney cells (Gallazzini et al., 2006). LPC is generated upon activation of PLA₂ (Macdonald et al., 2004) and also by lecithin:cholesterol acyltransferase (LCAT) (Santamarina-Fojo et al., 2000). High LPC concentrations are cytotoxic and associated with demyelination (Hall, 1972; Weltzien, 1979). LPC is also an effector molecule for the G protein-coupled G2A receptor (Parks et al., 2006). LPC is present in human plasma at

Abbreviations: IvcPLA₂, group IV cytosolic phospholipase A₂; DMEM, Dulbecco's Modified Eagle Media; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; FBS, fetal bovine serum; FP, fluorophosphonate; GFP, green fluorescent protein; HRP, horseradish peroxidase; IC₅₀, concentration inhibiting 50% of enzyme activity; I.S., internal standard; LC–MS, liquid chromatography–mass spectrometry; LPA, lysophosphatidic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MS, mass spectrometry; MSD, mass selective detector; NEAA, non-essential amino acids; NEST, NTE esterase domain; [³H]OBDPO, 2-[³H-*n*-octyl]-4*H*-1,3,2-benzodioxaphosphorin 2-oxide; OP, organophosphorus; OPIDN, OP-induced delayed neuropathy; PBS, phosphate buffered saline; PLA, phospholipase; PNPLA, patatin-like phospholipase; PNPLA7, NTE-related esterase; PV, phenyl valerate; rNTE, recombinant NTE; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SIM, single ion monitoring; TBST, Tris-buffered saline with Tween 20; t_R, retention time.

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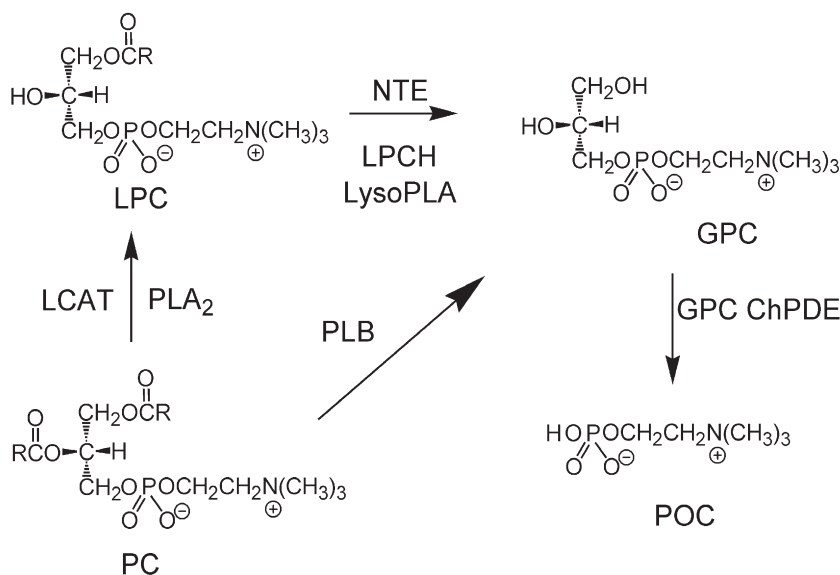


Fig. 1. Metabolic reactions related to NTE and LPC cellular function. Abbreviations: GPC, glycerophosphocholine; GPC ChPDE, glycerophosphocholine cholinephosphodiesterase; LCAT, lecithin:cholesterol acyltransferase; LPC, lysophosphatidylcholine; LPCH, lysophosphatidylcholine hydrolase; LysoPLA, lysophospholipase; NTE, neuropathy target esterase; PC, phosphatidylcholine; PLA₂, phospholipase A₂; PLB, phospholipase B; POC, phosphorylcholine.

concentrations up to 230 μM (Kishimoto et al., 2002), although it is mostly bound to albumin (Switzer and Eder, 1965) and unavailable for acting as a signaling effector.

The toxicology of organophosphorus (OP) insecticides and chemical warfare agents involves acetylcholinesterase as the primary target and several other serine hydrolases as secondary targets (Casida and Quistad, 2004; Gupta, 2006). Of these, NTE has received the greatest attention because its inhibition and aging leads to OP-induced delayed neuropathy (OPIDN), with about 70,000 cases in the last 70 years (Ehrich and Jortner, 2001; Johnson and Glynn, 2001). The steps from NTE inhibition to development of neuropathy are unclear but involve degeneration of long peripheral axons (Moretto and Lotti, 2006). A role of NTE in maintaining the integrity of peripheral motor axons is indicated by a M1012V mutation associated with a hereditary disease characterized by progressive lower-extremity weakness and wasting of distal extremity muscles (Rainier et al., 2008). NTE is also critical in embryonic development (Winrow et al., 2003; Moser et al., 2004). An understanding of the cellular role of NTE is an important step in evaluating OP toxicology.

The goal of this study was to determine the cellular function of NTE relative to LPC and OP action primarily by using recombinant NTE (rNTE). The first step was to express full-length rNTE (not NEST or YML059c) in Neuro-2a and COS-7 cells. rNTE was then characterized as to inhibitor sensitivity and specificity relative to LPHs in brain and blood. Next, the effect of expressed rNTE was examined on the sensitivity of the Neuro-2a and COS-7 cells to LPC. Finally the LPC pathway metabolites were compared in Neuro-2a cells without and with rNTE. The findings here (partly reviewed in Casida et al., 2008) show that NTE plays a key role in LPC homeostasis and action.

Methods

Chemicals and reagents. LPC-[¹⁴C]16:0 and PC-18:0/[¹⁴C]20:4 were from Amersham (Buckinghamshire, UK). 2-[³H-*n*-octyl]-4H-1,3,2-Benzodioxaphosphorin 2-oxide ([³H]OBDPO) was prepared in this laboratory (Yoshida et al., 1995). Chlorpyrifos oxon, paraoxon and glyphosate were from ChemService (Westchester, PA). Diisopropyl phosphonofluoridate and phenylmethanesulfonyl fluoride were from

Sigma-Aldrich (St. Louis, MO). Compounds available from previous or analogous syntheses in this laboratory were: *S*- and *R*-octyl-BDPO, 2-methylphenyl-BDPO, mipafox (Wu and Casida, 1992); isopropyl *n*-dodecylphosphonofluoridate, *n*-dodecanesulfonyl fluoride (Segall et al., 2003a); *n*-octanesulfonyl fluoride (Segall et al., 2003b); ethyl *n*-octylphosphonofluoridate (Wu and Casida, 1995); dipentyl-dichlorvos, phenyl benzylcarbamate (Wu and Casida, 1996); and dipentyl-chlorpyrifos oxon (Quistad et al., 2005a). Oksana Lockridge provided the fluorophosphonate (FP)-biotin probe (Schopfer et al., 2005). The internal standards (I.S.) LPC-17:0 and PC-14:0/14:0 were from Avanti Lipids (Alabaster, AL). Phospholipase A₂ (PLA₂), avidin-agarose beads, GPC, LPC and PC (from egg yolk), acid phosphatase potato extract (used as a source of patatin) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich. The LysoPLA I polyclonal antibody was from Abnova (Taipei City, Taiwan). The pNTE-GFP-N₁ (green fluorescent protein) plasmid encoding the full-length human NTE was provided by Paul Glynn (Lush et al., 1998).

Cell cultures, transfections and visualization. Neuro-2a cells were grown in Dulbecco's Modified Eagle Media (DMEM):Optimem (1:1) (Gibco Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS) and 1% non-essential amino acids (NEAA). Cells (40–60% confluency) were transfected with the pNTE-GFP-N₁ or empty pGFP-N₁ vector as a control using polyethylenimine (Polysciences Inc, Warrington PA). COS-7 cells were grown in DMEM supplemented with 10% FBS and 1% NEAA to 60% confluency and transfected with Polyfect (Qiagen, Valencia, CA). Cell cultures were maintained at 37 °C with 5% CO₂. Transfection complexes were removed after 24h. Cells 48h after transfection were washed with phosphate buffered saline (PBS), harvested in 100 mM Tris (pH 8.0) and subjected to analysis or frozen at – 80 °C until enzyme assays or sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Transfection efficiency was determined using an epifluorescence microscope. For confocal microscopy, cells were treated according to Li et al. (2003) and fluorescent images acquired using a Zeiss 510 meta confocal scanning microscope. For sorting based on GFP fluorescence, cells were harvested in TrypLE (Invitrogen) and resuspended in PBS containing 7% glycerol and 0.5 mM EDTA to prevent cell clumping.

Cells were processed using a Dako-Cytomation MoFlo high speed sorter (Dako, Glostrup, Denmark).

Western blotting and [³H]OBDPO labeling. Frozen cells from above were sonicated on ice and passed through a 25-gauge needle. Proteins were quantitated (Bradford, 1976) and then separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked for 1 h in 20 mM Tris, 500 mM sodium chloride, 0.01% Tween 20, pH 7.5 (Tris-buffered saline with Tween 20, TBST) containing 3% nonfat blotting grade milk (Apex, Kildare, Ireland), followed by incubation with an NTE polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or GFP monoclonal antibody (Clontech, Mountain View, CA) for 1 h. After washing three times (10 min each) with TBST buffer, membranes were incubated with goat anti-mouse horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA) for 30 min. Following three more washes with TBST, membranes were immersed in Immun-Star-HRP reagent (Bio-Rad, Hercules, CA) and exposed to film (Kodak, Rochester, NY). Alternatively, COS-7 cell preparations were incubated with 9 nM (final concentration) [³H]OBDPO in 100 mM phosphate buffer (pH 7.4) for 15 min. Following SDS-PAGE, the gel was sliced into 2 mm² pieces for determining their ³H content (Yoshida et al., 1995).

Enzyme assays. Sonicated cell preparations as above were added to 100 mM Tris, 1 mM calcium chloride, 0.01% Triton X-100, pH 8.0 (assay buffer) (490 μl total volume in 7.4-ml screw-top glass vials). Samples were treated with dimethyl sulfoxide (DMSO) alone (5 μl) or containing the test inhibitor and incubated for 15 min at room temperature. LPC-[¹⁴C]16:0 or PC-18:0/[¹⁴C]20:4 (each 70,000 dpm, 1.2 μM final concentration) was added in DMSO (5 μl). The reactions were incubated at 37 °C for 15 min. The radioanalysis extraction procedure in the incubation vials then varied with the substrate. The [¹⁴C]LPC reaction was terminated by adding isopropanol:hexane:1N sulfuric acid (20:5:1) (2.5 ml). After addition of ~ 100 mg silica gel the vial was vortexed for 10 s then hexane (1.5 ml) and water (1.5 ml) were added followed by vortexing for 15 s. The [¹⁴C]PC reaction was stopped by adding chloroform:methanol:hexane (1.25:1.4:1) (2.5 ml) followed by 0.83 ml 200 mM potassium carbonate and the samples were vortexed. Following 2–5 min for phase separation the top organic layer containing [¹⁴C]palmitic acid from [¹⁴C]LPC or [¹⁴C] arachidonic acid from [¹⁴C]PC was removed (1.8 ml) and the ¹⁴C content determined. To assay rNTE with phenyl valerate (PV) as the substrate, sonicated cell preparations in assay buffer (1 ml) were incubated with PV (1.4 mM final concentration in 50 mM Tris, 0.2 mM EDTA, 0.03% Triton X-100, pH 8.0) (1 ml) for 15 min at 37 °C (Quistad et al., 2003). The reaction was stopped by addition of 1% SDS and the color developed with 0.025% 4-aminoantipyrine in water (1 ml) followed by 0.4% potassium ferricyanide in water (0.5 ml). The liberated and derivatized phenol was measured colorimetrically at 490 nm using the Molecular Devices Versamax spectrophotometer (Sunnyvale, CA). Substrate hydrolysis by GFP-transfected cell preparations (controls) was subtracted from the hydrolysis by rNTE-containing preparations. Patatin hydrolysis of [¹⁴C]LPC and PV was measured as above in the linear range of protein level and time (Anderson et al., 2002). OP sensitivity is expressed as the IC₅₀ value (concentration which inhibits 50% of enzymatic activity) with the SD (*n* = 3).

Cytotoxicity assays. The cytotoxicity of LPC was determined for Neuro-2a and COS-7 cells at 70–80% confluency. Twenty-four h after transfection, LPC was introduced in Optimem (Neuro-2a cells) or DMEM with 10% FBS (COS-7 cells) and cytotoxicity was determined by adding MTT (0.5 mg/ml in the above media). After incubation for 3 h, media containing unreacted MTT was removed and 1 ml of DMSO and 0.5 ml of buffer (100 mM glycine, 100 mM sodium chloride, pH 10.5) were added to dissolve the blue formazan product

(Li and Casida, 1998). The absorbance was measured as above at 570 nm for comparison of LPC-treated cells to controls with no added LPC.

Phospholipid analysis. A reversed phase liquid chromatography–mass spectrometry (LC–MS) method was developed specifically for this study to analyze PC, LPC, GPC and phosphorylcholine (POC). The sonicated cells (0.9 ml) as above were added to chloroform:methanol:acetic acid (16:8:1) (1.5 ml) followed by the I.S. (LPC-17:0 and PC-14:0/14:0, 5 pmol each) in methanol (10 μl). After extraction, the organic layer contained PC and LPC and the aqueous phase GPC and POC. The organic layer was evaporated under nitrogen and the residue dissolved in DMSO (50 μl) and kept at – 20 °C. The aqueous layer was dried in a concentrator and the residual solid dissolved in water containing glyphosate (5 pmol) (100 μl) and kept at – 20 °C. The Agilent 1100 LC coupled with mass selective detector (MSD) system in the positive mode was used with a Kromasil C4 5 μM column (250 mm × 4.6 mm, Supelco, Sigma-Aldrich). The mobile phases, development conditions, retention times (*t_R*) and mass spectrometry (MS) data are given in Table 1. The predominant species of LPCs (16:0 and 18:0) and PCs (16:0/18:1 and 16:0/18:2) (Takatera et al., 2006) were chosen for quantitation. The scan and single ion monitoring (SIM) modes for MH⁺ were simultaneously used to quantitate by the I.S. method with LPC-17:0 for LPCs, PC-14:0/14:0 for PCs and glyphosate for GPC/POC. There was no interference from endogenous cell components with these I.S. compounds. The metabolites were normalized based on protein level. Statistical analysis was performed with R version 2.3.1 (<http://cran.cnr.berkeley.edu>).

Attempted characterization of erythrocyte LPCH. Erythrocytes from volunteers were washed three times with isotonic saline and sonicated in 100 mM phosphate buffer (490 μl, pH 7.0). Albumin was removed using ProteoExtract columns (EMD Chemicals, Gibbstown, NJ). Preparations were incubated with dipentyl-dichlorvos (0 or 100 μM, 15 min), followed by FP-biotin (10 μM) (OP additions in DMSO) for 2 h at room temperature. Excess FP-biotin was removed using Zebia columns (Pierce, Rockford, IL) and SDS was added to 0.5% (w/v). Samples were denatured by heating at 90 °C for

Table 1
Retention times (*t_R*) and MS data for analytes and internal standards

Compounds ^a	<i>t_R</i> (min) ^b	<i>m/z</i> (abundance %) ^c	
		MH ⁺	[M+Na] ⁺
<i>Organic layer</i>			
LPC			
LPC-16:0	11.0	496.6 (100)	518.7 (75)
LPC-17:0 (I.S.)	12.0	510.7 (100)	532.7 (33)
LPC-18:0	13.1	524.7 (100)	546.7 (22)
PC			
PC-14:0/14:0 (I.S.)	18.7	678.8 (100)	700.8 (33)
PC-16:0/18:2	20.8	758.9 (100)	780.9 (56)
PC-16:0/18:1	21.5	760.9 (100)	782.9 (34)
<i>Aqueous layer</i>			
GPC	3.7	258.5 (100)	280.4 (3)
POC	3.7	184.5 (100)	206.4 (7)
Glyphosate (I.S.)	3.5	170.4 (100)	192.5 (7)

^a See Fig. 1 for general structures. Glyphosate is *N*-(phosphonylmethyl)glycine.

^b Mobile phases: A – methanol: B – 10 mM ammonium acetate with 3% methanol and 0.05% formic acid. For LPC and PC separation the gradient was programmed at 80A:20B at 0 min increased linearly to 99A:1B at 19 min and held for 11 min at a flow rate of 0.8 ml/min. For GPC and POC analysis the gradient was programmed at 5A:95B at 0 min, increased linearly to 99A:1B at 19 min and held for 5 min at a flow rate of 0.8 ml/min.

^c MH⁺ masses were monitored in the positive mode by MSD from 100 to 300 amu for GPC and POC and 250 to 800 amu for LPCs and PCs. The SIM mode was programmed for LPCs at 496.6, 510.7 and 524.7 from 0 to 18 min and for PCs at 678.8, 758.9 and 760.9 from 18 to 30 min.

4 min and phosphate buffer as above was added to dilute the SDS to 0.2%. Avidin beads were incubated with the sample overnight at room temperature and washed three times with 50 mM Tris (pH 8.0) containing 0.2% SDS to reduce nonspecific binding. SDS 6× loading buffer was added to the beads and the samples subjected to SDS-PAGE. A dipentyl-dichlorvos-sensitive 75kDa band of possible interest was excised, subjected to tryptic digestion, and analyzed by tandem LC-MS using nano-electrospray ionization. In another study, erythrocytes were separated into membrane and cytosol fractions (Vose et al., 2007) and the membrane portion was solubilized by incubation with PLA₂ at 37 °C for 30 min. Solubilized membrane and cytosol fractions were separated by SDS-PAGE and the 150kDa bands excised for sequencing as above.

Results

rNTE expression in Neuro-2a and COS-7 cells

rNTE was expressed in Neuro-2a and COS-7 cells as a 182kDa GFP-fusion protein and identified by an NTE antibody (Fig. 2A) and a GFP antibody (Fig. 2B), while the control cells transfected with the empty vector expressed only the 27kDa GFP (Fig. 2B). The molecular mass of rNTE from COS-7 cells was verified by radiolabeling with [³H]OBDPO and SDS-PAGE giving a single radiolabeled protein band at the expected kDa (Fig. 2C). The transfection efficiencies were 25–35% and 50–60% for Neuro-2a and COS-7 cells, respectively. rNTE expressed as the GFP-fusion protein colocalized with calnexin (data not shown), indicating that it is an endoplasmic reticulum (ER)-associated enzyme.

rNTE hydrolysis of [¹⁴C]LPC and PV was assayed in the linear range of protein level and time (Fig. 3). The LPCH activities of control cells were less than 12% of those for the rNTE cells. Importantly, no NTE-dependent hydrolysis was observed for [¹⁴C]PC (Table 2).

Inhibitor sensitivity and specificity profiles for rNTE

The most potent inhibitors were OPs 1, 2 and 3, with IC₅₀ values of < 1nM when assayed with [¹⁴C]LPC (Table 3). The dipentyl analogs of both dichlorvos (4) and chlorpyrifos oxon (5) were also very active,

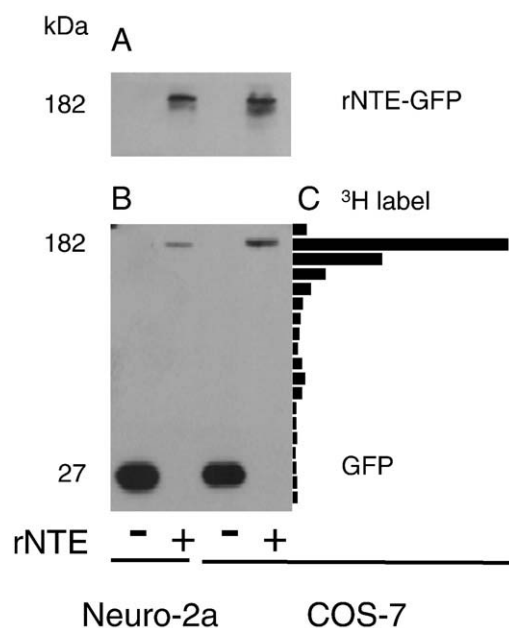


Fig. 2. rNTE from Neuro-2a and COS-7 cells detected by Western blotting with an NTE antibody (A) or GFP antibody (B) and by [³H]OBDPO labeling (C).

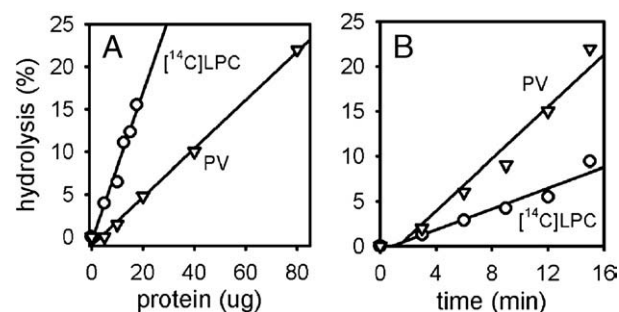


Fig. 3. [¹⁴C]LPC and PV hydrolysis by rNTE from COS-7 cells. (A) Dependence on protein level in assays for 15 min. (B) Dependence on time with protein levels (μg) of 15 for [¹⁴C]LPC and 75 for PV.

with IC₅₀ values < 25nM when assayed with either substrate. rNTE was moderately sensitive to compounds 6–11, while the other candidate inhibitors (compounds 12–15) were considerably less potent. rNTE showed the same profile of inhibitor sensitivity and specificity assayed with either [¹⁴C]LPC or PV ($r^2 = 0.84$, $n = 12$), with possible deviation from the correlation line only for dodecyl derivatives 2 and 9 (Fig. 4). On an overall basis the IC₅₀ ratio [¹⁴C]LPC/PV was 1.1 ± 0.7 ($n = 12$) indicating that assays with rNTE can be made with either substrate.

Inhibitor sensitivity and specificity profiles for rNTE compared to brain and blood LPCHs

Human brain, lymphocyte and erythrocyte LPCH activities show the same inhibitor sensitivity and specificity profiles (Vose et al., 2007). These earlier results can now be compared with the new findings on rNTE (Fig. 5). Data for rNTE were well correlated with mouse brain NTE ($r^2 = 0.89$, $n = 8$), human brain LPCH ($r^2 = 0.94$, $n = 12$), and human lymphocyte ($r^2 = 0.98$, $n = 12$) and erythrocyte ($r^2 = 0.97$, $n = 12$) LPCHs. This established that rNTE and brain, lymphocyte and erythrocyte LPCH enzyme(s) are very similar to each other with respect to a conserved inhibitor binding site conformation. With the same 12 compounds, human brain LPCH activity appeared to be 2.4- to 4.8-fold less sensitive than lymphocyte, erythrocyte and rNTE LPCH activities, whereas erythrocyte and lymphocyte LPCH activities were 1.8- to 2.3-fold more sensitive than rNTE (data from Fig. 5).

Effect of rNTE on the cytotoxicity of LPC

Neuro-2a and COS-7 cells expressing rNTE were more resistant than control cells to the cytotoxic effects of LPC (Fig. 6). With Neuro-2a, the IC₅₀ of LPC increased 2.6-fold from 50 μM for controls to 132 μM for cells expressing rNTE. The corresponding values for COS-7 were 153 and 192 μM for controls and rNTE cells, i.e. a protection factor of 1.3. The apparent magnitude of protection would presumably have been even greater with higher rNTE expression levels than the 25 to 60% in the present experiments. The lower protection factor with COS-7 cells might be related in part to 10% serum in the medium which contributed an added portion of LPC to the assay.

Table 2
Specific activities of rNTE expressed in Neuro-2a and COS-7 cells assayed with [¹⁴C]LPC, [¹⁴C]PC and PV

Cell line	rNTE	[¹⁴ C]LPC (pmol/min/mg)	[¹⁴ C]PC (pmol/min/mg)	PV (nmol/min/mg)
Neuro-2a	+	1679 ± 360	–	397 ± 67
	–	158 ± 45	–	0 ± 0
COS-7	+	427 ± 207	20 ± 20	121 ± 41
	–	50 ± 37	30 ± 60	0 ± 0

Table 3
Inhibitor sensitivity and specificity profiles for rNTE from COS-7 cells assayed with [¹⁴C] LPC and PV

Number	Inhibitor	IC ₅₀ , nM	
		[¹⁴ C] LPC	PV
1	S-octyl-BDPO ^a	0.57±0.01	0.78±0.06
2	Isopropyl n-dodecylphosphonofluoridate	0.21±0.04	19±3
3	Ethyl n-octylphosphonofluoridate	0.92±0.16	1.4±0.1
4	Dipentyl-dichlorvos	7.7±0.8	9.3±3.2
5	Dipentyl-chlorpyrifos oxon	13±1	24±2
6	R-octyl-BDPO ^a	67±2	62±4
7	Chlorpyrifos oxon	285±40	280±26
8	2-Methylphenyl-BDPO ^a	1967±375	1017±104
9	n-Dodecanesulfonyl fluoride	300±71	6333±943
10	Diisopropyl phosphonofluoridate	5950±1280	2667±125
11	n-Octanesulfonyl fluoride	4233±918	2167±94
12	Phenyl benzylcarbamate	63,000±9000	32,000±5000
13	Mipaflox	>50,000	<50,000
		(30±7) ^{b,c}	(61±2) ^{b,c}
14	Paraoxon	>40,000	>40,000
		(16±15) ^{b,c}	(17±2) ^{b,c}
15	Phenylmethanesulfonyl fluoride	>100,000	>100,000
		(17±9) ^b	(43±22) ^b

^a 1-Substituted-4H-1,3,2-benzodioxaphosphorin-2-oxide (BDPO) derivatives.

^b Inhibition (%) at indicated concentration.

^c In the traditional NTE assay, mipaflox is used at 50 to 200 μM and paraoxon at 40 μM with PV as the substrate in differentiating the NTE portion of the total hydrolytic activity (Quistad et al., 2003; Moretto and Lotti, 2006).

Effect of rNTE on LPC pathway metabolites in Neuro-2a cells

The cellular effect of NTE on levels of LPC pathway metabolites (Fig. 1) was examined by comparing Neuro-2a cells expressing rNTE with those containing only GFP. This required a suitable method for analyzing PCs and LPCs in the organic extract and another set of conditions for GPC and POC in the aqueous fraction. A typical LC-MS chromatogram in the combined SIM mode for lipid metabolites in the organic extract of control cells is shown in Fig. 7 with the *t_R* values and MS data given in Table 1. Good separation was achieved for the I.S. compounds and the endogenous LPCs and PCs, with PC-16:0/18:1 and PC-16:0/18:2 levels over 30-fold higher than LPC-16:0 and LPC-18:0 levels. As shown in Fig. 8, rNTE cells contained 26% lower levels (nmol/mg protein) than controls of LPC-16:0 (0.65±0.20 versus 0.88±0.14) and LPC-18:0 (0.17±0.04 versus 0.23±0.05) and significantly higher levels of GPC (0.76±0.19 compared to 0.28±0.06). The levels of POC, PC-16:0/18:1 and PC-16:0/18:2 were not significantly different between rNTE and control cells. In an attempt to correct for the 25–35% transfection efficiency, the rNTE cells were sorted to obtain a nearly pure population. PC levels (16:0/18:1 and 16:0/18:2) were still not significantly altered in rNTE cells (84±35% of controls) but

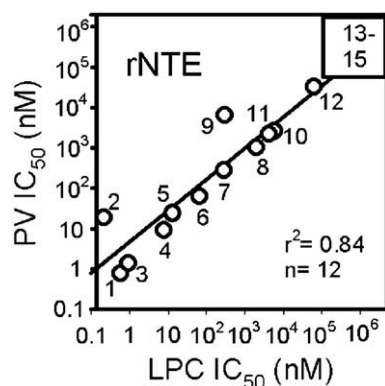


Fig. 4. Correlation of rNTE inhibitor sensitivity and specificity profiles with [¹⁴C]LPC and PV as substrates. The data are from Table 3. The correlation is improved on deletion of dodecyl derivatives 2 and 9 ($r^2=0.99$, $n=10$).

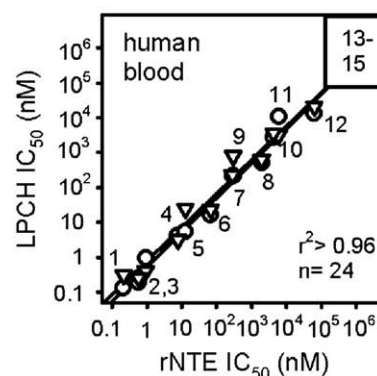
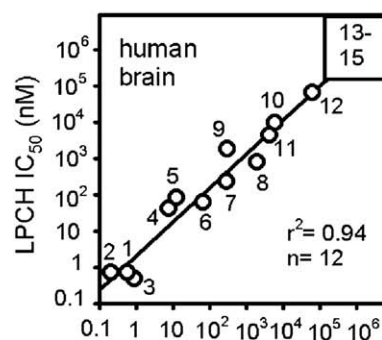
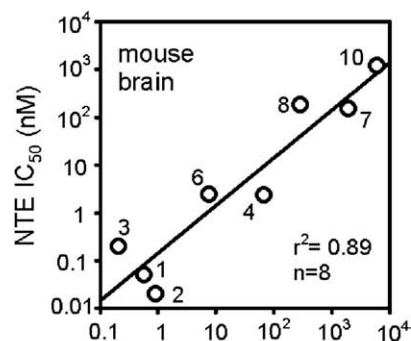


Fig. 5. Correlation of rNTE inhibitor sensitivity and specificity profiles with those of mouse brain NTE, human brain LPCH and human blood LPCH. Sources of data: rNTE with [¹⁴C]LPC substrate from Table 3; mouse brain NTE with LPC substrate (paraoxon-resistant and mipaflox-sensitive) from Quistad and Casida (2004); human brain, lymphocyte (▽) and erythrocyte (○) LPCHs with [¹⁴C]LPC substrate from Vose et al. (2007).

unfortunately the sample size was inadequate to quantitate LPC-16:0 and LPC-18:0 levels.

Other observations

Attempted characterization of erythrocyte LPCH

The FP-biotin probe at 10 μM inhibits erythrocyte LPCH activity, and might therefore be suitable for isolation of the derivatized enzyme with avidin beads followed by SDS-PAGE, tryptic digestion and sequencing. A dipentyl-dichlorvos-sensitive band at 75 kDa was identified as acylpeptide hydrolase. However, this did not account for the erythrocyte LPCH activity, since they have very different inhibitor sensitivity and specificity profiles (Quistad et al., 2005b). The erythrocyte membrane and cytosol proteomes were separated by SDS-PAGE and the 155 kDa region expected for NTE was excised, but no sequence related to NTE was obtained.

Relation of NTE to patatin

Patatin was considered as a possible model for NTE in studies of OP inhibitors. Potato extract was labeled with [³H]OBDDPO revealing

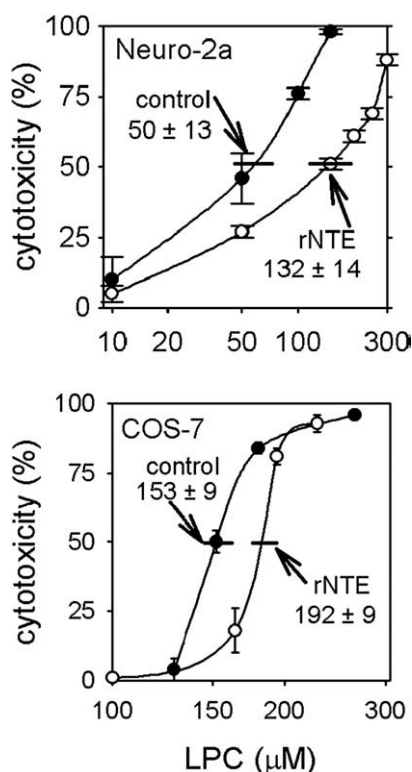


Fig. 6. Effect of rNTE on the cytotoxicity of LPC to Neuro-2a and COS-7 cells. IC₅₀ values are shown as µM levels.

a single band corresponding to the molecular mass (~40 kDa) of the patatin glycoproteins (data not shown). The OP sensitivity of the extract was evaluated with compounds 3, 4, 7, 8 and 10 using [¹⁴C] LPC and PV as substrates. Although the data are not shown, there was no correlation between the sensitivities indicating that different enzymes were hydrolyzing the two substrates. OP 3 was by far the most potent inhibitor with an IC₅₀ of 2.7±0.4 nM for [¹⁴C]LPC and 42±3 nM for PV.

Discussion

rNTE as a model for endogenous NTE

NTE originated as an OPIDN target defined by an assay with hen brain membranes using a discriminatory substrate (sequentially phenylphenyl acetate, PV and LPC) and selective inhibitors (para-oxon-resistant and mipafox-sensitive) (Johnson, 1988). This definition

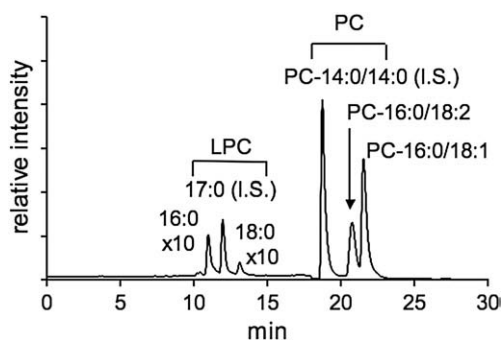


Fig. 7. Typical LC-MS chromatogram in the combined SIM mode of an organic extract of Neuro-2a control cells.

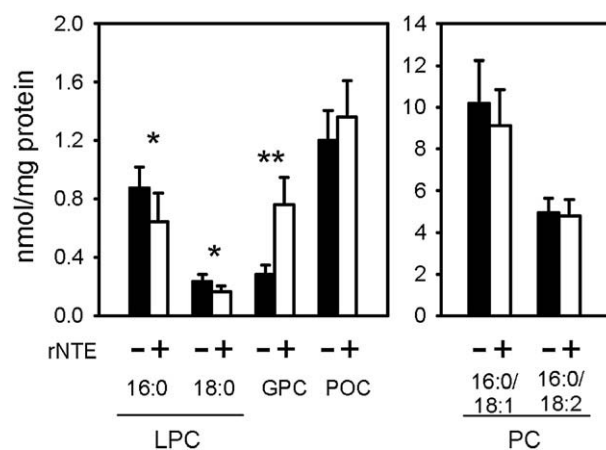


Fig. 8. Effect of rNTE on LPC pathway metabolites in Neuro-2a cells. The transfection efficiency for rNTE was 25–35%. Mean±SD, n=6. Significance of difference for rNTE – versus +: **p*<0.05, ***p*<0.01.

is experimentally cumbersome because several enzymes hydrolyze these substrates in most tissues. The studies were greatly facilitated by using NTE as a recombinant enzyme (Lush et al., 1998) in a background lacking interfering activities. rNTE in the present investigation was expressed as a 182 kDa GFP-fusion protein in both Neuro-2a and COS-7 cells which have little LPCH and insignificant PV endogenous activities. Both endogenous NTE (Quistad et al., 2003) and rNTE catalyze LPC and PV hydrolysis, are expressed in the ER (Li et al., 2003; this study) and have very similar inhibitor sensitivity and specificity profiles with either LPC or PV as substrates (Quistad et al., 2003; this study). However, it appears from the limited available data that dodecyl compounds 2 and 9 are somewhat more effective assayed with LPC than with PV. If this proves to be a consistent pattern it may be due to a long chain inhibitor competing better with a long chain substrate. Although either substrate can be used for specific NTE assays with the COS-7 expressed rNTE, LPC is examined here as the biologically-significant lipid and possibly the principal endogenous substrate.

NTE is one of many LPCHs

Human brain has at least four LPCHs (Ross and Kish, 1994; Quistad and Casida, 2004) and of these NTE may be the one most sensitive to OP inhibitors. When all of the inhibitor data are considered, LPCH activity from each source has essentially the same sensitivity and specificity profile, and rNTE is slightly more sensitive and brain somewhat less sensitive than the erythrocyte and lymphocyte enzymes; this may reflect the blend of enzymes or detoxification systems in the tissues.

Lymphocyte and erythrocyte LPCH activities are similar to rNTE in inhibitor sensitivity and specificity profiles. NTE is expressed in lymphocytes (Kaushik et al., 2005) and erythrocyte membranes (Pasini et al., 2006). Although erythrocyte membranes possess LPCH activity, 98% of the erythrocyte activity is cytosolic (Vose et al., 2007). Circulating erythrocytes expel their nuclei, ER and other intracellular organelles during maturation. It is possible that cytosolic erythrocyte LPCH enzymes have a similar structure to NTE, but without the N-terminal sequence that targets NTE to the ER membrane (Li et al., 2003). A splice variant of NTE is found in human liver (Winrow et al., 2003). Other soluble candidate LPCHs in erythrocytes are LysoPLA I (detected in this study by Western blotting) and group IV cytosolic phospholipase A₂ (IVcPLA₂) (Macdonald et al., 2004). IVcPLA₂ functions mainly as a PLA with specificity for PCs containing arachidonic acid at the sn-2 position,

and also exhibits LPCH activity (Ghosh et al., 2006) of unknown sensitivity to OPs. Attempts thus far to identify the erythrocyte LPCH have not been successful.

Cellular function of NTE in LPC action

NTE may protect cells from LPC accumulation and damage to membranes. It is associated with the ER (Li et al., 2003) near the inner bilayer of the cell membrane and may help maintain LPC homeostasis. Cells expressing rNTE and the corresponding control cells provide an excellent pair to determine the effect of NTE on the sensitivity to LPC as a preferred substrate of known toxicity (Weltzien, 1979). rNTE changed the sensitivity of the Neuro-2a and COS-7 cells to LPC by 2.6- and 1.3-fold, respectively, even though the expression level was ~30% indicating there might be an even greater potential difference between homogenous cell preparations with and without NTE. LPCHs are significant regulators of LPC and may also affect the levels of other lipid mediators, such as LPA.

Changes in the levels of PC, LPC, GPC or POC in Neuro-2a cells expressing rNTE would provide direct evidence on the cellular role of NTE. Neuro-2a cells were studied as the preferred model for examining the role of NTE in neurons. NTE is proposed to be a PLB capable of sequentially hydrolyzing both the *sn*-2 and *sn*-1 acyl moieties from PC (Glynn, 2006). However, in the present study, PC levels were not significantly different in Neuro-2a cells with and without rNTE. The increase in GPC with rNTE is greater than expected based on the decreased LPC levels; thus, more species of LPC in addition to 16:0 and 18:0 are probably hydrolyzed by rNTE to produce GPC. These results suggest that rNTE is primarily an LPCH in Neuro-2a cells. While the LPCH specific activity of rNTE from Neuro-2a cells acting on exogenous LPC is 10-fold higher than controls, the endogenous LPC levels are only decreased 26%, possibly due to cellular compartments of LPC of varying availability or a compensation mechanism of LPC synthesis. These results and others (Quistad et al., 2003; van Tienhoven et al., 2002) indicate that NTE is a LPCH *in vitro* and may perform the same function *in vivo*.

Relevance of NTE and LPC in OP toxicology

NTE is the primary target for OP delayed neurotoxicants that induce irreversible peripheral neuropathy (OPIDN) (Ehrich and Jortner, 2001). LPC, a principal substrate for NTE, plays a critical role in maintaining the integrity and fluidity of cellular membranes and initiates G protein receptor signaling. Deletion of NTE in mice results in embryonic lethality and of the homologous Swiss Cheese protein in *Drosophila* leads to neuronal cell death. LPC is capable of causing demyelination, and disruption of homeostasis can lead to impaired membrane integrity. Thus, major perturbations in LPC-regulating enzymes of the brain can have serious consequences. OPIDN is firmly associated with decreased activity and aging of NTE. The first step in its development could be an altered balance of LPC and other neurolipids, with further aging of NTE required in an unknown way for OPIDN. The present study shows the importance of characterizing localized LPCHs and LPC metabolism in understanding the progression of OPIDN.

Conflict of interest statement

The authors declare that there are no conflicts of interest in this study.

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