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# The homeostasis of phosphatidylcholine and lysophosphatidylcholine was not disrupted during tri-*o*-cresyl phosphate-induced delayed neurotoxicity in hens

Wei-Yuan Hou[a,b], Ding-Xin Long[a], Hui-Ping Wang[a], Qi Wang[a], Yi-Jun Wu[a,*]

[a] *Laboratory of Molecular Toxicology, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Datunlu Road, Beijing 100101, PR China*
[b] *Graduate School of the Chinese Academy of Sciences, Beijing 100039, PR China*

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ABSTRACT

Little is known regarding early biochemical events in organophosphate-induced delayed neurotoxicity (OPIDN) except for the essential inhibition of neuropathy target esterase (NTE). We hypothesized that the homeostasis of lysophosphatidylcholine (LPC) and/or phosphatidylcholine (PC) in nervous tissues might be disrupted after exposure to the organophosphates (OP) which participates in the progression of OPIDN because new clues to possible mechanisms of OPIDN have recently been discovered that NTE acts as lysophospholipase (LysoPLA) in mice and phospholipase B (PLB) in cultured mammalian cells. To bioassay for such phospholipids, we induced OPIDN in hens using tri-*o*-cresyl phosphate (TOCP) as an inducer with phenylmethylsulfonyl fluoride (PMSF) as a negative control; and the effects on the activities of NTE, LysoPLA and PLB, the levels of PC, LPC, and glycerophosphocholine (GPC), and the aging of NTE enzyme in the brain, spinal cord, and sciatic nerves were examined. The results demonstrated that the activities of NTE, NTE-LysoPLA, LysoPLA, NTE-PLB and PLB were significantly inhibited in both TOCP- and PMSF-treated hens. The inhibition of NTE and NTE-LysoPLA or NTE-PLB showed a high correlation coefficient in the nervous tissues. Moreover, the NTE inhibited by TOCP was of the aged type, while nearly all of the NTE inhibited by PMSF was of the unaged type. No significant change in PC or LPC levels was observed, while the GPC level was significantly decreased. However, there is no relationship found between the GPC level and the delayed symptoms or aging of NTE. All results suggested that LPC and/or PC homeostasis disruption may not be a mechanism for OPIDN because the PC and LPC homeostasis was not disrupted after exposure to the neuropathic OP, although NTE, LysoPLA, and PLB were significantly inhibited and the GPC level was remarkably decreased.



## 1. Introduction

Organophosphorous compounds (OP) are the principal class of insecticide and chemical warfare agents (Casida and Quistad, 1998; Marrs et al., 1996). Certain OPs such as TOCP, which is used in industry mainly as an additive to lubricating oil and a softener in the manufacture of plastic products, are able to induce a delayed neurodegenerative condition known as OP-induced delayed neurotoxicity (OPIDN) (Johnson, 1974; Craig and Barth, 1999; Winder and Balouet, 2002). OPIDN is an axonopathy which is characterized by distal degeneration of some long and large-diameter axons in the peripheral nerves and the spinal cord (Lotti, 1992; Johnson, 1993). The putative molecular target of OPIDN is a neural protein with esterasic activity called neuropathy target esterase (NTE), which is intimately associated with the cytoplasmic face of the endoplasmic reticulum (Li et al., 2003; Akassoglou et al., 2004). NTE is defined as the paraoxon-resistant and mipafox-sensitive esterase with phenyl valerate-hydrolyzing activity and the NTE assay is fully validated for toxicological relevance to OPIDN (Johnson, 1969, 1975, 1977). In sensitive species, OPIDN is initiated when >70% of the NTE is inhibited by neuropathic OPs and the clinical expression occurs 2–3 weeks later (Johnson, 1969). An aging reaction is also required for OPIDN (Johnson, 1990). Although OPIDN has been the subject of intense investigation for years, a definitive causal link between OP-modified NTE and nerve damage has not yet been established (Glynn, 2003; Read et al., 2007). Nevertheless, significant progresses have been achieved by studying the biochemical function of NTE. The purified recombinant catalytic domain of NTE has sequence similarity in the active site region to that of the calcium-independent phospholipase $A_2$, known to have lysophospholipase (LysoPLA) activity, and displays potent LysoPLA activity *in vitro* (van Tienhoven et al., 2002). Therefore, NTE may act as a

* Corresponding author. Tel.: +86 10 64807251; fax: +86 10 64807099.
*E-mail address:* wuyj@ioz.ac.cn (Y.-J. Wu).

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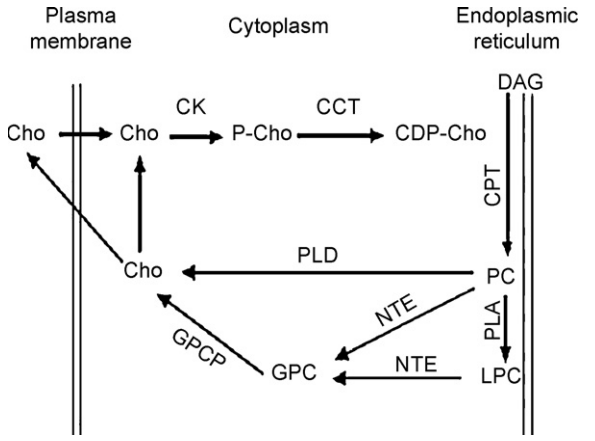


**Fig. 1.** The pathway of neuropathy target esterase (NTE) participating in phosphatidylcholine (PC) metabolism. Choline (Cho) is transported into cells and then phosphorylated by choline kinase (CK). Phosphocholine (P-Cho) reacts with cytidine triphosphate (CTP) in the pathway's rate-limiting step catalyzed by CTP:phosphocholine cytidylyltransferase (CCT) forming CDP-choline (CDP-Cho). CDP-Cho reacts with diacylglycerol (DAG) catalyzed by choline phosphotransferase (CPT) to form membrane-associated PC. PC can be hydrolyzed by phospholipase D, forming phosphatidic acid and Cho, PC can also be deacylated by NTE at the cytoplasmic face of the endoplasmic reticulum to form soluble products: free fatty acids and glycerophosphocholine (GPC). In addition, phospholipase A and NTE can sequentially deacylate PC to GPC. GPC is hydrolyzed by glycerophosphorylcholine phosphodiesterase (GPCP), forming Cho and glycerophosphate. Cho can be phosphorylated or secreted from the cell. The above information is combined from Quistad et al. (2003), Anfuso et al. (2003), Zaccheo et al. (2004), and Read et al. (2007).

kind of LysoPLA with lysophosphatidylcholine (LPC) as its physiological substrate in mouse (Quistad et al., 2003). Further study demonstrated that NTE also contains phospholipase B (PLB) activity which can convert phosphatidylcholine (PC) to glycerophosphocholine (GPC) in yeast and mammalian cells (Zaccheo et al., 2004). Collectively, these discoveries indicate that inhibition and aging of NTE during OPIDN may affect the homeostasis of PC and LPC (see Fig. 1 for the pathway of NTE participating in PC metabolism).

PC is the major phospholipid of eukaryotic cells, representing about 50% of the membrane phospholipids in animal cells (Cui and Houweling, 2002). Regulation of the PC biosynthesis, degradation, and relative distribution among different membranous structures is critical for cellular function (Fernández-Murray and McMaster, 2005). Inhibition synthesis or excessive breakdown of the PC has been associated with growth arrest and apoptotic cell death (Tercé et al., 1994; Cui et al., 1996; Anthony et al., 1999). Disruption of the PC homeostasis happens in many neurodegenerative disorders. For examples, breakdown of the PC has been observed in senile dementia while elevated PC levels can be found in specific brain regions of Alzheimer's patients (Klein, 2000; Soderberg et al., 1992). The PC and its related choline compounds have a neuronal-specific function, in that they have been shown to promote the synthesis and transmission of neurotransmitters (McDaniel et al., 2003). The LPC is another important phospholipid molecule in mammalian tissues. An elevated LPC level can induce neuronal sheath demyelination, together with a variable degree of axonal degeneration (Hall, 1972; Jean et al., 2002), which is similar to the pathological changes of OPIDN, suggesting inhibition of NTE may lead to demyelination. Myelin sheaths are extensions of the plasma membrane of Schwann cells in the peripheral nervous system, and of oligodendrocytes in the central nervous system. The myelin sheaths are specialized and unique membranes that have a high content of phospholipids and a relatively simple protein composition (Vance et al., 2000) and therefore correct composition of phospholipids is important for maintaining its proper function.

Together with the critical role of PC and LPC in the nerve system and the biochemical function of NTE, as a LysoPLA or PLB, disruption of PC and LPC homeostasis may be a mechanism for OPIDN. To confirm this hypothesis, we have induced OPIDN in hens with tri-*o*-cresyl phosphate (TOCP), an inhibitor of NTE and investigated the relationship between alteration of NTE-LysoPLA or PLA and NTE-PLB or PLB activities and the alteration of the homeostasis of the PC, LPC, and GPC.

## 2. Materials and methods

### 2.1. Reagents

TOCP (purity >99%) was purchased from BDH Chemicals Co. Ltd. (Poole, England). Coomassie brilliant blue G-250 and phenylmethylsulfonyl fluoride (PMSF) were purchased from Fluka Chemika (Buchs, Switzerland). Mipafox and phenyl valerate were synthesized in our laboratory as described previously (Johnson, 1982). Atropine sulfate was obtained from Minsheng Pharmaceutical Factory (Hangzhou, China). Benzenesulfonyl fluoride, paraoxon, *sn*-glycero-3-phosphocholine phosphodiesterase, choline oxidase, peroxidase (horseradish), PC, LPC, and GPC were purchased from Sigma (St. Louis, MO, USA). 3-(*N*-Ethyl-3-methylanilino)-2-hydroxypropanesulfonic acid sodium salt (TOOS) was obtained from Nanjing Robiot Company (Nanjing, China). 4-Aminoantipyrine was obtained from Beijing Xizhong Chemical Factory (Beijing, China) and silica gel 60 $F_{254}$ plates (20 × 20, 0.25-mm thick) were purchased from Merck (Darmstadt, Germany).

### 2.2. Animals

Adult Beijing white laying hens (8 months old and 1.5 kg in size) used in this study were purchased from the Dabei Poultry Farm (Beijing, China). They were housed in cages individually. The birds were acclimatized for at least 1 week prior to the start of the experiment. During the experiment periods, the temperature in the hen house was maintained at 22 °C and 50% humidity with a light/dark cycle of 12 h.

### 2.3. Administration

Thirty hens were divided into three groups (Control, TOCP-, and PMSF-treated) with 12 hens in each experimental group and 6 hens in the control group. Hens in the TOCP group were orally given a single dose of 750 mg/kg in a gelatin capsule while three hens in control group were given an empty gelatin capsule. The hens in the PMSF group were injected with PMSF (100 mg/kg, sc) dissolved in dimethyl sulfoxide (DMSO), while the other three hens in the control group received DMSO (0.8 mg/kg, sc) only. From the first day after exposure to the chemicals, the hens were examined daily (twice per day, in the morning and late afternoon) for the delayed neurotoxic signs until the 14th day. On completion of 4 h, 4 days, and 14 days post-dosing, four hens from chemical-treated groups at each time point and the hens from control group at the last time point were sacrificed, respectively, by cervical decapitation. The whole brain, spinal cord, and sciatic nerve were quickly dissected and frozen in liquid nitrogen before storing at −80 °C.

### 2.4. NTE activity assay

Nervous tissues were homogenized in TE buffer (50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0) and centrifuged at 100 × *g* at 4 °C for 2 min. NTE activity in the supernatant fraction was determined from colorimetric assay of the phenol formed by the absorbance difference for phenyl valerate hydrolysis between samples exposed to 40 μM paraoxon and those with both 40 μM paraoxon plus 50 μM mipafox according to Johnson (1977) with modification for reduced volume microassay as previously described in our lab and expressed as nanomoles of phenol formed per minute per milligram of protein with phenol as the standard (Chang et al., 2006). Concentration of protein was measured by the method of Bradford using bovine serum albumin as standard (Bradford, 1976).

### 2.5. NTE enzyme aging assay

The aging measurement procedure was previously described by Kellner et al. (2000) with modifications for reduced volume. Nerve tissue samples were homogenized in 10% (w/v) TE buffer. A fresh solution of potassium chloride (KCl) and potassium fluoride (KF) reactivation buffer was prepared (250 mM in 50 mM Tris-citrate, 0.2 mM EDTA, pH 5.2); and reactivation was initiated by adding 0.25 ml nerve tissue homogenate to 1.5 ml KF reactivation buffer. Another 0.25 ml aliquot was added to 1.5 ml KCl-containing buffer in plastic test tubes (buffer prewarmed to 37 °C in shaking water bath). After 30 min incubation at 37 °C, tubes were cooled on ice and 4.25 ml ice-cold distilled water was added to each to slow the reaction. The cooled tubes were centrifuged at 27,000 × *g* for 60 min, the supernatant was discarded, and the pellet was resuspended in 1 ml TE buffer. NTE activity was