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Purification and characterization of neuropathy target esterase (NTE) from rat brain

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Abstract

Neuropathy target esterase (NTE) is an integral membrane protein in vertebrate neurons and a member of a novel family of putative serine hydrolases. Neuropathic organophosphates react covalently with the active site serine residue of NTE, causing degeneration of long axons in spinal cord and peripheral nerves which becomes clinically evident 1–3 weeks after exposure to OPs, hence termed as organophosphate induced delayed neuropathy. The present study reports the isolation and characterization of NTE protein from rat brain. Rat brain microsomes were solubilized with phospholipase A_2 and they were fractionated by gel filtration chromatography in S-300 column. The sample was eluted in buffer containing polyoxyethylene W1 detergent, which yielded an active fraction of 200 kDa. The most enriched NTE active fraction was further purified by 3-9′-mercaptononylthio-1,1,1-trifluoropropan-2-one bound to sepharose CL4B. The SDS-PAGE confirmed the 155-kDa protein as the most likely candidate for NTE. Database searching of rat N-terminal protein revealed homology with variety of polypeptides from different organisms and suggested that NTE protein has function beyond the nervous system and mediates a biochemical reaction highly conserved through evolution.

Keywords: Organophosphate; NTE; Organophosphate induced delayed neuropathy; Axonopathies; Serine esterase

Introduction

Organophosphorus (OP) compounds represent a vast array of chemicals that are widely used as insecticides, additives, therapeutics agents and notoriously chemical warfare agents (Racke, 1992). Intoxication by OPs represents as much as 80% of pesticide related hospital admissions and deaths (Aldridge, 1993). The manifestations of OP poisoning are clinically divided into three types: the cholinergic syndrome, the intermediate syndrome, and the organophosphate induced delayed neuropathy (Mingxing et al., 2003). In humans, delayed chronic neurotoxic syndromes have been reported in Gulf war veterans, chronically exposed to organophosphates or combination of agents (Solberg and Belkin, 1997; Haley and Kurt, 1997, Haley et al., 1999; Enserink, 2001; Hitt, 2002). The most common and best-understood delayed syndrome is organophosphate induced delayed neuropathy (OPIDN), which becomes clinically evident 1–3 weeks after exposure to organophosphates. OPIDN is characterized by paralysis of the lower limb due to degeneration of long axons in the spinal cord and in peripheral nerves, hyperexcitability and flaccid extremities (Lotti, 2002). In addition to the tremendous differences on the clinical manifestations, these syndromes involve distinct molecular targets. While a variety of toxic agents can cause axonopathies, OP mediated neuropathy is unique in that its primary target protein neuropathy target esterase has been identified. The mechanism of action remains to be determined regarding the precise role of NTE in the development of OPIDN.

Neuropathy target esterase (NTE), the proposed target protein, is an integral membrane protein present maximally in vertebrate neurons. It is detected in vitro by its capacity to

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catalyze the hydrolysis of phenyl valerate although its physiological substrate is unknown (Lotti, 1992; Ray and Richards, 2001). Despite the worldwide use of very large quantities of OP pesticides, those with neuropathic potential have been screened out of the market, why then continue studies of NTE and OPIDN? First, it is now becoming apparent that the normal physiological function of NTE is in neuronal development and involves cell-signaling pathway (Glynn, 1999). Secondly, characterization of NTE and events subsequent to its modification by neuropathic OPs provide a singular opportunity to elucidate the molecular mechanisms of this neuropathy, which in turn may provide insight into mechanisms of normal axonal maintenance and of other neurodegeneration conditions. Thirdly, because NTE is an interesting protein in its own right as it is a member of newly discovered protein family, with a domain conserved through evolution, which may have a second function in addition to its capacity to hydrolyze esters (Glynn, 1999).

Elucidation of the structure, subcellular localization and normal functions of NTE are of interest not only from toxicology point of view but also for fundamental neurobiology, since the protein may play a role in normal axonal maintenance (Lotti, 2002). However, subsequent progress in this area had been hampered by difficulty in isolating NTE from brain tissue since it comprises only 0.03% of brain microsomal protein (Johnson, 1974). Moreover NTE has resisted purification and poor recovery from detergent solubilized NTE activity on chromatographic fractionation possibly due to loss of an activating factor (Glynn, 2000). Two forms of NTE have been isolated and characterized, particulate (P-NTE) and soluble (S-NTE) (Escudero and Vilanova, 1997). P-NTE is a membrane bound protein that constitutes the predominant form in chick brain (Glynn et al., 1994). Attempts to purify NTE by gel filtration, sucrose gradient centrifugation, ion exchange chromatography and isoelectric focussing have so far failed to yield significant amount of purified preparation of NTE. Previous methods to isolate active NTE by gel filtration chromatography have yielded a wide range of estimated sizes for the protein ranging from approximately 850 to 1800 kDa (Chemnitius et al., 1984; Pope and Padilla, 1989). Glynn et al. (1994) reported the isolation of P-NTE from chick brain with a molecular mass of 155 kDa using a biotinylated saligenin phosphate analog as an active site ligand (Thomas et al., 1989). Most of these purification studies have been concentrated on hen, which is the habitual model to study OPIDN, but in recent years, objections have been raised on the limited value of this animal in addressing the mechanistic aspects of OP neuropathies. Further rat too has been shown to be a model for OPIDN (Veronesi, 1984). In view of this, easy handling and abundant baseline data available in case of rat prompted us to attempt purification of NTE from rat brain.

The purpose of the present line of investigation was to isolate and characterize rat brain NTE using 3′-(9′-mercaptononylthio)-1,1,1-trifluoropropanone-2-one (MNTFP) bound to epoxy activated sepharose CL4B as an affinity ligand.

Materials and methods

Materials

Diethyl *p*-nitrophenyl phosphate (Paraoxon), sepharose (CL4B) DTT, EDTA, EGTA, polyoxyethylene W1 ether were obtained from Sigma; Sephacryl S-300 was purchased from Amersham Biosciences and 1-9, nonanedithiol, MNTFP, 1-4 butanediol diglycidyl ether was obtained from Fluka. Phenyl valerate was provided as a kind gift from Defence Research and Development Establishment, Gwalior, India.

Microsomal preparation

Microsomes were prepared from male Wistar rat brain. Rats were decapitated and the whole brain was placed on ice, weighed and washed with 0.33 M sucrose. Twelve grams of fresh brain tissue was homogenized with mechanically driven Teflon fitted potter Elvejhem type homogenizer in a 50 mM Tris–HCl buffer pH 8.0. The homogenate was centrifuged at 10,000×*g* for 20 min at 4 °C. The supernatant was removed and the pellet resuspended in the same volume of buffer and again centrifuged at 100,000×*g* for 60 min at 4 °C.

NTE solubilization

The pellet obtained above was suspended in 50 mm Tris–HCl buffer pH 8.8. To this suspension, the equivalent of 12.5 U/ml phospholipase A_2 (isolated from apis mellifera) was added and microsomal suspension was incubated at 37 °C for 30 min (Richardson et al., 1979; Dessen, 2000). After incubation, the suspension was placed on ice and EDTA, EGTA, DTT were added to yield final concentration of 1 mM, 1 mM and 250 μM respectively. This suspension was again centrifuged at 100,000×*g* for 60 min at 4 °C and the resulting microsomal supernatant (Seifert and Wilson, 1994) was collected and tested for NTE activity.

Gel filtration chromatography

Gel filtration chromatography was performed using a 2× 100 cm S-300 HR column (Amersham Biosciences)—using peristaltic pump at a constant output of 3.5 ml/min (Fig. 1). The column's exclusion volume was determined using dextran blue (avg. mass 2000 kDa) and standardized using known commercial protein standards within a range of 663 kDa to 12.5 kDa (thyroglobulin to cytochrome *c*). The PLA_2 solubilized NTE extracts were combined with gel filtration buffer and incubated at 4 °C for 1 h. An aliquot of 500 μl of PLA_2 solubilized sample was retained for NTE activity, protein determination and rest of the sample applied to the column. The sample was eluted in 50 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 0.1% (w/v) polyoxyethylene W1, 500 mM NaCl buffer pH 8.2. Eluates were collected in 3.5 ml aliquots. The reproducibility of the chromatograms was controlled by monitoring the absorbance at 280 nm. The total esterase activity, NTE activity and protein were measured in each