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In vivo and in vitro regional differential sensitivity of neuropathy target esterase to Di-*n*-butyl-2,2-dichlorovinyl phosphate*

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Abstract. Organophosphate-induced delayed polyneuropathy (OPIDP) is initiated by inhibition/aging of more than 70–75% of neuropathy target esterase (NTE). Di-*n*-butyl-2,2-dichlorovinyl phosphate (DBDCVP) (1 mg/kg s.c.) inhibited 96%, 86% and 83% of NTE in brain, spinal cord and peripheral nerve, respectively, and induced a typical central peripheral distal axonopathy in hens. A lower dose (0.45 mg/kg s.c.) caused 90%, 83% and 54% NTE inhibition in the same organs; by contrast, hens developed a spastic ataxia with axonal degeneration in spinal cord but not in peripheral nerve. With a dose of 0.2 mg/kg s.c., a suprathreshold inhibition of NTE was produced in brain (78%) but not in spinal cord (56%) and peripheral nerve (33%) and no morphological or clinical signs of neuropathy developed in hens. With doses up to 4.0 mg/kg s.c., acetylcholinesterase (AChE) inhibition was similar throughout the nervous system. In vitro time-course inhibition studies showed a different sensitivity to DBDCVP of NTE from peripheral nerve ($k_a = 5.4 \times 10^6$) relative to that from spinal cord ($k_a = 13.9 \times 10^6$) or brain ($k_a = 20.6 \times 10^6$). In vitro I_{50}s of DBDCVP for AChE were similar in brain, spinal cord and peripheral nerve (11–17 nM). These data support the hypothesis that the critical target for initiation of OPIDP is located in the nerve fiber, possibly in the axon and also suggest that peripheral nerve NTE has a different sensitivity to DBDCVP than the brain enzyme. Moreover, they confirm data showing that the degree of NTE inhibition in brain after dosing with organophosphates may not be a good monitor for the enzyme in parts of the nervous system where axonal degeneration actually develops. Therefore, direct assay of peripheral nerve NTE yields data which closely correlate with degree of axonal degeneration.

Key words: Acetylcholinesterase – Di-*n*-butyl-2,2-dichlorovinyl phosphate – Neuropathy target esterase – Organophosphates – Polyneuropathy

Introduction

Single doses of certain organophosphorus (OP) esters induces a central-peripheral distal axonopathy (known as organophosphate-induced delayed polyneuropathy, OPIDP) (Johnson 1982). The biochemical mechanisms underlying initiation of OPIDP involve the phosphorylation of neuropathy target esterase (NTE) followed by a further non-enzymatic reaction ("aging") in which a chain is cleaved from the bound phosphorus atom, leaving a charged monosubstituted phosphoric residue at the active site. The threshold for initiation of OPIDP is 70–75% of inhibition/"aging" of NTE. Routinely, only the inhibition of NTE is measured and, on the basis of the chemical structure of the phosphorylating agent, the amount of inhibited/aged NTE is assumed to be equivalent (Johnson 1982). Recent evidence, however, suggests that this assumption is not always true as in the case of NTE inhibited by different chiral isomers of OPs (Johnson 1987). For more than 200 OP esters there is a close correlation between suprathreshold inhibition of hen brain NTE within 40 h after dosing and development of clinical ataxia 10–20 days later. For convenience, the effect on NTE is then usually monitored only in brain, despite the fact that the pathological lesions occur in spinal cord and peripheral nerve axons and that the critical OP-NTE interaction occurs in the nerve fiber (presumably in the axon) rather than in the cell body (Caroldi et al. 1984; Lotti et al. 1987). Furthermore, measurement of the effect of some dimethyl phosphates on brain NTE was found not to be a good biochemical monitor for OPIDP (Johnson 1978) because the inhibition of spinal cord NTE was lower than that of brain NTE. This was interpreted as a different access of the inhibitor to the target.

We report on a compound Di-*n*-butyl-2,2-dichlorovinyl phosphate (DBDCVP), which differentially inhibited NTE, but not acetylcholinesterase (AChE), in brain, spinal cord and peripheral nerve in vivo and in vitro.

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Materials and methods

Chemicals

DBDCVP was kindly supplied by Dr R. J. Richardson (Ann Arbor, MI, USA). Paraoxon (diethyl *p*-nitrophenyl phosphate), diisopropyl phosphorofluoridate (DFP) and phenylmethanesulphonyl fluoride (PMSF) were purchased from Sigma Chem. Co. (St. Louis, MO, USA); paraoxon was purified as previously described (Johnson 1977). Mipafox (N,N-diisopropyl phosphorodiamidofluoridate) was kindly supplied by Dr M. K. Johnson (Carshalton, UK). Phenylvalerate was synthesized and purified as described by Johnson (1977).

Animals

Treatment. Experiments were performed on randomly bred adult White Leghorn hens (1.5–2.3 kg body wt). Animals were divided into groups and treated as follows:

1. glycerol formal 0.1 ml/kg subcutaneously (s.c.) (controls);
2. DBDCVP 0.20 mg/kg s.c.;
3. DBDCVP 0.45 mg/kg s.c.;
4. DBDCVP 1.00 mg/kg s.c.;
5. DBDCVP 2.00 mg/kg s.c.;
6. DBDCVP 4.00 mg/kg s.c.

DBDCVP was dissolved in glycerol formal immediately before use and injected s.c. in the anterothoracic region in a volume of 0.1 ml/kg.

Biochemistry. Animals were decapitated 24 h after dosing. Brain, lumbar spinal cord and sciatic were excised immediately and either placed in ice-cold 50 mM Tris/HCl buffer (pH 8.0) containing 0.2 mM EDTA and assayed for NTE and AChE or stored at −80°C for not more than a week prior to assay. NTE and AChE are unaffected by this procedure. NTE activity was determined according to Johnson (1977) (brain and spinal cord) and Caroldi and Lotti (1982) (sciatic nerve); the peripheral nerve assay was modified by spinning of whole homegenate (30% w/v) at 100 g for 5 min at 4°C. The supernatant used in assay mixtures gives a differential $\overline{OD}$ of about 0.400. AChE activity was determined by the method of Ellman et al. (1961), slightly modified.

Clinical evaluation. The clinical evaluation for neurological dysfunction was performed on each bird of groups 1–4 daily from day 7 until sacrifice (day 21). Walking performance was evaluated according to the 0–4 point scale of Johnson and Barnes (1970) (0 = no defect; 1 = slightly abnormal gait; 2 = severely abnormal gait; 3 = animal can stand but frequently collapses; 4 = animal unable to stand). The "leg-retraction" reflex, namely the ability of the bird to retract its legs from the dangling position when lifted under the breast, was evaluated as described by Lotti et al. (1987); the legs characteristically remain extended and flaccid in animals with typical OP neuropathy. Reported clinical scores refer to day 15 after dosing.

Histopathology. Animals were anesthetized with ketamine (30 mg/kg intramuscularly) and ether, the thoracic cavity opened and the tissues fixed by vascular perfusion with Sorensen's fixative (phosphate-buffered 4% paraformaldehyde/glutaraldehyde, pH 7.4) via a cannula inserted into the left ventricle of the heart. Perfusion was continued for 5 min, when muscles became rigid (volume of 500–700 ml fixative). The sciatic and tibial (medialis and lateralis) nerves, and the cervical, thoracic and lumbo-sacral spinal cord, were excised and post-fixed in Dalton's 2% chrome osmium tetroxide, dehydrated stepwise in increasing concentration of ethanol and embedded in epoxy resin. One-micrometer sections were cut from hardened blocks, stained with toluidine blue and examined by bright-field microscopy. The degree of axonal degeneration was blindly scored on a 0–4 point scale (0 = no degeneration; 1 = occasional degenerating fibers, questionable significance; 2 = moderate number of degenerating fibers; 3 = pronounced degeneration; 4 = severe degeneration). Degeneration in spinal cord was limited to those regions typically associated with OPIDP, i.e. dorso-lateral tracts in cervical spinal cord, lateral and ventral tracts in thoracic spinal cord and ventral tracts at lumbosacral level.

In vitro inhibition studies

Nervous tissues were obtained from randomly inbred red hens.

For time-course studies on NTE, paired tissue samples, conventionally called P and M, were prepared and used as described by Clothier and Johnson (1980): these samples are identical, except that P contains active NTE while M does not. An acetone solution of DBDCVP or DFP was added (1% final solvent concentration) to give the desired inhibitor concentration. The slopes (k′) of each-semi-log plot (log% remaining activity versus inhibition time) were calculated by linear regression analysis of the data (2–3 different concentrations, 6–8 time-points). All plots passed through the origin at zero-time. The second-order rate constant (k_a) was then calculated by dividing $2.303 \times k'$ by inhibitor concentration.

About 65% of peripheral nerve NTE is recovered in fraction P; the remaining 35% of activity is recovered in the supernatant after 30000 *g* (30 min, 4°C) centrifugation. On the other hand, 85–90% of brain NTE is recovered in fraction P. However, sensitivities to inhibition by DBDCVP (20 min, 37°C, pH 8.0) of sedimented and non-sedimented NTE were found to be similar. Sedimented and non-sedimented NTEs of peripheral nerve of one hen, 24 h after treatment with 1 mg/kg s.c. of DBDCVP, were equally inhibited.

NTE and AChE sensitivites to inhibition (I_{50}) were also derived from fixed-time incubations. Inhibitor, dissolved in acetone (1% final concentration of solvent), was added to the reaction mixture and incubated for 20 min at 37°C, pH 8.0.

Results

In vivo studies. Table 1 reports the effects of in vivo administration of DBDCVP. Single doses of DBDCVP caused a differential inhibition of NTE, but not AChE, in brain, spinal cord and peripheral nerve.

A dose of 0.2 mg/kg s.c. DBDCVP inhibited NTE above the threshold in brain only; these animals did not display walking defects or axonal degeneration in either spinal cord and peripheral nerve.

DBDCVP (0.45 mg/kg s.c.) inhibited NTE in brain and spinal cord above the threshold, but not in peripheral

Table 1. Biochemical, morphological and clinical effects of DBDCVP in different areas of the nervous system

Dose of DBDCVP (mg/kg)	Peripheral nerve			Spinal cord			Brain		Clinical score[a]	
	NTE[b]	AChE[b]	morphological score[c]	NTE[b]	AChE[b]	morphological score[c]	NTE[b]	AChE[b]	Walk	Reflex
0.00	100±11	100± 9	0.3±0.1	100±7	100±4	0.3±0.2	100±7	100±7	0	(+)
0.20	67± 6	92± 3	0.3±0.1	44±4	86±7	0.4±0.1	22±6	81±5	0	(+)
0.45	46± 9	73± 2	0.3±0.1	17±3	68±1	2.6±0.2[d]	10±1	72±3	1.8±0.4	(+)
1.00	17± 3	51± 2	1.8±0.3[e]	14±2	43±2	2.9±0.2[d]	4±1	42±2	3.5±0.5	(−)
2.00	ND	27±11	ND	ND	21±3	ND	ND	15±5	ND	ND
4.00	ND	12± 3	ND	ND	15±3	ND	ND	9±2	ND	ND

All data are expressed as mean ± SEM

[a] Assessed 15 days after treatment ($n = 3$). (+) or (−) refer to the presence or absence of the leg-retraction reflex in all animals bilaterally.

[b] Activities (3–4 animals per group) were measured 24 h after dosing and expressed as percentages of the activities of corresponding tissues obtained from control birds dosed with vehicle on the same day. NTE activity (μmol/min/g of tissue) of 4 controls was 2.58 ± 1.9 in brain, 0.72 ± 0.05 in spinal cord and 0.18 ± 0.02 in peripheral nerve. AChE activity (μmol/min/g of tissue) of 5 controls was 24.1 ± 1.7 in brain, 4.9 ± 0.2 in spinal cord and 1.1 ± 0.1 in peripheral nerve

[c] Assessed 21 days after treatment ($n = 3$). Sciatic and tibial (medialis and lateralis) nerves were scored for each animal

[d] $p < 0.001$ vs vehicle and 0.20 mg/kg dose groups with Kruskall-Wallis test

[e] $p < 0.001$ vs vehicle, 0.20 mg/kg and 0.45 mg/kg dose groups with Kruskall-Wallis test

ND = not done

Table 2. Sensitivities of NTE and AChE from different nervous tissues to inhibition by DBDCVP and DFP

Tissue	DBDCVP			DFP	
	NTE		AChE	NTE	
	$k_a 10^6$ ($M^{-1} \cdot min^{-1}$)	I_{50} (nM)	I_{50} (nM)	$k_a 10^3$ ($M^{-1} \cdot min^{-1}$)	I_{50} (nM)
Brain	20.6±2.4	1.7[a] 2.0[b]	14.7[b]	44.2±4.2	780[a]
Spinal cord	13.9±0.8	2.5[a] 2.7[b]	11.5[b]		
Peripheral nerve	5.4±0.4	6.4[a] 8.0[b]	17.4[b]	30.9, 42.7	1118[a], 809[a]

Data are expressed as mean ± SEM ($n = 3-4$)

[a] These I_{50} values are referred to 20 m preincubation time as derived from k_a

[b] These I_{50} values were derived from fixed time (20 min) incubations (pH 8.0, 37°C). The same homogenate was used for AChE and NTE inhibition experiments

nerve. Animals showed a syndrome characterized by spasticity and difficulty in balance with no loss of the "leg-retraction" reflex; axonal degeneration was present in spinal cord, but not in peripheral nerve.

A higher dose of DBDCVP (1.00 mg/kg s.c.), causing pronounced inhibition of NTE in brain, spinal cord and peripheral nerve, produced the typical clinical signs of OPIDP, with histological evidence of axonal degeneration both in spinal cord and peripheral nerves (sciatic and tibial).

In vitro studies. In vitro NTE sensitivities of inhibition by DBDCVP were found to differ according to the tissue source of the enzyme: peripheral nerve NTE was less sensitive to DBDCVP than brain and spinal cord NTEs (Table 2). In concurrent experiments, sensitivity of NTE to DFP inhibition was shown to be comparable in brain and peripheral nerve. AChE sensitivity to DBDCVP inhibition was also found to be comparable regardless of the tissue source of AChE.

Discussion

Table 3 shows all available data on comparative sensitivities of NTEs from different nervous tissues, both in vitro and in vivo.

The NTE I_{50}s of compounds 2, 4 and 5 are the same in brain, spinal cord and peripheral nerve. Also mipafox, an inhibitor not included in this table, has the same I_{50} (7 μM) in all tissues (unpublished results). Therefore the differences on NTE inhibition between brain and spinal cord found after dosing with compounds 2 or 4 and between brain and peripheral nerve after compound 5, might be interpreted as a differential access of the compounds to the target enzymes. The dosing with compounds 6, 7 or 8 also causes regional differences in NTE inhibition, but comparative in vitro data for the compound itself or for its active metabolite are not available. Therefore, for the time being, the above explanation for a different access cannot be applied. Compounds 9 and 10 do not show differences. Data for compound 1 are limited and inhibition too high to draw any conclusion.

Only DBDCVP (compound 3) shows a differential inhibition of NTE in brain, spinal cord and peripheral nerve both in vitro and in vivo. In vitro data are similar also for human enzymes (unpublished results). The concurrent AChE inhibitions obtained after dosing birds with DBDCVP (Table 1) are not different, suggesting that the ultimate inhibitor has the same access to all nervous tissues.

Some differences between brain and peripheral nerve NTE have been recently reported (Vilanova and Barril 1989). In brain and peripheral nerve the percentages of total NTE recovered in sedimented and non-sedimented fractions after centrifugations of crude homogenates are different. Considering however that the absolute amounts of non-sedimented NTE in both organs are similar, these differences probably reflect the fact that much less mem-

Table 3. Comparative inhibition of NTE in brain, spinal cord and peripheral nerve after dosing hens with several OPs

Compound	Dose & Route (mg/kg)	NTE (% Activity of controls, 24 h after dosing)			References
		Brain	Spinal cord	Peripheral nerve	
1. 0,0-diethyl 0-3, 5, 6-trichloro-2-pyridyl phosphorothioate[a]	150 p.o.	3, 12	21, 6	25, 11	Lotti 1987
2. 2,2-dichlorovinyl dimethyl phosphate	100 s.c.	8, 9, 10, 11 [20]	30, 19, 28, 22	10, 11, 14, 21 [20]	Caroldi & Lotti 1981
3. 2,2-dichlorovinyl di-n-butyl phosphate	0.2 s.c.	22 ± 6	44 ± 4	67 ± 6	This paper
	0.45 s.c.	10 ± 1	17 ± 3	46 ± 9	This paper
	1.00 s.c.	4 ± 1	14 ± 2	17 ± 3	This paper
4. di-isopropyl fluorophosphate	0.6 s.c.	25, 27, 29 [0.7]	36, 35, 48	27, 27, 28 [0.7]	Caroldi & Lotti 1982
	0.25 i.v.	61, 62	56, 77	65, 67	Caroldi et al. 1984
	0.185 i.v.	35, 47	39, 49	28, 29	Lotti et al. 1987
5. phenylmethane sulphonyl fluoride	30 s.c.	16, 11, 11 [90]	21, 17, 19	43, 27, 21 [90]	Caroldi & Lotti 1982
	30 s.c. ($n = 4$)	12 ± 4	16 ± 1	18 ± 4	Moretto et al. 1987
6. mono-0-cresyl diphenylphosphate	10 p.o.	16, 27	33, 34	30, 42	Caroldi & Lotti 1981
	2.5 p.o. daily for 10 weeks[b]	about 40	–	about 60	Lotti & Johnson 1980
	2.5 p.o. daily for 10 weeks +50 p.o.[b]	6, 7, 9	–	17, 22, 24	Lotti & Johnson 1980
7. 0,0-dimethyl-0-dichlorophenyl phosphoroamidate	100 p.o.	52	66	84	Johnson et al. 1989
	200 p.o.	17, 27	36, 41	37	Johnson et al. 1989
8. 0,0-diethyl-0-dichlorophenyl phosphoroamidate[c]	10 p.o.	14, 12, 14	20, 20, 16	36, 57, 2	Johnson et al. 1989
9. 0,0-di-n-butyl-0-dichlorophenyl phosphoroamidate	70 p.o.	44, 32	43, 30	42, 33	Johnson et al. 1989
10. 0,0-di-n-hexyl-0-dichlorophenyl phosphoroamidate[c]	15 p.o.	42	47	53	Johnson et al. 1989

[a] NTE activity was measured 4 days after dosing
[b] NTE activity was measured 24 h after the last dose and 24 h after the further single dose
[c] NTE activity was measured 22 h after dosing
[in brackets] in vitro I_{50} s data (μM) from our laboratory were measured for each compound on the same experiment, and calculated according to Lotti & Johnson (1978)

brane-bound NTE is present in sciatic nerve, due to the anatomical differentiation of the neurite. Furthermore, the in vitro (unpublished data) and in vivo sensitivities of sedimented and non-sedimented peripheral nerve NTE to DBDCVP are similar. Therefore it can be concluded that the reasons of the differences here reported cannot be due to a different physical form of NTE in the axons.

The suggestion that NTE might be not entirely homogeneous throughout the nervous system was made by several authors. Chemnitius et al. (1983) reported that kinetic analysis of NTE inhibition curves allowed the identification of two isoenzymes in hen brain. Carrington and Abou-Donia (1986) with similar experiments proposed an alternative model based on the formation of Michaelis complex between one enzyme and the inhibitor. The relevance, however, of these differences in mechanistic terms was not investigated in the above-mentioned studies. Furthermore, on the basis of heat inactivation experiments of brain NTE the presence of two isoenzymes was ruled out, because the biphasic kinetics was explained by the formation of one enzymic intermediate (Reiner et al. 1987). It was also reported that a small component of NTE (3–6% of total brain NTE) can be detected with some inhibitors. However, many of the compounds which do not inhibit this small component of total NTE can cause OPIDP or protect from it, depending on their chemistry (Johnson 1988). Therefore if this component really represents NTE, it is mechanistically irrelevant, and, of course, cannot be related to either of the two "components" of peripheral nerve NTE.

In conclusion there is conflicting evidence, obtained by different means, that different NTE isoenzymes can be detected in the nervous system. These data on DBDCVP, however, suggest that peripheral nerve NTE is different from that in the brain and confirms that doses which do not cause NTE supra-threshold inhibition in the peripheral nerve are not associated to OPIDP.

Comparative in vitro and in vivo data on NTE inhibition in brain, spinal cord and peripheral nerve are very limited and perhaps other OPs will display a behaviour similar to that of DBDCVP.

These data further confirm that brain NTE activity is not always a good monitor for enzyme acitivity in spinal cord and peripheral nerve, where axonal degeneration ac-

tually develops. In fact when the threshold for NTE inhibition is reached in the spinal cord and not in peripheral nerve, a "spastic spinal cord syndrome" is produced, whereas suprathreshold inhibition of NTE in brain only is not associated with OPIDP (Lotti et al. 1987). The practical implications of these results should be therefore considered, when measuring nervous tissues NTE inhibition in OPIDP experiments.

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