Biochemical Pharmacology, Vol. 33, No. 20, pp. 3213–3217, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

INTRA-ARTERIAL INJECTION OF DIISOPROPYLFLUOROPHOSPHATE OR PHENYLMETHANESULPHONYL FLUORIDE PRODUCES UNILATERAL NEUROPATHY OR PROTECTION, RESPECTIVELY, IN HENS*

STEFANO CAROLDI,† MARCELLO LOTTI† and ALBERTO MASUTTI‡

†Università di Padova, Istituto di Medicina del Lavoro, and ‡Centro Traumatologico Ortopedico, Via Facciolati 71, 35127 Padova, Italy

(*Received* 13 *February* 1984; *accepted* 2 *May* 1984)

Abstract—Hens injected in one sciatic artery with diisopropylfluorophosphate (DFP) (0.184 mg/kg) developed monolateral ataxia on the injected side 10–12 days later. The inhibition of neuropathy target esterase (NTE) was 85% in the sciatic nerve of the injected leg and less than 60% in the contralateral sciatic nerve, in spinal cord and in brain. Other hens injected in the wing vein with the same dose of DFP showed low inhibition of NTE in the nervous system and did not develop delayed neuropathy. Hens injected in one sciatic artery with phenylmethanesulphonyl fluoride (PMSF) (1 mg/kg) and 24 hr later with high subcutaneous dose of DFP (1.1 mg/kg) developed monolateral ataxia 10–12 days later on the side not injected with PMSF. The level of NTE inhibition after PMSF was greater than 40% in the sciatic nerve on the injected side compared with less than 20% in other parts of the nervous system. The same dose of PMSF injected in the wing vein produced low NTE inhibition in the nervous system and failed to protect the animals from the same high systemic dose of DFP. We conclude that both toxic and protective effects of NTE inhibitors for delayed neuropathy are better related to the level of NTE inhibition in the peripheral nerve on the site of injection than to NTE inhibition in other parts of the nervous system. Furthermore we suggest that NTE inhibition should also be measured in the peripheral nerve in the standard toxicity testing for organophosphate-induced delayed neurotoxicity.

Certain organophosphorus esters (OP) induce a symmetrical delayed neuropathy after single or repeated doses in several susceptible species. This distal axonal degeneration occurs in peripheral nerves and in selected tracts of the central nervous system [1]. An inhibition of neuropathy target esterase (NTE), the proposed target for delayed neuropathy, of greater than 70%, measured in hen brains 1–24 hr after a single dose of several neurotoxic OPs, was shown to correlate with the development of neuropathy 10–15 days later. However, delayed neuropathy does not depend directly on the loss of the enzymatic activity of NTE. A further step is required after phosphorylation of the active site, called 'aging'. This non-enzymatic reaction is usually rapid and involves the loss of a group attached to the phosphorus, leaving a negatively charged phosphorus group attached to the protein; groups lost during aging might have a wide range of reactivity. The aging depends only on the chemistry of the OP. Inhibitor–NTE complexes that are able to age are those formed by phosphates, phosphonates and phosphoroamidates: those formed by phosphinates, carbamates and sulphonates are not able to age. The former compounds might cause delayed neuropathy when the threshold of inhibition of NTE *in vivo* is reached, whereas the latter will never cause delayed neuropathy. Conversely, occupying the catalytic site of NTE (inhibition greater than 30–40%), these compounds protect the animals against a subsequent dose of a neurotoxic OP [2].

Most data about the biochemistry of NTE in hens and humans, as well as the association of NTE inhibition with delayed neuropathy, have been obtained from studies in brain which show the highest NTE activity throughout the whole nervous system [1, 3]. However, neuropathic lesions are seen principally in parts of the nervous system other than the brain [4].

Moreover, some dimethylphosphates can produce inhibition of NTE in brain to a greater degree than in spinal cord and in such cases the inhibition in brain is not related to the development of ataxia [5, 6].

The present study was aimed to investigate further the anatomical site in the nervous tissue where NTE inhibition is associated with the development of delayed neuropathy. A single dose of either a neurotoxic or a protective inhibitor was injected into one sciatic artery of hens to produce different levels of NTE inhibition between the corresponding sciatic nerve and the rest of the nervous system. The levels of NTE inhibition were measured throughout the nervous system and correlated to the anatomical localization of clinical symptoms.

MATERIALS AND METHODS

OP inhibitors. Diisopropylfluorophosphate (DFP) was purchased from Fluka AG Chem Fabrik (CH 9470 Buchs, Switzerland). Two different samples

* Presented in part at the Workshop 'Delayed Neurotoxicity' held in Urbana, Illinois, U.S.A., June 1982.

of DFP from the same source were used in these experiments (samples A and B): sample A was stored at laboratory conditions of light and temperature in a desiccator for about one year before starting the experiments while sample B was immediately used after purchase. Phenylmethanesulphonyl fluoride was purchased from E. Merck (Darmstadt, F.R.G.). Purified Mipafox (*N,N*-diisopropyl phosphorodiaminofluoridate) and Paraoxon (diethyl *p*-nitrophenyl phosphate), both used in the NTE assay, were gifts of Dr M. K. Johnson (MRC Laboratories, Carshalton, U.K.).

Animals. Experiments were performed on non-inbred red hens (*Gallus domesticus*) (1.8–2.2 kg body wt) which were caged in groups of three and allowed to drink and eat *ad libitum*. Birds were ranked in paired groups according to the treatment as follows:

(a) DFP intravenously (i.v.).
(b) DFP intra-artery (i.a.),
(c) PMSF i.v. plus DFP subcutaneously (s.c.),
(d) PMSF i.a. plus DFP s.c.

Surgery and injections. Anesthesia was first induced with 30 mg/kg of Ketamine i.m. (2-(*O*-chlorophenyl)-2-(methylamino) cycloexanone hydrochloride) (Parke-Davis S.p.A. Lainate, Milan, Italy). After 10–15 min hens were transferred from cages to the operating table and anesthesia was continued with ether/air by inhalation. Sciatic arteries were visualized after cutting (about 2 cm) the ileofibularis and ilcotibialis lateralis muscles. Injections were performed no more than 0.5 cm below the point where the artery comes out of the foramen ischiaticum. Hemostasis was obtained successfully by manual pressure for 1–2 min on the hole of the injection. The trauma was minimal and no aseptic presidia were used. Recovery from anesthesia occurred in about 1 hr and the birds were standing and walking 6–8 hr thereafter.

DFP was dissolved in saline immediately before use and was injected at a final volume up to 2.5 ml. PMSF was dissolved in glycerol formal and injected at a final volume up to 0.5 ml. The final volume of glycerol formal injected into the sciatic artery of hens which were kept alive for the clinical assessment of ataxia was further reduced to 0.05 ml to avoid problems of local toxicity. Control birds were unaffected by the intra-arterial injection of glycerol formal. Experimental animals were injected bilaterally with either DFP or PMSF on one side, and solvent only on the other. Intravenous injections were performed on unanesthetized hens into the wing vein at final volumes of solvent similar to those injected into the sciatic arteries. For the assessment of protection, groups of hens were dosed 24 hr after having been injected either i.v. or i.a. with PMSF, with a highly neurotoxic dose of DFP (1.1 mg/kg of DFP sample B dissolved in 0.2 ml of glycerol formal given s.c. in the anterothoracic region). This systemic dose of DFP causes more than 90% inhibition of NTE in the whole nervous system. These birds showed, soon after DFP injection, severe cholinergic symptoms and were treated with atropine sulphate (E. Merck, Darmstadt, F.R.G.), 20 mg/kg i.p. Clinical recovery was almost complete within 6 hr.

The groups of surviving hens (DFP only either i.a. or i.v. and PMSF either i.a. or i.v. plus DFP s.c.) were observed daily for abnormal gait assessed according to the four-point scale of Johnson and Barnes [7].

Neuropathy target esterase assay. Animals were killed by decapitation 24 hr after dosing. Sciatic nerves (outlet from spinal cord as far as tarsus), thoracic and lumbar spinal cord and brain were immediately dissected and cooled in ice-cold 50 mM Tris–HCl buffer pH 8.00 containing EDTA 0.2 mM. NTE assay was performed on the same day as previously described [8]. Percentages of NTE inhibition in brain, spinal cord and sciatic nerves were calculated in respect to the mean of all the control values. In each experiment a control bird was used. The values were: brain 2625 ± 50 nmole/min/g tissue (12); spinal cord 510 ± 12 nmole/min/g tissue (12); sciatic nerve 106 ± 3 nmole/min/g tissue (14) (mean ± S.E.M., number of animals in brackets).

Table 1. Percentage of NTE inhibition in the nervous system of hens after dosing with DFP

Dose (mg/kg)	Route*	No. of hens	NTE percentage of inhibition†			
			Brain	Spinal cord	Sciatic nerve‡ (S)	(I)
DFP (sample A)						
0.600	i.a.	1	89	NM	86	93
0.300	i.a.	1	58	NM	56	76
0.250	i.a.	5	29 ± 8	33 ± 4	33 ± 7	93 ± 3
0.250	i.v.	2	38,39	23,44	33,35§	
DFP (sample B)						
0.250	i.a.	1	83	76	84	93
0.184	i.a.	2	51,51	52,59	49,46	85,86
0.150	i.a.	1	30	22	29	65

* Intra-arterial injection in the sciatic artery (i.a.), intravenous injection in the wing vein (i.v.).
† Measured 24 hr after injection. Numbers are either the percentage of inhibition on a single animal or the mean ± S.E.M.
‡ Side injected with the solvent (S) and side injected with DFP (I).
§ Percentage of inhibition in one sciatic nerve.
NM: not measured.

RESULTS

Unilateral neurotoxicity

Hens were injected into one sciatic artery with DFP doses up to 0.60 mg/kg (sample A) and up to 0.25 mg/kg (sample B). Systemic cholinergic symptoms were not detectable. At the same nominal doses of DFP, NTE inhibition in all the regions of the nervous system was lower with sample A than that with sample B (Table 1). Nevertheless the comparative inhibition of NTE among different areas of the nervous system was the same. Samples were not analyzed for their purity (when differences were detected, sample A was no longer available). We concluded that either some decay of the DFP activity (sample A) occurred during storage in our laboratory or the two samples were originally different.

The levels of NTE inhibition were always higher in the sciatic nerve on the injected side than in the contralateral sciatic nerve or in spinal cord or brain. The gap between the percentage of NTE inhibition in the sciatic nerve on the injected side and in the rest of the nervous system was reduced by increasing the DFP dose. At 0.25 mg/kg DFP i.a. (Sample A) and at 0.184 mg/kg DFP i.a. (Sample B), the inhibition of NTE was greater than 80% only in the sciatic nerve on the injected side. After i.v. injection of 0.25 mg/kg of DFP the inhibition of NTE was less than 50% in the whole nervous system. Hens treated with these doses of DFP either i.v. or i.a. have been observed for the development of neurological symptoms for three weeks and the results are reported in Table 2. Both groups of hens dosed i.v. were negative for neurological symptoms. The hens

Table 2. Grade of ataxia in hens dosed with DFP

DFP (mg/kg)	Route	No. of hens	Grade of ataxia*† (S)	(I)
0.250 (sample A)	i.a.	3	0,0,0	2,1,0
0.184 (sample B)	i.a.	5	0,0,0,0,0	3,2,2,1,1
0.250 (sample A)	i.v.	3	0,0,0‡	
0.184 (sample B)	i.v.	3	0,0,0‡	

* Assessed 15 days after DFP injection according to the four-point scale of Johnson and Barnes [7].
† Injected with the inhibitor (I) and injected with the solvent (S).
‡ Bilateral assessment.

Table 3. Percentage of NTE inhibition in the nervous system of hens after dosing with PMSF

Dose (mg/kg)	Route	No. of hens	NTE percentage of inhibition* Brain	Spinal cord	Sciatic nerve† (S)	(I)
8	i.a.	1	72	68	67	94
2	i.a.	1	33	22	44	77
1	i.a.	6	16 ± 1	13 ± 4	8 ± 4	42 ± 7
1	i.v.	2	0,20	0,8	11,9‡	

* Measured 24 hr after PMSF injection. Numbers are either the percentage of inhibition in a single animal or the mean ± S.E.M.
† Side injected with the solvent (S) and side injected with the inhibitor (I).
‡ Percentage of inhibition measured in one sciatic nerve.

Table 4. Grade of ataxia after PMSF/DFP* dosing in hens

PMSF (dose & route)	DFP (dose & route)	No. of hens	Grade of ataxia†‡ (S)	(I)
1 mg/kg i.a.	1.1 mg/kg s.c.	3	2,4,4	0,0,1
1 mg/kg i.v.	1.1 mg/kg s.c.	2	4,4	

* DFP (sample B) given 24 hr after PMSF.
† Assessed 15 days after DFP according to the four point scale of Johnson and Barnes [7]. First, second and third numbers represent grade of ataxia in hen No. 1, 2 and 3 respectively.
‡ PMSF injected side (I) and solvent injected side (S).
§ Bilateral assessment.

dosed with DFP i.a. showed, starting days 10–12, monolateral signs of ataxia only on the side injected with DFP, while contralateral legs were completely unaffected. These hens were then killed at day 21, without any improvement being apparent.

Unilateral protection. Hens were injected into one sciatic artery with PMSF doses up to 8 mg/kg. The percentage of NTE inhibition, measured 24 hr after dosing, was at any dose higher in the sciatic nerve on the injected side than in the rest of the nervous system (Table 3). At 1 mg/kg of PMSF i.a. the level of NTE inhibition was greater than 40% only in the sciatic nerve on the injected side. The same dose of PMSF but injected into the wing vein inhibited homogeneously NTE throughout the whole nervous system to a lesser extent. Hens dosed with PMSF 1 mg/kg either i.a. or i.v. were given a s.c. neurotoxic dose of DFP 24 hr later. The hens which received PMSF in the vein before the challenging DFP dose, showed bilateral signs of ataxia starting days 10–12, and at day 15 they were completely paralyzed (Table 4). Hens dosed with PMSF in the sciatic artery showed asymmetrical symptoms more severe on the side not injected with PMSF before DFP. At day 15 two of these hens showed a complete paralysis on the solvent-injected side but they were still able to move by jumping on the leg protected by the PMSF injection. All five hens were killed at day 21 without any apparent improvement.

DISCUSSION

Unilateral neuropathy can be produced in hens by injecting DFP into one sciatic artery and protection against neuropathy by injecting PMSF before DFP; both effects are related to the level of inhibition of NTE in the sciatic nerve on the injected side. Doses of DFP or PMSF equal to those which caused the unilateral syndromes but injected intravenously produced an homogeneous low inhibition of NTE throughout the whole nervous system and failed to cause neuropathy or protection.

Inhibition of NTE greater than 70%, measured 1–24 hr after a single dose of a neurotoxic OP in hen brains, correlates with the development 10–15 days later of delayed neuropathy. The reported critical level for the protective effect is 30–40% [2]. At doses of DFP or PMSF which produced the unilateral syndromes in these experiments, the percentage of NTE inhibition measured 24 hr after the intra-arterial injection of either inhibitor, was above the critical levels only in the sciatic nerve on the injected side where eventually signs of ataxia or protection were evident. The different levels of NTE inhibition measured in the nervous system after intra-arterial injections of DFP agree with the reported data on the distribution of labeled DFP in the nervous tissue of cats after injection into one femoral artery. These experiments showed a higher DFP concentration in the sciatic nerve on the injected side than in the rest of the nervous system [9]. Unilateral neuropathy has been reported also in cats after injection of DFP directly into one femoral artery [10]. This neuropathy was quantified electrophysiologically by a severe loss of the capacity of soleus motor nerve terminals to generate stimulus-bound repetition 21 days after DFP dosing. Pretreatment with systemic PMSF increased this repetitive capacity; protection was complete on clinical testing even though the repetitive capacity was still less than the one in the animals treated with PMSF alone [11].

Present results confirm the reported critical levels of NTE inhibition which have been shown to be associated in hen brains with the development of neurotoxicity or protection. They indicate, however, that both effects of NTE inhibitors are related to these level of inhibition in the target organ (the peripheral nerve) rather than in other parts of the nervous system. Therefore whatever the route of administration and dosage regime in toxicity testing for OP-delayed neurotoxicity, the biochemical test should include the peripheral nerve NTE assay. Indeed NTEs in brain, spinal cord and sciatic nerve have similar sensitivity to inhibitors [8], but the toxic OP cannot be homogeneously distributed among different areas of the nervous system. These results can also explain the anomalous behavior of some dimethylphosphates in the biochemical test for delayed neuropathy [6]. After a high single dose of Dichlorvos (dimethyl 2,2-dichlorovinyl phosphate) high levels of NTE inhibition were found in the brain but not in the spinal cord and no neuropathy developed. After a higher single dose, however, ($10 \times LD_{50}$, hens protected against cholinergic symptoms by pretreatment with eserine, atropine and 2-PAM) high inhibition of peripheral nerve NTE was measured and neuropathy developed [5]. These differences may reflect a non-homogeneous access of Dichlorvos to different parts of the nervous system. Furthermore, pharmacokinetic problems might be relevant also during chronic exposures to OP compounds. Different studies led to the conclusion that neuropathy can be associated during a chronic exposure to a lower NTE inhibition (50–60%) in brain and spinal cord compared with that found in the single dose situation [12, 13]. The effects of NTE inhibitors can be more cumulative in the peripheral nerve rather than in brain and spinal cord: measurement of NTE inhibition in peripheral nerve during a chronic dosing regime will ascertain if the biochemical correlation for delayed neuropathy is really different from that of the single dose situation.

The physiological functions of the protein NTE are unknown. The catalytic site is clearly not essential and several hypotheses about the consequences of "aging" have been made [2]. In this study we dissociated the effect of OPs on axonal NTE and on cell body NTE. Since the critical level of NTE inhibition must be reached in the axon to cause either neuropathy or protection, we suggest that NTE is associated with some axonal functions which are relevant in maintaining the health of the neuron.

REFERENCES

1. C. S. Davis and R. J. Richardson, in *Experimental and Clinical Neurotoxicology* (Eds. P. S. Spencer and H. H. Schaumburg), p. 527. Williams & Wilkins, Baltimore (1980).
2. M. K. Johnson, *Rev. Biochem. Toxicol.* **4**, 141 (1982).
3. M. Lotti and M. K. Johnson, *J. Neurochem.* **34**, 747 (1980).

4. J. B. Cavanagh, *J. Neurol. Neurosurg. Psychiat.* **17**, 163 (1954).
5. S. Caroldi and M. Lotti, *Toxicol. Lett.* **9**, 157 (1981).
6. M. K. Johnson, *Arch. Toxicol.* **41**, 107 (1978).
7. M. K. Johnson and J. M. Barnes, *Biochem. Pharmac.* **19**, 3045 (1970).
8. S. Caroldi and M. Lotti, *Toxic. appl. Pharmac.* **62**, 498 (1982).
9. R. D. Howland, H. E. Lowndes, T. Baker and R. J. Richardson, *Brain Res.* **184**, 248 (1980).
10. H. E. Lowndes, T. Baker and W. F. Jr. Riker, *Eur. J. Pharmac.* **29**, 66 (1974).
11. T. Baker, H. E. Lowndes, M. K. Johnson and I. C. Sandborg, *Arch. Toxicol.* **46**, 305 (1980).
12. M. Lotti and M. K. Johnson, *Arch. Toxicol.* **45**, 263 (1980).
13. G. L. Sprague, L. L. Sandvik and A. A. Bickford, *Neurotoxicology* **2**, 523 (1980).