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Decrease of Phosphofructokinase Activity in Relation to the Pathogenesis of Triorthocresyl-Phosphate Induced Delayed Neuropathy

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The *in vivo* effect of a single dose of the neuropathic compound triorthocresyl-phosphate (TOCP) on phosphofructokinase (PFC, E.C. 2.7.1.11) and its relation with the initiation step (inhibition and aging of neuropathy target esterase, NTE) in the TOCP-induced delayed neuropathy have been studied. Hens were treated with a neurotoxic dose of TOCP (500 mg/kg, p.o.) and with a protective compound (Phenylmethanesulfonyl fluoride, PMSF, 30 mg/kg s.c.) in different combinations: TOCP, TOCP+PMSF, PMSF+TOCP and PMSF. PFK activity was determined in brain and sciatic nerve 1, 3, 7 and 15 days after treatment. PFK activity decreased in sciatic nerve 15 days after dosing with TOCP or TOCP+PMSF. When animals were dosed with the protective agent (PMSF) alone or before administering the neurotoxic compound, PFK activity was unaltered and clinical signs of neuropathy were absent. The data presented here suggest that phosphofructokinase is involved in the pathogenesis of the neuropathy induced by TOCP.

Key words: Triorthocresyl-phosphate, Organophosphate-induced delayed neuropathy, Phosphofructokinase, Sciatic nerve.

Accidental or suicidal intake of certain organophosphorus (OP) esters by humans can cause neuropathy with a characteristic delay from 10 to 14 days after poisoning. This syndrome is referred to as organophosphorus ester-induced delayed neuropathy (OPIDN) that primarily involves a progressive inhibition of neuropathy target esterase (NTE) (14). An essential step in the genesis of delayed neuropathy is a further reaction called «aging» which involves the generation of a charged monosubstituted phosphoric acid residue on the protein (6, 7, 29). Predosing hens with

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phosphinates, carbamates or sulfonates such as phenyl-methanesulfonyl fluoride (PMSF) protects them against the clinical neuropathic effects of OP-esters (12, 16). The characteristic common to all the protective agents is that they produce inhibited NTE with no residual bonds suitable for further transformation to a charged species (aging). The threshold for initiation of OPIDN is 70-80 % inhibition of NTE. Therefore, if 30 % or more of NTE is occupied by a protective compound, a subsequent challenge dose of a neuropathic OP fails to cause neuropathy.

The critical OP-NTE interaction occurs in the nerve fiber rather than in the cell body (5, 18). However, the nature and sequence of events that occur after the two-step initiation mechanism (within hours of dosing) and precede the clinical expression of OPIDN are unknown. No data are available on the biochemical events leading from the inhibition of NTE to clinical manifestations.

SABRI and SPENCER (27) suggested a common mechanism for toxic distal axonopathies —depletion of chemical energy in the nerve fiber— although a causal relationship between toxic inhibition of glycolysis and axonal degeneration remains unclear. This hypothesis is supported by *in vitro* data obtained mainly with acrylamide (22) and neurotoxic hexacarbons (24, 25) but very few *in vivo* studies have been made.

In a previous report (9) it was shown that glycolytic enzymes are not inhibited *in vitro* by some OP-esters which induce OPIDN. Recently, an *in vivo* decrease in phosphofructokinase (PFK) activity in hen sciatic nerve 15 days after treatment with 500 mg/kg of triorthocresyl-phosphate (TOCP) was reported (10). The purpose of this work was to determine the relationship between the decrease in the activity of the glycolytic enzyme phosphofructokinase (PFK, E.C. 2.7.1.11) and the initiation step of the TOCP-induced delayed neuropathy.

Materials and Methods

Animal care and treatment. — Experiments were performed on randomly chosen adult hens (*Gallus gallus domesticus*) Dekalb^{R} G-Link (1.7-2.5 kg b.w.). The hen has been used as the test animal of choice to study OPIDN for several reasons (1): (a) all organophosphorus esters involved in OPIDN also cause delayed neurotoxicity in hens; (b) the delayed histopathological lesions and clinical signs are similar in humans and hens; (c) data on OPIDN in hens are widely available making the comparison of experimental results possible.

Seventy-two birds were used and distributed in four groups of treatment. In each group three animals were assigned to control and to each of the different times assayed (1, 3, 7 days) except for the 15 day group where six animals were employed.

Birds were caged in groups of three in an air-conditioned room (21-26 °C) on a 12 h light-dark cycle and allowed to drink and eat *ad libitum* except for predose fasting. However, treated hens showed significant weight loss, and had to be force-fed daily by stomach tube on a liquid mixture prepared from a standard hen diet (Sanders, S.A.). This was not necessary for control birds.

The animals were divided into groups and treated as follows: 1) TOCP 500 mg/kg p.o.; 2) TOCP 500 mg/kg p.o. + PMSF 30 mg/kg s.c.; 3) PMSF 30 mg/kg s.c. + TOCP 500 mg/kg p.o.; 4) PMSF 30 mg/kg s.c. TOCP (99 % purity, Eastman Kodak, Liverpool, UK) was dissolved in sunflower oil (45 % w/v). Phenylmethanesulphonyl-fluoride, PMSF (Sigma) was dissolved in glycerol formal immediately before use and injected subcutaneously in anterolateral region of breast. It was given 24 h after TOCP *in set* 2) and 7 h before TOCP *in set* 3). Appropriate negative controls were dosed only with an equivalent volume of vehicle.

Tissue preparation. — Hens were killed by cervical dislocation 1, 3, 7 and 15 days after treatment. Blood was collected in heparinized tubes and plasma was separated by centrifugation. Brain and sciatic nerve were excised immediately and placed in ice-cold 20 mM triethanolamine buffer, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA and 0.1 mM DL-dithiothreitol (Buffer A) (3). The following steps were performed at 4 °C.

Brains were weighed and homogenized in 10 ml of ice-cold buffer A using a power-driven close-fitting perspex/glass homogenizer. After removing all the adhering tissue from the nerves they were blotted dry, minced with fine scissors and weighed. Sciatic nerves were homogenized in 3.5 ml of buffer A in an all-glass Potter-Elvehjem homogenizer. Aliquots of these homogenates were kept for the assay of NTE and AChE activities. Then, the rest of the homogenate was centrifuged at 29000 × *g* for 20 min, and this supernatant was divided in aliquots for the assay of the phosphofructokinase activity. All the aliquots were stored at −40 °C. Enzyme activities were unaffected by this procedure.

Separation of lymphocytes from red cells and granulocytes was performed with the Pharmacia Ficoll-Paque kit following the manufacturer's indicated procedure on 2 ml of heparinized blood samples freshly collected by venopuncture (2).

Enzyme assays. — NTE activity in brain was determined as described by JOHNSON (13), NTE activity in sciatic nerve by the method of CAROLDI and LOTTI (4) and NTE activity in lymphocytes by the method of MARONI and BLECKER (21). Butyrylcholinesterase (BuChE, E.C.3.1.1.8) and acetylcholinesterase (AChE, E.C.3.1.1.7) activities were measured according to ELLMAN *et al.* (8). PFK activity (E.C. 2.7.1.11) was assayed according to SABRI *et al.* (24). All enzyme determinations were performed in duplicate and are expressed as specific activity or units per gram of wet tissue. Protein was measured by the method of LOWRY *et al.* (20).

Clinical evaluation. — A clinical evaluation for delayed neuropathy was performed on each bird daily, from day 10 until death (day 15). Walking performance was evaluated according to the four-point scale of JOHNSON and BARNES (15) (0: no defect; 1: slight abnormal gait; 2: severely abnormal gait; 3: animal can stand but frequently collapses; 4: animal unable to stand). The «leg-retraction» reflex was also evaluated before death as described by LOTTI *et al.* (18). The legs remain extended and flaccid in animals with typical organo-phosphorus neuropathy.

Statistical analysis. — Significant differences between control and dosed animals were determined by analysis of variance and comparison of means by the Dunnet test, and the non-parametric Kruskall-Wallis test.

Results

Animals treated with TOCP or TOCP + PMSF showed the first clinical signs of OPIDN on day 11-12 after dosing. These signs included gait abnormalities which became progressively more severe and by day 15 the functional deficit was fully developed. A summary of biochemical and clinical results of all 4 experimental groups is presented in table I.

NTE was inhibited in brain and sciatic nerve by all treatments with inhibition values of 76-95 % for brain and 72.5-98 % for sciatic nerve 24 h after dosing. The lowest inhibition was always obtained with PMSF and the highest one when TOCP and PMSF were administered together. From the moment of its peak inhibition, NTE showed a good recovery of its activity with values of 56-84 % in brain

Table I. *Biochemical and clinical effects following different treatments with TOCP and PMSF.*
Each value represents the mean ± SD of 3 animals. Significant difference between control and experimental values: * $p < 0.05$, ** $p < 0.01$.
N.D. = Not done.

Treatment	NTE activity (% control)[a]			AChE activity (% control)[a]			Plasma BuChE activity (% control)[a]	Clinical Score[b]	
	Brain	Sciatic nerve	Lymphocytes	Brain	Sciatic nerve	Erithrocytes		Walk	Reflex
TOCP (500 mg/kg)	7.5±5.3**	9.0±4.5**	24.3±18.2**	88.9±1.5	95.5±9.1	91.8±6.4	35.3±5.8*	1-3	(±)
TOCP (500 mg/kg)+ PMSF (30 mg/kg)	5.3±0.6**	2.1±3.7**	N.D.	96.3±6.7	80.3±11.8	114.4±6.5	18.7±4.5**	2.3	(−)
PMSF (30 mg/kg)+ TOCP (500 mg/kg)	4.8±1.2**	10.2±9.3**	N.D.	78.2±3.9	77.7±10.7	98.8±11.7	14.3±5.6**	0	(+)
PMSF (30 mg/kg)	23.7±6.7**	28.6±20.6**	N.D.	90.7±5.4	91.3±14.1	93.1±3.8	102.2±16.3	0	(+)

[a] Measured 24 h after treatment and expressed as percentage of activities from control animals. In group 3 enzyme activities were determined 24 h after TOCP treatment.
[b] Assessed on day 15 after treatment. Clinical signs of neurotoxicity: 0: no defect; 1: slight abnormal gait; 2: severely abnormal gait; 3: animal can stand but frequently collapses; 4: animal unable to stand. Leg retraction reflex: (+) = present; (−) = absent.

Table II. *Phosphofructokinase activity[a] in hen sciatic nerve following different treatments with neuropathic and/or protective agents.*
Significant difference between control and experimental values: * $p < 0.01$.

Treatment	Control	Days after treatment			
		1	3	7	15[b]
TOCP[c] (500 mg/kg)	0.172 ± 0.021	0.178 ± 0.004	0.157 ± 0.021	0.176 ± 0.031	0.100 ± 0.048*
TOCP + PMSF	0.173 ± 0.074	0.168 ± 0.017	0.156 ± 0.021	0.198 ± 0.085	0.102 ± 0.085
PMSF + TOCP	0.173 ± 0.036	0.198 ± 0.074	0.154 ± 0.013	0.168 ± 0.045	0.162 ± 0.008
PMSF (30 mg/kg)	0.173 ± 0.037	0.170 ± 0.031	0.197 ± 0.051	0.190 ± 0.016	0.168 ± 0.017

[a] Enzyme activities are expressed as U/mg protein. Each value represents the mean ± SD of 3 animals except when otherwise indicated.
[b] Six animals were used on 2 independent sessions.
[c] Data from Hernández *et al.* (10).

by day 15 and > 70 % in sciatic nerve. Furthermore, all birds treated with 500 mg/kg of TOCP had lymphocyte NTE inhibition above 70 %, but 7 days after dosing lymphocyte NTE activity was completely restored.

Acetylcholinesterase (AChE) was not affected in brain, sciatic nerve and erythrocytes in all the experiment sets (table I), which allowed elimination of cholinergic interferences. However, plasma cholinesterase activity (BuChE) was inhibited by TOCP, TOCP+PMSF and PMSF + TOCP (64.7, 81.3 and 85.7 % respectively), whereas treatment with only PMSF was unable to change it. At the time of sacrifice (15 days after dosing) the BuChE activity had been fully restored.

Table III. *Relationship between the initiation of OPIDN and the decrease of PFK activity.*

Treatment	NTE	PFK activity decrease	Neuropathy
	Inhibition/ «Aging»		
TOCP	+/+	+	+
TOCP + PMSF	+/+	+	+
PMSF + TOCP	+/−	−	−
PMSF	+/−	−	−

(+): Effect observed; (−): Effect not observed.

Phosphofructokinase activity (PFK) in brain was unaltered by all treatments. By contrast, PFK activity showed a significant decrease after dosing with TOCP (500 mg/kg) as well as with TOCP (500 mg/kg) + PMSF (30 mg/kg) although in the latter case no statistical significance was found, in spite of the evident decrease in the mean values after 15 days (table II). The reason was undoubtedly the greatly variable results as reflected by the standard deviation values. Animals dosed only with the protective agent (PMSF, 30 mg/kg) did not show changes in PFK activity during the period studied. Furthermore, when the protective agent (PMSF) was administered seven hours before the neuropathic compound (TOCP) no signs of neurotoxicity were developed and PFK activity in sciatic nerve was unaffected (table III).

Discussion

The present results confirm and extend our previous observation concerning the involvement of the glycolytic pathway in the pathogenesis of OPIDN (10).

Table I shows the validity of the experimental model used in this work. The minimal threshold of NTE inhibition required for the development of OPIDN

(14) is reached in brain, sciatic nerve and lymphocytes in all treatments 24 h after dosing. Measurement on NTE in lymphocyte has been proposed as a test for prediction of OPIDN in humans (17). The inhibition of lymphocytic NTE is correlated here with the clinical effects observed after 15 days. Data of AChE activity show lack of cholinergic effect under treatment with TOCP and/or PMSF, which allows for the elimination of cholinergic interferences in the interpretation of the present results. The BuChE activity was inhibited by all treatments except by PMSF alone, which is irrelevant.

The activity of PFK in hen sciatic nerve dosed with 500 mg/kg of TOCP remains unaltered until 7 days after treatment. The decrease in PFK activity was evident 15 days after dosing (58 % of the controls) and the decrease in enzyme activity persisted 21 days after poisoning (data not shown). The purpose of this study was to check the possible relation of the decrease in PFK activity and the pathogenesis of OPIDN. Therefore, birds were dosed with a protective agent (PMSF) which inhibits NTE but does not cause neuropathy. This compound was used alone or in different combinations with TOCP. The treatment with PMSF or PMSF + TOCP allows us to detect effects produced by inhibition of NTE (PMSF) and other effects of TOCP not related to inhibition and aging of NTE (PMSF + TOCP). The experiment with TOCP + PMSF, dosing the protective agent 24 h after TOCP, was used to rule out possible effects of PMSF independently of its inhibitory effect on NTE. This treatment seems to be basically equivalent to the administration of TOCP alone.

The decrease in PFK activity was obtained only with TOCP and TOCP + PMSF, but the effect was not observed when hens were protected with PMSF before administering a neurotoxic dose of TOCP. In birds which received PMSF (30 mg/kg, s.c.) only 24-29 % of NTE was available for phosphorylation by a subsequent dose of TOCP (500 mg/kg, p.o.) administered 7 h after. These animals also failed to develop any sign of OPIDN.

It is evident that the decrease in PFK activity is not a direct effect on the enzyme. Compared to NTE inhibition the action of TOCP is different: the maximal inhibition of NTE was observed at 24 h although there was a delay of several days before the decrease in PFK activity. This fact could be an effect of the sequence of biochemical events leading from inhibition/aging of NTE to clinical manifestations.

PFK was not affected in brain 15 days after treatment which supports the interpretation mentioned above in relation to metabolic changes that could occur in peripheral nerve and not in brain.

Our results suggest that the decrease in PFK activity is associated with the development of OPIDN, since it seems a consequence of inhibition and aging of NTE. Only when inhibition and aging of NTE was achieved OPIDN developed and PFK activity decreased.

The significance of PFK activity decrease in nerve fiber degeneration is not known. In a sequence of catalytic reactions, the most vulnerable site is the rate-limiting step, one of which in the case of glycolysis is PFK (19). An important consequence of PFK decrease would be a reduction in high-energy phosphate available to neurons. According to SABRI and OCHS (26) the reduction of about 40 % in PFK activity could be critical for the maintenance of fast axoplasmic transport. Glycolytic enzymes are transported by fast axoplasmic transport (22). If this common mechanism is assumed changes in other glycolytic enzymes would be expected. However, no changes were detected (10). On the other hand, membrane-bound enzymes are transported by that mechanism (28) and consequently its blockage would affect such enzyme activities (e.g. AChE, NTE). The time-course

of these activities is not consistent with an alteration of fast axoplasmic transport. The relation of a retrograde transport deficit reported by MORETTO *et al.* (23) with the decrease in PFK activity is unlikely for it would lead to an increase in enzyme activities rather than to a decrease in activity as observed in our experiments.

It must be further considered if this change is specific for OPIDN or if it is a feature common to other toxic axonopathies. There are very few *in vivo* studies about the effect of neurotoxic compounds on glycolytic enzymes. SABRI *et al.* (24) reported an inhibition of 31 % in PFK activity in 2,5-hexanedione-intoxicated rats 12 weeks after treatment, when clinical signs were evident. They concluded that those results, as well as *in vitro* data (24, 25) were consistent with the hypothesis that hexacarbon compounds inhibit the activity of enzymes required for the energy production and the maintenance of integrity in the nerve fiber. On the other hand, PFK activity on brain, spinal cord, sciatic nerve and muscle of cats intoxicated with acrylamide was unaltered in nerve tissues 10-12 days after treatment, when neuropathy was clinically evident (11).

To sum up, the present results suggest that the decrease in PFK activity can be related to the biochemical mechanism that follows inhibition and aging of NTE.

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Resumen

Se estudia en gallinas el efecto *in vivo* de una dosis única de tri-*o*-cresil fosfato (TOCP), compuesto neurotóxico sobre la actividad fosfofructoquinasa (PFK, E.C.2.7.1.11) y su relación con la fase de iniciación (inhibición y «envejecimiento» de la esterasa diana de la neuropatía, NTE) de la neurotoxicidad retardada inducida por compuestos organofosforados. Los animales se tratan con una dosis neuropática de TOCP (500 mg/kg p.o.) y con el compuesto protector fenilmetanosulfonil fluoruro (PMSF, 30 mg/kg s.c.) en diferentes combinaciones: TOCP, TOCP+PMSF, PMSF + TOCP y PMSF. La actividad PFK se determina en cerebro y nervio ciático 1, 3, 7 y 15 días después del tratamiento. Se observa un descenso de la actividad en nervio ciático a los 15 días después de la administración de TOCP o TOCP + PMSF. La administración del PMSF sólo o antes que del TOCP, no produce alteración de la actividad PFK ni aparecen signos clínicos de neuropatía. Los resultados sugieren que la actividad PFK podría estar involucrada en la patogénesis de la neuropatía retardada inducida por TOCP.

Palabras clave: Tri-*o*-cresil fosfato, Neuropatía retardada por organofosfatos, Fosfofructoquinasa, Nervio ciático.

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