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Neurofilaments degradation as an early molecular event in tri-ortho-cresyl phosphate (TOCP) induced delayed neuropathy

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ABSTRACT

Tri-ortho-cresyl phosphate (TOCP), an organophosphorus ester, is capable of producing organophosphorus ester-induced delayed neuropathy (OPIDN) in human being and sensitive animals. In the present study, adult hens were treated with TOCP by gavage at single dosage of 750 mg/kg, and sacrificed by decapitation on the corresponding time points of 1, 5, 10, and 21 day post-dosing, respectively. The tibial nerves were dissected, homogenized, and centrifuged at 100,000 × *g*. The level of neurofilaments protein in both pellet and supernatant fractions was determined. Western blot analysis showed a nearly depletion of NF-M and a dramatic decrease of NF-L in both fractions of tibial nerves. These changes were observed within 24 h of TOCP administration and then followed by an obvious recovery. In contrast, a progressive reduction in NF-H was observed in tibial nerves of TCOP-treated hens throughout the period of experiment. With the reduction of NF-L level, the rate of NF-L degradation demonstrated a significant increase in both fractions of tibial nerves. Furthermore, the expression of μ-calpain in tibial nerves was increased following TOCP. Taken together, these results demonstrated that NFs changes occurred much earlier than the clinical appearance of ataxia in TOCP-induced delayed neuropathy, indicating that disruption of NF homeostasis in peripheral nerves might be an early molecular event in the development of OPIDN.

1. Introduction

Organophosphorus compounds (OPs) are widely used in agriculture and industry as defoliants, fungicides, herbicides, insecticides, industrial fluids, flame retardants, nerve agents, and therapeutic agents. The acute neurotoxicity of OPs has been documented in accidental human poisoning, epidemiological studies, and animal models. Some OP compounds, such as tri-ortho-cresyl phosphate (TOCP), may also cause another type of toxicity, known as organophosphate-induced delayed neuropathy (OPIDN) (Abou-Donia, 1981). Signs and symptoms include tingling of the hands and feet, followed by sensory loss, progressive muscle weakness and flaccidity of the distal skeletal muscles of the lower and upper extremities, and ataxia (Lotti, 1992; Lotti and Moretto, 2005).

OPIDN can be classified as a distal sensorimotor axonopathy. Typically, the delayed-neuropathy occurs 2–3 weeks after a single exposure when signs of both the acute cholinergic and the intermediate syndromes have subsided (Abou-Donia, 1981, 2003). The morphological pattern of OP distal axonopathy consists of symmetrical, distal axonal degeneration of ascending and descending nerve fiber tract located in central and peripheral nervous systems. Primarily, long, large diameter fibers are affected. In the peripheral nervous system, the longer nerve fibers to the hindlimbs undergo axonal degeneration before the shorter fibers to forelimbs. Concurrently, the long spinal cord tracts, such as dorsal columns, especially the fasciculus gracilis, the corticospinal pathways, and the spinocerebellar tract, show distal axonal degeneration (Cavanagh, 1964; Abou-Donia and Graham, 1979; Tanaka and Bursian, 1989; Classen et al., 1996). Ultrastructural studies have shown that axonal swelling containing aggregations of neurofilaments (NFs), microtubules, multivesicular vesicles, and proliferation of smooth endoplasmic reticulum are presented in early stage, and followed by partial matting, and disappearance of NFs from swollen axons (Bischoff, 1967, 1970).

Although incidents of OPIDN have been documented for over a century, no therapeutic regimen is available to counter this syndrome due to the lack of complete understanding of the mechanisms for OPIDN. Some scholars believed neurotoxic esterase (NTE) inhibition as the earliest event of OPIDN while others regard the alteration of Ca^{2+}/calmodulin kinase II (CaM kinase II) activity as the earliest event that leading to the subsequent series of pathologic changes in the development of OPIDN (Johnson, 1993; Abou-Donia and Lapadula, 1990). Moreover, many morphological

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studies indicated that the NF disorganization might be involved in the occurrence and development of OPIDN. Ultrastructural alterations in OPIDN were seen mostly as aggregation and accumulation of neurofilaments, followed by their dissolution and disappearance (Abou-Donia, 2003).

The hen (female of *Gallus gallus domesticus*) classically is the species of choice for demonstration of delayed neuropathy following a single dose of OP compounds, particularly TOCP (Abou-Donia and Lapadula, 1990). The NF disruption in TOCP or other organophosphorus compound-induced delayed neuropathy has been studied by several investigators. In our previous study, Zhao et al. (2004) observed a significant decrease of NF-H and NF-M in sciatic nerves of hens treated with 750 mg/kg TOCP on day 21 post-dosing. Regrettedly, the one time-point assay could not identify the peak effect of NF alteration. Furthermore, whether any difference in NF alteration between proximal (sciatic nerve) and distal (tibial nerve) region of axons has not been reported. The initial pathological lesion of OPIDN appeared primarily in tibial nerve of OP-treated hen, hence it seemed particularly important to elucidate the events occurring in the latent period before onset of clinical signs in OP-dosed hens. Considering the characteristic of NF alteration pathologically in OPIDN, we hypothesized that a disturbance of NF degradation produced by OP might participate in the progression of OPIDN. However, the relationship between NF degradation and OPIDN has not been investigated thoroughly so far.

Therefore, in the present investigation, we have investigated the time course of NFs alteration in tibial nerves of hens induced by TOCP, and studied the alteration of NF degradation during the development of OPIDN.

2. Materials and methods

2.1. Materials

Tri-ortho-cresyl phosphate (TOCP, purity >99%) and Protease Inhibitor Cocktail set III were purchased from Merck Biosciences, Inc. (Darmstadt, GER). Monoclonal antibodies anti-NF-H (clone NE-14), anti-NF-M (cloneNN-18), anti-NF-L (clone NR-4), anti-β-actin (clone AC-15), anti-m-calpain (clone 107–82), anti-μ-calpain (clone 15C10), and HRP-conjugated goat-anti-mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BCA™ Protein assay Kit and SuperSignal® West Pico Chemiluminescent Substrate Kit were purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). Film was obtained from Eastman Kodak Co. (Rochester, NY, USA). All other chemicals were of highest quality commercially available.

2.2. Animal treatment and neurological testing

Roman hens, 10 months old and weighing 1.5–2.0 kg, obtained from Institute of Poultry, Academy of Agriculture of Shandong (Jinan, CN) were used in this study. Drinking water and complete-value hen powder food were available ad libitum. The hens were housed one per cage made of stainless steel wire. The animal room was maintained at approximately 22 °C and 50% humidity with a 12 h light/dark cycle. All experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and followed the principles in the "Use of Animals in Toxicology". After 7 days for acclimatization, the animals were randomly divided into five groups, i.e. four experimental groups (1-day, 5-day, 10-day, and 21-day groups; $n = 6$ each group) and one control group ($n = 6$). In order to determine the time course of NFs changes induced by TOCP, hens in experimental groups were treated with TOCP by gavage at single dosage of 750 mg/kg. TOCP was dissolved in corn oil and administered at 0.65 ml/kg. The control group received an equivalent volume of corn oil by gavage. Hens in different groups were anesthetized with ether and sacrificed by decapitation on the corresponding time points of 1, 5, 10, and 21 day post-exposure, respectively. The tibial nerves were quickly dissected and frozen in liquid nitrogen. Subsequently nerve samples were kept at −80 °C until used for the determination of NFs proteins.

During the course of this experiment, the hens were weighed twice a week and examined daily for the clinical signs of delayed neurotoxicity after the administration of TOCP. Neurological evaluation was performed by a blinded observer who was not involved in animal care and administration. OPIDN neurological signs were assessed by an eight-point graded scale (0, normal ambulation; 1–2, slight and infrequent hindlimb incoordination; 3–4, moderate but definite hindlimb incoordination; 5–6, severe and frequent difficulty in walking and standing erect; 7–8, virtual to complete hindlimb paralysis) (Pope and Padilla, 1990).

2.3. Tissue preparation, electrophoresis and immunoblotting

Tibial nerves were broken into powder with pestle in liquid nitrogen, and homogenized in ice-cold buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 25 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM dithiothreitol, Protease Inhibitor Cocktail (50 μl/g tissue) and phosphatase inhibitors (5 mM Na_3VO_4, 10 mM $Na_4P_2O_7$ and 1 mM iodoacetic acid). Nerve homogenates were centrifuged at 100,000 × *g* for 1 h to yield a high-speed pellet (P) and a high-speed supernatant (S) fraction (Tsuda et al., 1997; Shea et al., 1997; LoPachin et al., 2005). Protein concentration of two fractions was determined by using BCA™ Protein assay Kit, and used for Western blotting analysis of NFs. As for detecting calpains expression, tibial nerves were broken into powder with pestle in liquid nitrogen, and homogenized in a tissue extraction buffer (Tris–HCl pH 6.8 containing 1 mM EGTA, 1 mM EDTA, and 1% Triton X-100), 20 mM β-glycerophosphate, 20 mM sodium fluoride, 1 mM sodium vanadate, and protease inhibitor cocktail (50 μl/g tissue). The samples were centrifuged at 30,000 × *g* for 30 min. The supernatants were used for Western blotting analysis of calpains.

To assess relative changes of protein content in tissue preparations, corresponding protein samples from both control and experimental animals were subjected to SDS-PAGE on 4% stacking and 7.5% or 10% resolving gel. Following electrophoresis, proteins were transferred electrophoretically to nitrocellulose membranes. Then the membranes were blocked with 4% fat-free milk for 45 min and incubated with primary antibody diluted in 0.1% BSA for 3 h. Following primary antibody, membranes were washed in TBS and incubated with horseradish peroxidase-conjugated secondary antibody for 3 h at the room temperature. After being washed again, the membranes were incubated by using the SuperSignal West Pico Chemiluminescent Substrate reagents for 5 min, and then exposed to film for 15 s. Immunoreactive bands of proteins were scanned with Agfa Duoscan T1200 scanner and digitized data were quantified as integrated optical density (IOD) using Kodak Imaging Program and Image-Pro Plus software.

2.4. Statistical analysis

The data of body weight, gait score and IOD value of immunoreactive bands were expressed as mean ± S.D., statistical analysis was performed with one-way analysis of variance (ANOVA), followed by LSD's post-hoc tests, which was provided by SPSS 10.0 statistical software. The differences were significant at $P < 0.05$.

3. Results

3.1. Clinical signs

Hens did not show any signs of acute cholinergic toxicity after administration of a single dose of 750 mg/kg TOCP. The TOCP-treated hens began to show slightly abnormal gait on day 5. As the time went on, the symptoms aggravated progressively. Most of the hens reached hindlimb paralysis on day 15 post-dosing (mean clinical score = 7.5). By the end of 21-day experimental period, all birds showed complete paralysis gait (mean clinical score = 8.00) (shown in Fig. 1). In contrast, no any clinical signs of delayed neurotoxicity were observed in control hens. Furthermore, no severe symptoms of acute intoxication were observed in all hens of experimental groups although they exhibited a trend of reductions in body weights. On

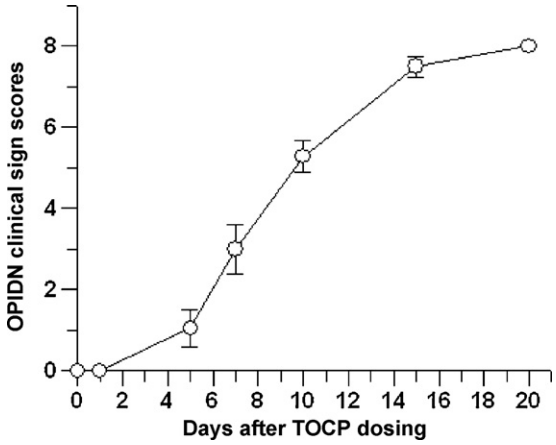

Fig. 1. The mean scores of delayed neuropathy following TOCP administration. The clinical evaluation of delayed neuropathy was estimated in hens by an eight-point scale method daily for 21 days.