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Verapamil abolished the enhancement of protein phosphorylation of brainstem mitochondria and synaptosomes from the hens dosed with tri-*o*-cresyl phosphate

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Abstract

To explore the changes of the endogenous phosphorylation of brainstem mitochondrial and synaptosomal proteins in adult hens dosed with tri-*o*-cresyl phosphate (TOCP) following the development of organophosphate-induced delayed neurotoxicity (OPIDN). Verapamil (7 mg/(kg day), i.m.) was given for 4 days. A dose of TOCP (750 mg/kg, p.o.) was administrated in second day after verapamil. Phosphorylation of the proteins from brainstem mitochondria and synaptosomes was assayed *in vitro* by using [γ-^{32}P]ATP as phosphate donor. Radiolabeled proteins were separated by SDS-PAGE and visualized by autoradiography. The results showed that TOCP administration enhanced the phosphorylation of the cell organelle proteins (mitochondria: 60, 55, 45, and 20 kDa; synaptosomes: 65, 60, and 20 kDa), while verapamil abolished the enhancement induced by TOCP. Additionally, the reaction for the phosphorylation is catalyzed by the calcium/calmodulin protein kinase. Therefore, TOCP can enhance the phosphorylation of the brainstem mitochondrial and synaptosomal proteins from the hens with OPIDN; however, protection from the enhancement of the phosphorylation should be involved in the mechanisms of the amelioration of TOCP-induced delayed neurotoxicity by verapamil.

Keywords: Verapamil; Tri-*o*-cresyl phosphate; Hen; Mitochondrion; Synaptosome; Protein phosphorylation

1. Introduction

Organophosphorus compounds (OPs) are a diverse group of chemicals used primarily as pesticides, plasticizers, plastic softeners, flame-retardants, antioxidants, and hydraulic fluids (Craig and Barth, 1999; Winder and Balouet, 2002). Most OPs can induce acute toxicity in human and animals due to inhibition of acetylcholinesterase. Some OPs can additionally induce delayed effects, so-called organophosphate-induced delayed neurotoxicity (OPIDN), which is characterized by distal axonal degeneration and secondary demyelination of the central and peripheral axons in 7–14 days before onset of clinical pathological symptoms by single or multiple doses (Johnson, 1974, 1982; Abou-Donia, 1981). One organophosphate, tri-*o*-cresyl phosphate (TOCP) has been the archetype of organophosphates inducing a delayed neurotoxicity, and adult hens are usually used as the animal model (Cavanagh, 1973; Abou-Donia, 1993).

Although the inhibition of neuropathy target esterase (NTE) has been reported to be an initial biochemical event associated with the development of OPIDN (Johnson, 1987, 1993), the exact causality between pesticide exposure and emergence of clinical symptoms has not yet been precisely defined (Randall et al., 1997). Investigation to other neuropathies has suggested that intraaxonal elevation of free calcium is associated with the breakdown of cytoskeletal elements and degeneration of myelin (Abou-Donia, 1993). Furthermore, it has been shown that some calcium channel blockers can ameliorate the clinical symptoms and histological lesion of nerves during the development of OPIDN (El-Fawal et al., 1989; Wu and Leng, 1997a,b). However, the mechanisms of both OPIDN development and protective effect of the blocker on the neuropathy in OPIDN are unclear (Glynn, 2003).

Mitochondria and individual mitochondrial proteins are known to participate in processes involving signal transduction pathways, including programmed cell death, neoplastic prolif-

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eration and oxidative stress (Rama Rao et al., 2005; Youdim et al., 2005). Protein phosphorylation has been recognized to cover very important functions in signal transduction (Salvi et al., 2002). However, there are few studies on mitochondrial protein phosphorylation in the field of OPIDN. Other work has showed that prominent axonal lesions occur in the brainstem (Huggins and Richardson, 1999). We reported here that TOCP can stimulate the brainstem mitochondrial and synaptosomal protein phosphorylation and calcium channel blocker verapamil can abolish the effect.

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP (185 PBq/mol) was obtained from the Beijing Yahui Company of Biotechnology (Beijing, China). TOCP and physostigmine sulfate were purchased from BDH Chemicals Co. Ltd. (Poole, England). Atropine sulfate was obtained from Minsheng Pharmaceutical Factory (Hangzhou, China). Coomassie brilliant blue (G-250, R-250), 2-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), and adenosin-5′-triphosphate disodium salt (5′-ATP-Na_2) were purchased from Fluka Chemiabiochemika (Buchs, Switzerland). Tris, protein molecular weight marker (MW-70-2), *N*,*N*,*N*′,*N*′-methylene bisacrylamide (bis), bovine serum album (BSA), and verapamil was purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and ammonium persulfate were purchased from Bio-Rad Laboratories (Richmond, CA). Acrylamide (Acr) and sodium dodecyl sulfate (SDS) were obtained from Serva Fine Chemicals Co. (Heidelberg, Germany). Bovine brain calmodulin (CaM) was obtained as a gift from the Department of Biology, Hebei Normal University (Shijiazhuang, China).

2.2. Animals treatment

Adult Haisai laying hens (about 12 months old and weighing about 1.5 kg, purchased from the Beijing Dabei Poultry Farm, Haidian, Beijing, China) were used in this study. They were housed with one bird per cage. The birds were acclimatized for at least 1 week prior to the start of a 21-day study. Hens were divided into three groups (control, TOCP + verapamil, and TOCP alone). During the experiment periods, the temperature in the hen house was maintained at about 20 °C with a light/dark cycle of 12 h each. The hens from two groups (TOCP + verapamil; TOCP alone) were given a single oral dose of TOCP (750 mg/kg) in a gelatin capsule while the control hens were given an empty gelatin capsule. Verapamil dissolved in saline was injected into the breast muscle of the hens (TOCP + verapamil group) daily at a dose of 7.0 mg/kg administrated for 4 days according to the method of El-Fawal et al. (1989) with a slight modification.

2.3. Preparation of subcellular fractions

The hens were killed by decapitation on days 7, 14, 21 after administration of the treatments. Following sacrifice, brainstems (including portions of the diencephalons and medulla oblongata, which were defined as brainstem in this study) were removed immediately and immersed in ice-cold the homogenization buffer (0.32 M sucrose, 50 mM Tris–HCl, pH 7.4). Meninges and superficial blood vessels of the brainstem were stripped off carefully and the brainstem was homogenated with a Teflon-glass homogenizer using 10 up-and-down strokes in 10 volumes per brain of the homogenization buffer.

Mitochondria and synaptosomes were isolated as described previously by Gray and Whittaker (1962) with some modifications. The homogenate was centrifuged at 1000 × *g* for 10 min to yield a supernatant (S_1) and a crude nuclear pellet. S_1 was then centrifuged for 55 min at 17,000 × *g* to yield a supernatant and a crude pellet (P_2). After suspension in 50 mM Tris–HCl buffer (pH 7.4, containing 0.32 M sucrose), P_2 was loaded on a discontinuous density gradient sucrose and then separated into three fractions by centrifuging samples at 100,000 × *g* for 2 h. The layer between 0.8 and 1.2 M sucrose is synaptosomes. The pellet below 1.2 M sucrose is mitochondria. The samples were resuspended in assay buffer, and then frozen and stored at −80 °C until assay or assayed immediately.

2.4. Endogenous protein phosphorylation assays

The phosphorylation reaction was undertaken duplicately according to Patton et al. (1983) with a slight modification. The standard assay mixture, in a final volume of 200 μl, contained 75 μg protein of mitochondria or synaptosomes, 50 mM PIPES, pH 6.5, 0.3 mM PMSF, 10 mM $MgCl_2$, 5 μM [γ-^{32}P]ATP. Additionally, the proteins were incubated in the absence or presence of 300 μM $CaCl_2$ or 300 μM $CaCl_2$ + 1 μg calmodulin (CaM) (final concentration). Proteins were incubated for 2 min at 35 °C; the phosphorylation reaction was then initiated by the addition of [γ-^{32}P]ATP. The reaction was terminated after 30 s by adding 100 μl of SDS-PAGE sample buffer (containing 0.125 M Tris–HCl, pH 6.8, 4.5% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) bromophenol blue), followed by heating for 3 min in a water bath at 90 °C.

2.5. Protein determination

Protein concentrations were determined by the method of Bradford (1976) using Coomassie brilliant blue G-250 with bovine serum albumin as a standard.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography and microdensitometry

The crude mitochondrial or synaptosomal proteins were resolved on 10-well SDS-polyacrylamide vertical slab gels (70 mm × 80 mm × 1 mm) using the Bio-Rad Mini-protein II. Electrophoresis was performed in the buffers described by Laemmli (1970) with 4% (w/v) and 10% (w/v) acrylamide in the stacking and resolving gels, respectively. Aliquots of the samples (7.5 μg/well) were subjected to electrophoresis under conditions of constant voltage (200 V) until the dye front reached the bottom of the gels. The apparent electrophoretic mobilities of the resolved proteins were determined from molecular standards of MW-70-2 (Sigma) that had been subjected to electrophoresis under conditions identical to the brainstem mitochondrial and synaptosomal samples and were presented in kilodaltons. Following the electrophoresis, the gels were fixed and stained for proteins with 0.1% Coomassie brilliant blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid for 4 h. The gels were then distained in 40% methanol and 10% acetic acid for 2–4 h and dried naturally between two sheets of dialysis membrane for 18–24 h. Autoradiographs of ^{32}P-labeled proteins were obtained by placing the dried gels in close contact with Fuji X-ray medical film at −20 °C for about 3 days.

3. Results

3.1. Variation in clinical symptoms with time after TOCP and TOCP plus verapamil administration

Development of delayed neurotoxicity in the tested hens was monitored each day up to 21 days after dosing. Adult hens were treated orally with TOCP at time zero. On day 9, the TOCP-treated hens began to show symptoms of neurological dysfunction in the form of mild ataxia, characterized by diminished leg movement and reluctance to walk. Clinical symptoms progressed to disturbances in gait, unsteadiness, and frequency of falling on the floor. By day 14 most of the hens were unable to walk from a standing position. On day 21, they were completely paralyzed. However, during the entire experimental period, no apparent symptoms of ataxia were evident in the hens treated with vehicle, and the ataxia of the hens given TOCP alone was more severe than that of the hens given TOCP plus verapamil.

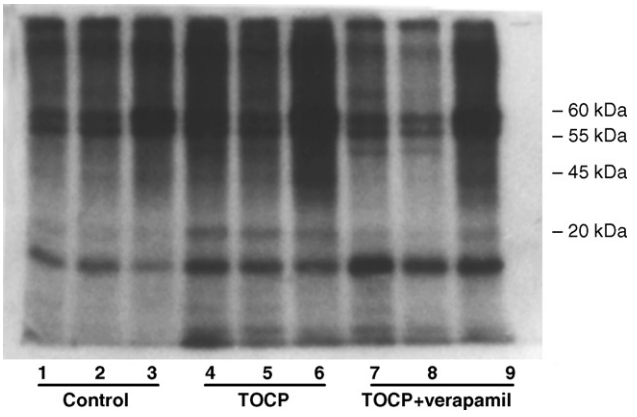

Fig. 1. Autoradiographs of ^{32}P incorporation into mitochondrial proteins from the brainstem of control and TOCP-treated hens on day 7 (*in vitro*). Lanes 1, 4 and 7: none added to the reaction medium; lanes 2, 5 and 8: in the presence of 300 μM $CaCl_2$; lanes 3, 6 and 9: in the presence of 300 μM $CaCl_2$ and 1 μg calmodulin.

3.2. Changes in brainstem mitochondrial and synaptosomal protein phosphorylation following the administration of TOCP and TOCP plus verapamil

The proteins from control, TOCP-treated alone, TOCP plus verapamil-treated hens on days 7, 14, 21 after treatment were phosphorylated with [γ-^{32}P]ATP *in vitro* by endogenous kinases. Subsequent separation by SDS-PAGE revealed the molecular weights of the phosphorylated substrate proteins. There was no variation among the samples, which are equal in mass from the three groups, in the relative abundance of any band in the Coomassie brilliant blue protein-staining pattern.

Differences in neural protein phosphorylation among the three groups were analyzed qualitatively from the autoradiographic patterns of the dried gels (Figs. 1–4). On day 7, mitochondrial proteins from TOCP-treated group were phosphorylated to a greater degree *in vitro* than those from the control hens (Fig. 1). The phosphorylation was prominent especially for proteins of apparent molecular weights of 60, 55, 45 and 20 kDa. The mitochondrial proteins from hens treated with TOCP plus verapamil, however, showed no obvious enhancement of phosphorylation (Fig. 1). Clearly, verapamil had prevented the increase of phosphorylation *in vitro* caused by TOCP. On day 14, there are no obvious differences among the three groups (Fig. 2).

On day 7, the phosphorylation level of synaptosomal proteins from TOCP-treated group were higher than that from control group, especially the proteins with molecular weights of 65, 60, 20 kDa (Fig. 3). It is also showed that the synaptosomal protein phosphorylation level from the hens treated with TOCP plus verapamil was close to that from control group. It seems that verapamil suppressed the enhancement of phosphorylation. On day 21, the differences of phosphorylation level among the three groups are similar to those on day 7.

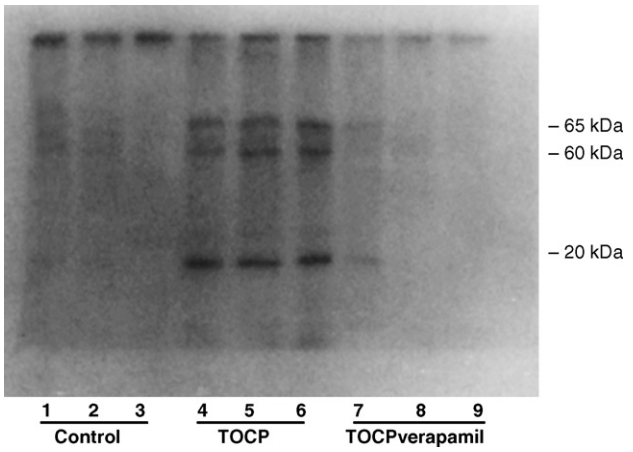

Fig. 3. Autoradiographs of ^{32}P incorporation into synaptosomal proteins from the brainstem of control and TOCP-treated hens on day 7 (*in vitro*). Lanes 1, 4 and 7: none added to the reaction medium; lanes 2, 5 and 8: in the presence of 300 μM $CaCl_2$; lanes 3, 6 and 9: in the presence of 300 μM $CaCl_2$ and 1 μg calmodulin.

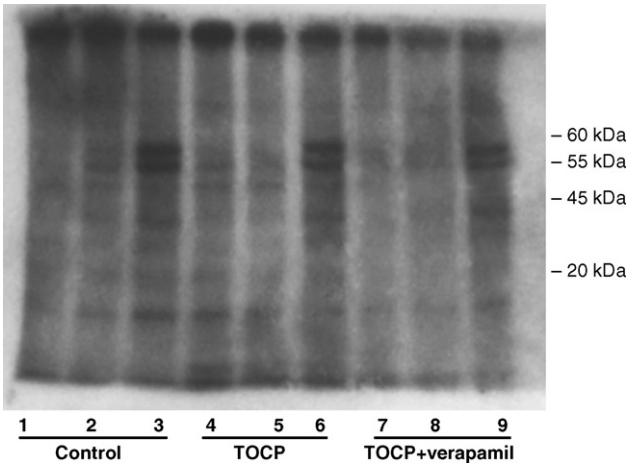

Fig. 2. Autoradiographs of ^{32}P incorporation into mitochondrial proteins from the brainstem of control and TOCP-treated hens on day 14 (*in vitro*). Lanes 1, 4 and 7: none added to the reaction medium; lanes 2, 5 and 8: in the presence of 300 μM $CaCl_2$; lanes 3, 6 and 9: in the presence of 300 μM $CaCl_2$ and 1 μg calmodulin.

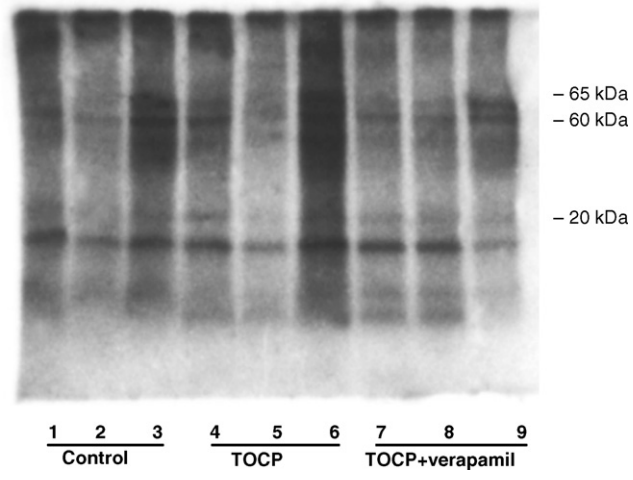

Fig. 4. Autoradiographs of ^{32}P incorporation into synaptosomal proteins from the brainstem of control and TOCP-treated hens on day 21 (*in vitro*). Lanes 1, 4 and 7: none added to the reaction medium; lanes 2, 5 and 8: in the presence of 300 μM $CaCl_2$; lanes 3, 6 and 9: in the presence of 300 μM $CaCl_2$ and 1 μg calmodulin.

When 300 μM $CaCl_2$ or 300 μM $CaCl_2$ plus 1 μg CaM was added to the phosphorylation reaction medium, the results showed that the phosphorylation of substrate proteins were catalyzed by a calcium/calmodulin protein kinase (CCPK) that is dependent on calcium and CaM. Calcium alone enhanced the level of phosphorylation of proteins from the control group in a small degree, while calcium plus CaM enhanced even more.

4. Discussions

The results showed that a single neurotoxic dose of TOCP enhanced the level of mitochondrial and synaptosomal endogenous protein phosphorylation intensively. In comparison, the proteins from hens dosed with TOCP plus verapamil showed no obvious enhancement of phosphorylation, even the level of protein phosphorylation approaches to or lower than that of the control group. Also, the protein phosphorylation reaction was catalyzed by a calcium/calmodulin protein kinase, which indicates that TOCP may induce the enhancement of the intrasynaptosomal calcium concentration. Meanwhile, it has been found that the clinical symptoms of TOCP plus verapamil-treated hens were ameliorated in the experiment period, compared with those of TOCP-treated hens.

It is known that calcium plays a key role as a messenger linking external stimuli to intracellular environment in the nerve. Ca^{2+} is involved in the degeneration and death of nerve cells (Quinton et al., 1996). Cell plasma membrane and different intracellular pools, including the endoplasmic reticulum and mitochondria, participate in regulating Ca^{2+} signals in neuronal cells. Calcium can enter into the cell from both extracellular solution and endoplasmic reticulum as well as mitochondria, two main intracellular calcium stores through a variety of calcium channels in their membranes, respectively. In addition, the intracellular calcium in the cytoplasm can be removed into the extracellular solution or the calcium pools in the cells by the Na^+/Ca^{2+}exchanger and/or Ca-ATPase pumps (Gaffin, 1999). A great deal of evidence showed that protein phosphorylation is involved in regulating the permeability of the calcium ion channel in the nervous system (Ismailov and Benos, 1995).

In this experiment, verapamil, a calcium channel blocker, was demonstrated to prevent the enhancement of mitochondrial and synaptosomal protein phosphorylation. So, it is speculated that TOCP induced the mitochondrial and synaptosomal protein phosphorylation, thereby causing the calcium channel to open; calcium, on the one hand, thus may enter into the cytoplasm from extracellular solution; the mitochondria, on the other hand, may release calcium into cytoplasm. In this way, the intrasynaptosomal calcium concentration rises.

Our previous studies have indicated that TOCP can also enhance the levels of protein phosphorylation in brain synaptosomes and microsomes; and verapamil can prevented the enhancement (Wu and Leng, 1997b; Li et al., 2002). Taking into account the two results, we might safely conclude: an increase of phosphorylation of synaptosomal, mitochondrial, and microsomal proteins from chicken central nerve system is a result of TOCP neurotoxicity; the mechanisms of verapamil to ameliorate the symptoms of OPIDN might be protection from the enhancement of the protein phosphorylation.

Further work will be needed to determine the identity of the proteins and the reasons for their increased phosphorylation in response to TOCP. It remains to be determined whether the enhanced protein phosphorylation capability observed during OPIDN results from covalent organophosphorylation of neural proteins.

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