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Calcium and calmodulin stimulated in vitro phosphorylation of rooster brain tubulin and MAP-2 following a single oral dose of tri-o-cresyl phosphate

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The effect of a single oral administration of 750 mg/kg tri-o-cresyl phosphate (TOCP) on endogenous phosphorylation of specific brain cytosolic proteins has been studied in roosters following the development of delayed neurotoxicity. In vitro phosphorylation assay using [γ-^{32}P]ATP was carried out. Proteins were then resolved on one dimensional 8% SDS-PAGE and two-dimensional gel electrophoresis, stained with Coomassie blue and autoradiographed. The amount of proteins, as well as the amount of ^{32}P incorporation, were quantified by microdensitometry, TOCP-administration enhanced the phosphorylation of cytosolic proteins of M_r 52–59 kDa, 70 kDa, and 300 kDa by as much as 155%, 199% and 166%, respectively. Two-dimensional gel electrophoresis confirmed the 52–59 kDa proteins as α and β tubulin and the 300 kDa protein as microtubule-associated protein-2.

Some organophosphorus esters, including tri-o-cresyl phosphate (TOCP), produce a delayed neurotoxicity (organophosphorus compounds-induced delayed neurotoxicity, OPIDN) which develops 6–14 days following a single dose in many susceptible species, such as humans, dogs, cats, chickens and water buffalo[1,18]. The clinical condition is characterized by ataxia followed by paralysis. Degeneration of the axons with subsequent secondary degeneration of the myelin is seen in both the central and peripheral nervous systems. Although no studies to date have defined the mechanism of OPIDN, Patton et al.[2,16,17] have suggested that Ca^{2+}-calmodulin-regulated phosphorylation may play a role in the pathogenesis of OPIDN. The increase of chicken brain protein phosphorylation correlated with the time of the development of ataxia and paralysis[2,17].

A number of Ca^{2+}-calmodulin protein kinase activities have been identified in various preparations of brain[6,9,11,14,20]. Burke and DeLorenzo[5] also reported a Ca^{2+}-calmodulin-stimulated tubulin kinase system that phosporylates tubulin. This kinase was later found to also phosphorylate some other proteins such as microtubule associated protein-2 (MAP-2) and myelin basic protein[8]. Using a similar in vitro brain protein phosphorylation system, we have found in vivo TOCP treatment to increase in vitro phosphorylation of rooster brain tubulin and MAP-2, as well as a 70 kDa protein.

Roosters, obtained from Featherdown Farms (Raleigh, NC), weighing ca. 4 kg, were housed singly in cages in 40–60% humidity and temperature controlled rooms (20–23 °C) with a 12 h light cycle. They were given food (Ralston-Purina, St. Louis, MO) and water ad libitum. Animals were divided into two groups of 6 roosters: Group I served as control and Group II was given a single oral dose of 750 mg/kg TOCP (99% pure, Eastman Kodak, Rochester, NY) in gelatin capsules. Body weights were monitored weekly and animals were observed for neurological dysfunction. Animals were killed after 18 days. Immediately following sacrifice, brains were rapidly removed and immediately homogenized at 4 °C in extraction buffer (100 mM PIPES, pH 6.94, 2 mM EGTA, 1 mM $MgCl_2$ and 0.3 mM PMSF) in a ratio of 1 g brain/1 ml buffer[8].

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The homogenate was then centrifuged at 100,000 *g* for 50 min. The resulting supernate (referred to as 'brain cytosol') was used for protein phosphorylation. The standard phosphorylation reaction was initiated by the addition of a 25 μl protein aliquot containing 100 μg of brain cytosol to the reaction mixture. The standard reaction mixture also contained a final volume of 200 μl: 50 mM PIPES, pH 6.5, 5 μM [γ-^{32}P]ATP (2900 Ci/mmol, New England Nuclear, Boston, MA) and the following components: presence or absence of 10 mM $MgCl_2$, presence of 10 mM $MgCl_2$ plus 5 μM or 50 μM $CaCl_2$, 50 μM $CaCl_2$ and 5 μg calmodulin, 50 μM $CaCl_2$ and 50 μM trifluoperazine (TFP), presence of 2 mM EDTA in the absence of $MgCl_2$, where indicated. Reactions were incubated at 37 °C and terminated after 1 min by the addition of 100 μl of 'stop solution' (0.125 M Tris-HCl, pH 6.8, 4.5% SDS, 20% glycerol and 10% β-mercaptoethanol).

The de-ionized water used in the phosphorylation assays as well as in the homogenization was passed through a Chelex 100 column to ensure thorough removal of cations[3]. The assays were performed in plastic (polyethylene) test tubes and all solutions in contact with the tissue samples before the addition of stop solution were stored in plastic containers.

Samples were resolved on 20-well 0.1% SDS-polyacrylamide vertical slab gels. 1-Dimensional electrophoresis was performed by the method of Laemmli[12] on 8% resolving and 4% stacking gels, with the following modifications: 2 mM EDTA was added to resolving, stacking and tank buffers[8]. The pH of resolving buffer as well as the tank buffer were adjusted to 9.1 (ref. 13) and 8.6 (ref. 8), respectively. Aliquots of the samples (50 μl) were subjected to electrophoresis under conditions of constant current (25 mA/gel) until the tracking dye reached the bottom of the gels. For 2-dimensional electrophoresis[15], 100 μl aliquots of samples were spiked with 5 μg of twice cycled, P-11 column purified chicken brain tubulin[4], lyophilized and were then dissolved in lysis buffer (9.5 M urea, 2% NP-40, 2% ampholines, 5% β-ME and 2% SDS). Isoelectric focusing was run on 4% acrylamide tube gels at 400 V for 18 h, followed by 550 V for 2 h. The second dimension was carried out by placing the tube gel on top of the stacking gel overlayed with 1% agarose solution and electrophoresis was performed the same way as in 1-dimensional. Following electrophoresis, gels were stained with Coomassie brilliant blue, dried under vacuum, and subjected to autoradiography. Autoradiographs of ^{32}P-labeled proteins were obtained by placing the dried gels in close contact with Kodak X-OMAT RP X-ray film at −70 °C using intensifying screens (Dupont Cronex Lighting Plus, CGR Corp., Raleigh, NC). The amount of proteins as well as the amount of phosphoproteins in each autoradiographic band were quantified by integration of the area under the corresponding peak in the densitometric scan, obtained by using an LKB Ultrascan Laser Densitometer interfaced with a Recording Integrator (LKB Instruments, Gaithersburg, MD).

TOCP-treated roosters suffered 15% weight loss during the 18-day experiment, while the control birds had 2.9% weight gain. Mild ataxia was observed at day 7 in treated animals and by day 12 they were completely paralyzed.

Autoradiography of the phosphorylated brain cytosolic proteins differed significantly between the control and TOCP-treated roosters. There was an in-

TABLE I

Stimulation of specific brain cytosolic proteins by TOCP

The incorporation of phosphate into specific brain cytosolic proteins under the standard phosphorylation assay with the indicated co-factors. Values are in arbitrary units ± S.E.M. of at least 12 independent observations. Significance was determined by a Student's two-tailed *t*-test, $P < 0.05$. n.d., bands were not detectable by densitometer; n.s., not significantly different from control.

M_r	*Protein staining*			*Autoradiography 50 μM Ca^{2+}, 10 mM Mg^{2+} and 5 μg calmodulin*		
	Control	*Treated*	*% of control*	*Control*	*Treated*	*% of control*
52–59 kDa	3.43±0.15	3.21±0.13	n.s.	26.1±8.5	40.5±5.93	155
70 kDa	n.d.	n.d.	—	2.28±0.62	4.53±1.24	199
300 kDa	0.62±0.06	0.55±0.05	n.s.	5.13±1.4	8.53±1.91	166

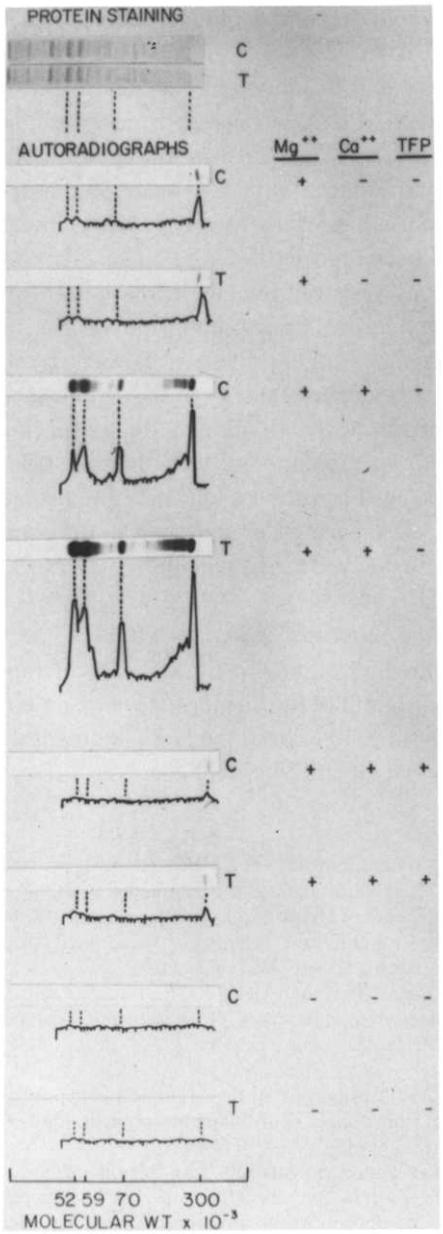

Fig. 1. Protein staining, autoradiography and microdensitometry of rooster brain cytosolic proteins. Brain cytosol from control (C) and TOCP-treated (T) roosters was incubated under standard phosphorylation assay condition in the presence or absence of the indicated cofactors (10 mM Mg^{2+}, 50 μM Ca^{2+} and 5 μg calmodulin or 50 μM trifluoperazine). Seventeen μg of protein samples were subjected to gel electrophoresis and autoradiography. The amount of proteins as well as the amount of phosphate incorporated into specific proteins, from at least 12 independent observations, were quantified by microdensitometry. A significant increase in the phosphorylation of 52–59 kDa, 70 kDa and 300 kDa proteins in the presence of Mg^{2+}, Ca^{2+} and calmodulin are shown here.

creased phosphorylation of 52–59 kDa, 70 kDa and 300 kDa proteins by as much as 155%, 199% and 166%, respectively (Table I). The enhancement in phosphorylation of brain proteins in treated animals was not artifactual since the concentration of the corresponding protein bands showed no change between the control and TOCP-treated groups. The 3 proteins were phosphorylated in a Ca^{2+}- and Mg^{2+}-dependent manner (Fig. 1). There was no significant increase in ^{32}P-incorporation into the proteins by adding EDTA, a divalent cations chelator, into the sample mixture. Phosphorylation was also enhanced with the addition of 50 μM Ca^{2+} compared to addition of 5 μM Ca^{2+}. This effect was also calmodulin-dependent; adding trifluoperazine, a calmodulin inhibitor, significantly decreased the amount of phosphorylated protein.

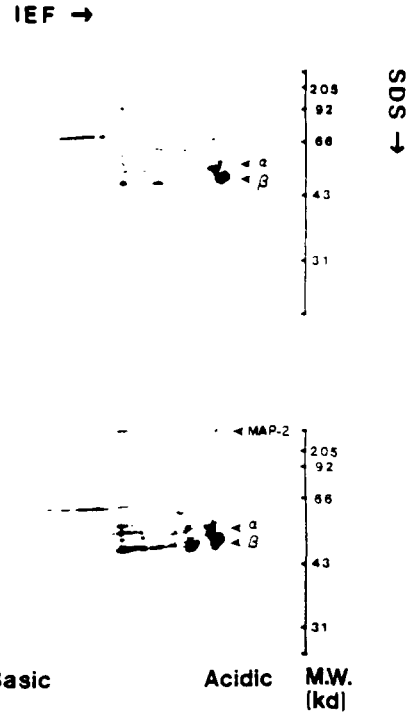

Fig. 2. Autoradiography of 2-dimensional gel electrophoresis of rooster brain cytosolic proteins. Brain cytosol from control or TOCP-treated rooster was incubated under standard phosphorylation assay in the presence of 10 mM Mg^{2+}, 50 μM Ca^{2+} and 5 μg calmodulin. Thirty-four μg protein samples were spiked with 5 μg P-11 column purified chicken brain tubulin and subjected to 2-D gel electrophoresis and autoradiography. The 52–59 kDa proteins co-migrated with the α and β P-11 purified tubulin, while the 300 kDa protein appeared to be the MAP-2. The incorporation of phosphate into the tubulin and MAP-2 seemed to be more in treated (lower) than in control (upper).

Two-dimensional gel electrophoresis was used to identify the 3 proteins. Spiking the samples with P-11 column purified chicken brain tubulin confirmed the proteins with M_r 52–59 kDa as α and β tubulin, while the 300 kDa protein was found to be MAP-2 (Fig. 2). Although the existence of multiple phosphorylation sites on the neurofilament subunits has been reported[10], 2-dimensional electrophoresis showed that the 70 kDa protein was unlikely to be the lower molecular weight subunit of neurofilament. The identity and the functional significance of this 70 kDa protein is unknown at present.

The enhanced phosphorylation of tubulin has been reported to be specific for organophosphorus compounds capable of causing delayed neurotoxicity (TOCP, leptophos) and not for non-delayed neurotoxic organophosphorus compounds (parathion, tri-*p*-cresyl phosphate or TPCP)[2]. Tubulin and MAP-2 have been reported to be substrates of tubulin-associated calmodulin-dependent kinase[8] as well as of a 640 kDa kinase[7]. The increased phosphorylation of the two proteins may be due to an increased activity of either one, if not both, of the kinases involved. This, in turn, may have a significant effect on the physicochemical properties of the microtubule proteins. Increase Ca^{2+}-calmodulin-dependent phosphorylation of MAP-2 has been reported to inhibit microtubules assembly[19]. The disassembly of microtubules when incubated with Ca^{2+}-calmodulin-dependent protein kinase in the presence of ATP and Mg^{2+} has also been reported[21].

In conclusion, we have found an increase in the in vitro phosphorylation of brain tubulin and MAP-2, as well as a cytosolic 70 kDa protein in TOCP-treated roosters. A further understanding of the significance of the enhanced phosphorylation of these specific proteins on the functional integrity of the cytoskeletal proteins, as well as of the axon, may shed some light on the mechanism of organophosphorus-compounds-induced delayed neurotoxicity.

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