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Oral administrations of methamidophos and TOCP have different effects on *in vitro* protein phosphorylation levels from subcellular fractions of hen brain

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Abstract

The effects of methamidophos and tri-*o*-cresyl phosphate (TOCP) on the endogenous phosphorylation of specific brain proteins were studied in Beijing white laying hens during the early stage of delayed neurotoxicity. Phosphorylation of mitochondrial and synaptosomal proteins was assayed *in vitro* by using [γ-^{32}P]ATP as phosphate donor. Tri-*o*-cresyl phosphate (TOCP) administration enhanced the phosphorylation of synaptosomal proteins with molecular weight of 40 and 55 kDa by as much as 36% and 65%, respectively, and decreased the phosphorylation of mitochondrial protein (35 kDa) by 33%. A single dose of methamidophos enhanced the phosphorylation of 32-kDa synaptosomal protein by 44%; however, it had no effect on brain mitochondrial proteins. The activity of neuropathy target esterase (NTE) in dosed hens' brain and spinal cord was assayed for the effects of methamidophos and TOCP. These results showed that methamidophos inhibited brain NTE by 41% compared with that of control after 7-day exposure, while TOCP inhibited brain NTE by 66%. Moreover, NTE activity from spinal cord in treated hens also exhibited a similar trend of activity inhibition. Together, these results suggested that methamidophos and TOCP induced changes of protein phosphorylation level from hen brain and resulted in different kinds of neurotoxicity.

Keywords: Methamidophos; Tri-*o*-cresyl phosphate (TOCP); Phenylmethylsufonyl fluoride (PMSF); Delayed neurotoxicity; Protein phosphorylation

1. Introduction

Organophosphorus pesticide (OP) is the most widely used pesticide in the world. Although the immediate action of OP is the inhibition of acetylcholinesterase, some of these compounds also produce a neurodegenerative disorder known as organophosphate-induced delayed neurotoxicity (OPIDN) [1]. OPIDN is characterized by a central-peripheral distal axonopathy after a delay period of 7–14 days in sensitive species [2]. Although OPIDN has been the subject of intense investigation, its pathogenesis remains to be elucidated. Tri-*o*-cresyl phosphate (TOCP) has been the prototype neurotoxicity-inducing agent, and adult hens are usually used as the animal model for experimental studies of OPIDN.

Neuropathy target esterase (NTE), an enzyme expressed in nervous tissues, is considered as the molecular target for OPIDN. It has been long known that certain non-neuropathic NTE inhibitors do not initiate OPIDN but can modify the clinical effects of both toxic and traumatic insults [3,4]. For example, phenylmethylsulfonyl fluoride (PMSF) protects the animal from OPIDN when given before the neurotoxic OP [5], yet exacerbated the polyneuropathy when given after OP administration. Prophylaxis by

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pretreatment with PMSF is related to the fact that NTE, inhibited by PMSF, does not undergo the aging reaction [6]. However, it is likely that the potentiation of OPIDN by PMSF does not involve NTE [3].

Current studies have demonstrated the involvement of endogenous protein phosphorylation of subcellular fractions from nervous system in the molecular pathogenesis of chemical-induced neurotoxicity [7]. An aberrant increase in phosphorylation of cytoskeletal proteins, such as MAPs, neurofilament proteins, and myelin basic protein after administration of TOCP to the hens has been found, which is mediated by calcium/calmodulin kinase II (CaM-K II) [7]. In addition to the changes of endogenously phosphorylated proteins described in animal models with OPIDN, toxicity on the cellular level has also been demonstrated in a diverse array of immortal cell lines *in vitro* [8]. *In vitro* systems showed that organophosphorus compounds can induce structural and functional alterations in mitochondria. This implicates mitochondria as primary subcellular targets for these compounds [9].

Methamidophos, the most widely used OP in China, can cause acute cholinergic toxicity, and OPIDN, which has been reported in man but seldom seen in the hen [10,11]. The effect of oral administration of methamidophos on the endogenous phosphorylation of specific proteins from subcellular fractions is investigated here, in order to explain the species selectivity for methamidophos-induced neurotoxicity.

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP (3000 Ci/mmol) was purchased from the Beijing Furui Company of Biotechnology (Beijing, China). TOCP and physostigmine sulfate were purchased from BDH Chemicals Co. Ltd (Poole, England). Methamidophos (MET) was obtained from the Shandong Pesticide Factory (Jinan, China). Atropine sulfate was obtained from Minsheng Pharmaceutical Factory (Hangzhou, China). Coomassie brilliant blue G-250 and R-250, phenylmethylsulfonyl fluoride (PMSF), adenosine-5′-triphosphate disodium salt (5′-ATP-Na_2), and 2-mercaptoethanol were purchased from Fluka Chemika (Buchs, Switzerland). Tris, *N*,*N*,*N*′,*N*′-methylene bisacrylamide, bovine serum album (BSA), and PIPES were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and ammonium persulfate were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Acrylamide and sodium dodecyl sulfate (SDS) were obtained from Serva Fine Chemicals Co. (Heidelberg, Germany). Calmodulin (CaM) was a gift from Dr. Juan Bai of the Department of Biology, Hebei Normal University (Shijiazhuang, China).

2.2. Animal treatment

Adult Beijing white laying hens (about 8 months old and weighting about 1.5 kg, purchased from the Dabei poultry farm, 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 the experiment. Hens were divided into four groups (control, MET, TOCP, and PMSF), with three hens in each group. 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. All treated hens were administrated with atropine (10 mg/kg, normal saline, s.c.) and physostigmine sulfate (0.1 mg/kg, normal saline, s.c.) 15 min before the administration of MET, TOCP, or PMSF to protect hens from the possible acute cholinergic effects of the OPs. TOCP- and MET-group hens were given a single oral dose of 750 and 25 mg/kg, respectively, in a gelatin capsule while the control hens were given an empty gelatin capsule. PMSF group hens were injected with PMSF (90 mg/kg, s.c.) dissolved in DMSO, while the control hens received DMSO (0.5 mg/kg, s.c.). After exposure to TOCP, MET and PMSF on the first day, the hens were examined daily for the delayed neurotoxic signs.

2.3. Isolation of mitochondria and synaptosome

The hens were sacrificed by decapitation on day 7 after administration of the OPs. The mitochondria and synaptosomes were prepared from hen brain and spinal cord by the discontinuous sucrose gradient centrifugation method of Whittaker [12]. In brief, hen brain or spinal cord tissue was homogenized in 10 volumes of 20 mM Tris–HCl buffer (pH 7.4) containing 0.32 M sucrose and centrifuged at 1000*g* for 20 min at 4 °C. The resultant supernatant (S1) was then centrifuged at 17,000*g* for 30 min at 4 °C. The supernatant (S2) including microsomes and ribosomes was obtained to assay NTE activity and the residual pellet of crude synaptosomes was resuspended in the same buffer containing 0.32 M sucrose, and layered upon a gradient containing 0.8, 1.0, and 1.2 M sucrose. The gradient was centrifuged at 100,000*g* for 2 h at 4 °C to get the synaptosomal fraction at the interface over the 1.2 M sucrose layer. The mitochondria are in the bottom pellet. The two fractions were resuspended in PIPES buffer, and stored at −80 °C until use or assayed immediately. Protein concentrations were determined by the method of Bradford using Coomassie brilliant blue G-250 with bovine serum albumin as a standard [13].

2.4. Endogenous protein phosphorylation assays

The phosphorylation reaction was undertaken according to Patton et al. [14] with a slight modification. The standard assay mixture, in a final volume of 200 μl, contained 75 μg protein, 50 mM PIPES, 0.3 mM PMSF, 10 mM $MgCl_2$, 5 μM [γ-^{32}P]ATP, pH 6.5. Additionally, proteins were incubated in the absence or presence of 300 μM $CaCl_2$ + 1 μg CaM. 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 1 min 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. Phosphoprotein separation and detection procedures

SDS–polyacrylamide gel electrophoresis was performed by standard methods with the Bio-Rad Mini-protein II in the buffers described by Laemmli [15] with 4 and 10% (w/v) acrylamide in the stacking and resolving gels, respectively. 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 destained in 40% methanol and 10% acetic acid for 2–4 h and dried naturally between two sheets of dialysis membrane for 18–24 h. The dried gel was then placed in an exposure cassette with phosphor imaging screen (Amersham Biosciences, USA) and autoradiographs of ^{32}P-labeled proteins were obtained. The amount of phosphorylated proteins in each autoradiographic band was quantified by determining the area under the corresponding peak in the densitometric scan obtained in Typhoon 9400 imaging system (Amersham Biosciences, USA).

2.6. NTE assay

Neuropathy target esterace (NTE) was assayed by the method of Kayyali [16]. The S2 fraction was incubated for 20 min at 37 °C with 40 μM paraoxon and either (a) TE buffer (50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0) or (b) mipafox (50 μM) in a final volume of 2 ml. The substrate phenyl valerate (1 ml) was then added to a final concentration of 1 mM to all samples except the blank, and the samples were incubated for another 15 min at 37 °C followed by the addition of SDS (3.33%, w/v), 4-aminoantipyrene (0.025%), and potassium ferricyanide (0.4%). The resultant red color was read at 510 nm. NTE activity was calculated as the difference between (a) and (b), and is expressed in terms of nanomoles of phenol formed per minute per milligram protein.

2.7. Statistical analysis

The results are presented as means ± SE and were analyzed by one-way analysis of variance (ANOVA) followed Dunnett's multiple comparison test and Student's unpaired two-tailed *t*-test. The value of $P < 0.05$ was considered significant.

3. Results

3.1. Clinical signs

TOCP treated hens suffered mild ataxia when sacrificed on day 7, but no symptom of delayed neurotoxicity was found in control, MET- or PMSF-treated hens during the whole experiment period.

3.2. Brain and spinal cord NTE activity

Inhibition of NTE has been reported to be a sensitive marker of the potential of OP in producing delayed neurotoxicity. Serial estimations of NTE activity were carried out in the S2 fraction from brain and spinal cord 7 days after exposure to MET, TOCP, and PMSF. As evident in Fig. 1, the inhibition rate of the NTE from brain is much higher than that of the one from spinal cord. Moreover, a 25 mg/kg dose of MET inhibited brain NTE only by 41% compared with that of the control after 7-day exposure, lower than TOCP and PMSF did. The NTE activity from spinal cord also exhibited a similar trend of inhibition.

3.3. Changes in brain mitochondrial and synaptosomal proteins phosphorylation following the administration of MET, TOCP, and PMSF

Mitochondrial and synaptosomal proteins of brain from control, MET-, TOCP-, and PMSF-treated hens were phosphorylated with [γ-^{32}P] ATP *in vitro* by endogenous kinases on day 7 after the treatment. Subsequent separation by SDS–PAGE revealed the molecular weights of the phosphorylated substrate proteins. There was no significant difference in Coomassie brilliant blue-stained protein bands between the control and each of the treated groups, which were equal in mass from the four groups and in the relative abundance of any band. Differences in neural protein phosphorylation among the each group were analyzed

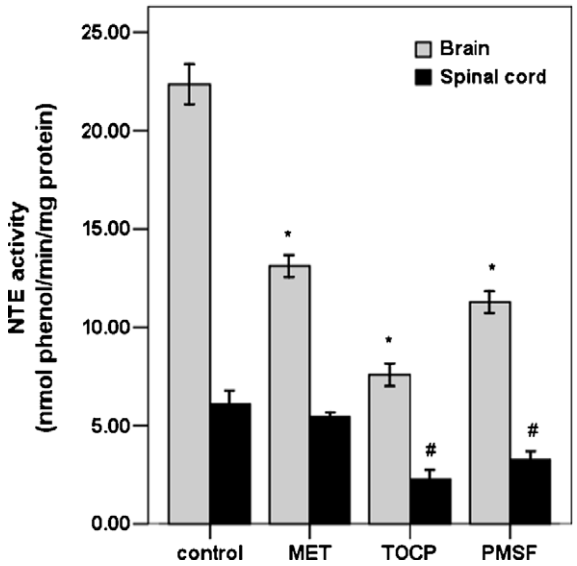

Fig. 1. Effect of MET, TOCP, and PMSF on NTE activities of hen's brain and spinal cord *in vivo*. The hens were treated as described in Section 2.2. The data are represented as mean ± SE ($n = 3$). $^{*}P < 0.05$, compared with control of brain; $^{\#}P < 0.05$, compared with control of spinal cord.

qualitatively from the autoradiographic patterns of the dried gels. A 300 μM $CaCl_2$ and 1 μg CaM were added to the phosphorylation reaction medium to determine if these chemicals affect the level of protein phosphorylation of brain proteins from the tested hens.

3.3.1. Changes in mitochondrial proteins phosphorylation

A decreased phosphorylation of 35-kDa protein was observed in brain mitochondrial fraction from TOCP- and PMSF-treated hens after seven days of administration, reaching the level of 67% and 66% of the control (Fig. 2, Table 1). In contrast, proteins from hens treated with MET showed no statistically significant changes of phosphorylation in the experiments. Moreover, as shown in Fig. 2 and Table 1, calcium plus CaM did not increase the level of phosphorylation of 35-kDa substrate protein from the hens treated with TOCP and PMSF, only 58% and 62% of the control hens, respectively.

3.3.2. Changes in synaptosomal proteins phosphorylation

Autoradiography of synaptosomal proteins resolved by SDS–PAGE indicated an enhanced phosphorylation of several proteins in response to MET and TOCP, respectively (Fig. 3). As shown in Table 1, the molecular weights (Mr) and maximal phosphorylation (percent of control, $P < 0.05$) for the most prominently affected bands were as follows: MET—32 kDa (144%), TOCP—40 kDa (136%), 55 kDa (165%), while the corresponding band from the other groups exhibited no obvious changes. There was no change in phosphorylation level in the synaptosome of PMSF-treated hens. Furthermore, the phosphorylation of protein mentioned above was catalyzed by a kinase that is dependent on calcium and CaM. In the presence of exogenous calcium and CaM, the level of phosphorylation of

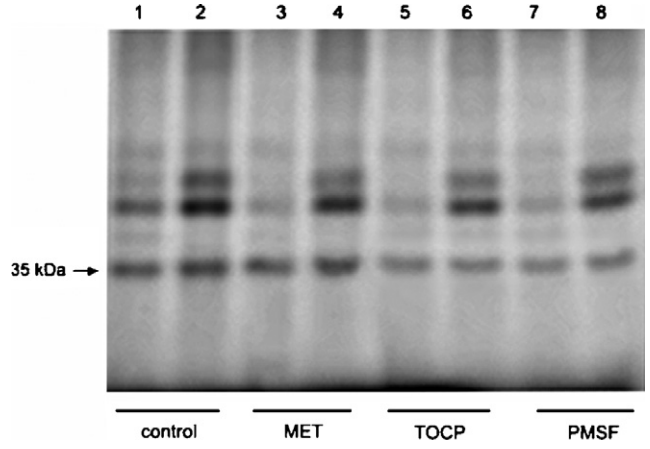

Fig. 2. Phosphorylaiton of endogenous brain mitochondrial proteins from control and treated hens. Autoradiographic files of mitochondrial proteins obtained from the hens sacrificed seven days after administration. Lanes 1, 3, 5, and 7 represent the samples in the absence of calcium and CaM; lanes 2, 4, 6, and 8 are sample obtained in the presence of 300 μM $CaCl_2$ and 1μg CaM. Data are representative of triplicates of three independent experiments.

Table 1
Changes of brain protein phosphorylation in hens treated with organophosphates or PMSF

Subcellular fractions	M_r (kDa)	None added				300 μM $CaCl_2$ + 1μg CaM			
		Control	MET	TOCP	PMSF	Control	MET	TOCP	PMSF
Mitochondria	35	287 ± 46	315 ± 42 (110%)	192 ± 53 (67%)*	189 ± 29 (66%)*	355 ± 39@	373 ± 44 (105%)	205 ± 57 (58%)#	220 ± 33 (62%)#
Synaptosome	32	241 ± 48	348 ± 40 (144%)*	268 ± 16 (111%)	244 ± 35 (101%)	293 ± 24	378 ± 51 (129%)#	284 ± 58 (97%)	265 ± 64 (90%)
	40	250 ± 54	267 ± 19 (107%)	340 ± 35 (136%)*	259 ± 47 (104%)	311 ± 33	338 ± 29 (109%)@	502 ± 63 (161%)#@	299 ± 45 (96%)
	55	108 ± 29	125 ± 64 (116%)	178 ± 51 (165%)*	121 ± 119 (112%)	173 ± 37	145 ± 57 (84%)	208 ± 50 (120%)#	131 ± 121 (76%)

The incorporation of ^{32}P into brain proteins was measured under the standard phosphorylation assay conditions with the indicated cofactors. Values are in arbitrary units, means ± SE of three independent observations. The numbers in parentheses represent the percentage of the control. There was no significant difference in Coomassie blue protein staining between control and the treated. *$P < 0.05$, compared with control added none. #$P < 0.05$, compared with control added calcium and CaM. @$P < 0.05$, compared within the same chemical treated group with none added.

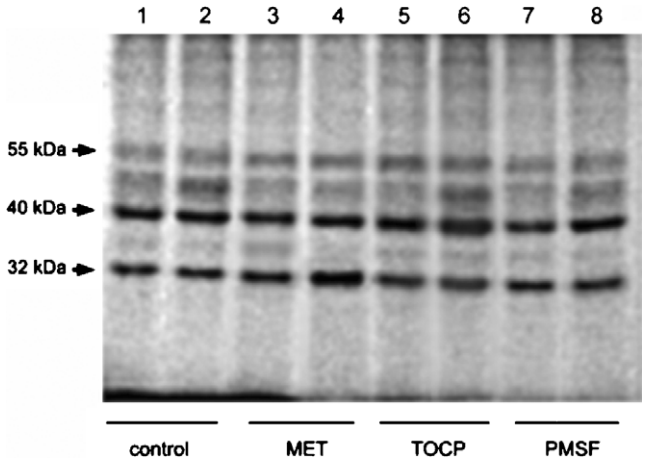

Fig. 3. Phosphorylaiton of endogenous brain synaptosomal proteins from control and treated hens. Autoradiographic files of synaptosomal proteins obtained from the hens sacrificed seven days after administration. Lanes 1, 3, 5 and 7 represent the samples in the absence of calcium and CaM; lanes 2, 4, 6 and 8 represent samples obtained in the presence of 300 μM $CaCl_2$ and 1μg CaM. Data are representative of triplicates of three independent experiments.

32 kDa (MET) and 40, 55 kDa (TOCP) synaptosomal increased to as much as 129%, 161%, and 120%.

4. Discussion

Methamidophos (MET) has been reported to cause delayed neurotoxicity in humans following severe intoxication incidents, but tests of this compound in hens at doses up to twice the LD_{50} with prophylaxis and therapy against acute cholinergic toxicity produced no clinical neuropathy [17]. Therefore, questions arise as to whether there is any special feature of the molecular mechanism of initiation of neuropathy by MET. The purpose of this study was to investigate different changes in the levels of phosphorylated proteins in delayed neurotoxicity induced by MET or TOCP during the early stage.

NTE was identified as the preferred candidate target of OP that cause delayed neurotoxicity over 30 years ago [18]. Here we studied the NTE activity of brain and spinal cord from hens dosed with approximately an LD_{50} MET. The data obtained *in vivo* suggested that MET is a relatively weak inhibitor of NTE compared to a potent NTE inhibitor TOCP and a non-aging NTE inhibitor PMSF. Although impurities could contribute to OPIDN potential [19], MET did not produce 70–80% NTE inhibition in this study, which is considered as threshold value for initiation of OPIDN [1,7]. As such, it suggests that OPIDN may occur through some other mechanism besides the inhibition of NTE.

It is generally recognized that phosphorylation/dephosphorylation of target proteins is involved in nearly all cellular functions, such as proliferation, differentiation, apoptosis, and degeneration [20]. Abou-Donia suggested that Ca^{2+}/CaM-dependent protein kinase may play a pivotal role in the pathogenesis of OPIDN [7]. Autophosphorylation of CaM-K II and protein kinase-mediated phosphorylation of cytoskeletal proteins led to the anomalous accumulation of phosphorylated neurofilament aggregates in the central and peripheral axons of hens treated with TOCP [3].

Although no delayed neurotoxicity was found in the hens treated with MET, our *in vivo* test showed that there are different effects of MET and TOCP on protein phosphorylation of subcellular fractions. In synaptosome fraction, TOCP stimulated the phosphorylation of 40- and 55-kDa proteins while MET seemed only to enhance the phosphorylation of 32-kDa protein. Addition of Ca^{2+} and calmodulin further enhanced the phosphorylation level of these proteins.

It has been proposed that NTE inhibition by OP is associated with an increase in [Ca^{2+}], which, in turn, may lead to aberrant protein kinase-mediated phosphorylation of cytoskeletal proteins and their accumulation [7]. A previous research indicated that MET could inhibit the calcium uptake of brain synaptosomes in hens, while the effect of TOCP on the calcium uptake was dependent on its concentration [21]. This leads to the speculation of proteins found in our study being involved in calcium regulation, which is perhaps one of the reasons why the signs of the neurotoxicity was different in hens exposed to MET and TOCP.

In mitochondrial fraction, only TOCP inhibited the phosphorylation of 35-kDa mitochondrial protein, while MET performed no function on it. These data are consistent with our results from *in vitro* test (unpublished data). Mitochondria are involved directly in cell survival and death. Mitochondrial dysfunction and free radical-induced oxidative damage have been implicated in the pathogenesis of several different neurodegenerative diseases [22–24]. The initial phase of mitochondrial-induced apoptosis in neuronal cell death is triggered by a variety of insults, such as xenobiotics, Parkinsonism-endogenous and exogenous neurotoxins, radiation, oxidative stress, and glucose, and oxygen deprivation [25]. Recent studies in a human neuronal cell line (neuroblastoma SH-SY5Y cell) and primary dorsal root ganglia (DRG) cells suggested that mitochondria are an important early target for OPs, with exposure resulting in depletion of ATP production [8]. Neuronal apoptosis in anterior horn of spinal cord of hens appeared in OPIDN, suggesting that cellular apoptosis may play an important role in the pathogenesis of OPIDN [26]. TOCP is a typical toxicant that can induce delayed neurotoxicity in several species including man; however, while MET can induce delayed neurotoxicity in humans, it does not do so in the hen. Our data suggest that the differential neurotoxicity of these two organophosphorus compounds may be due to the unique effects of MET and TOCP on the protein phosphorylation of mitochondrial proteins. After 7-day administration, TOCP inhibited the phosphorylation of 35-kDa mitochondrial protein, which is perhaps the cause of the progression of the neuronal apoptosis. However, MET had no effect on this protein, which is perhaps one of the reasons that the sign of delayed neurotoxicity are not observed in hens exposed to MET. It has been reported that TOCP induced significant concentration-dependent

mitochondrial hyperpolarization followed by gradual depolarization [27]. Pretreatment with cyclosporin A (500 nM, 30 h), a mitochondrial permeability transition pore (PTP) inhibitor, decreased the hyperpolarization [27]. Hence, we speculate that TOCP might cause changes in mitochondrial phosphoprotein resulting in mitochondria-mediated apoptosis. Further research is required to identify the function and structure of relevant proteins during the development of the delayed neurotoxicity induced by MET.

PMSF was chosen in this experiment because it is the most potent NTE inhibitor available, and has so far been used in most promotion and protection studies. A number of observations led to the hypothesis that promotion of OPIDN involves a target molecule other than NTE [28] and might directly affect the compensation/repair mechanism(s) of the nervous system [29]. Our endogenous protein phosphorylation assays showed that PMSF decreased the phosphorylation level of 35-kDa brain mitochondrial protein in a manner similar to TOCP, yet PMSF had little effects on synaptosomal protein phosphorylation. As such, the data in this study showed that the modification of OPIDN by PMSF might correlate with the phosphorylation of proteins from subcellular fractions.

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