Potentiation of Organophosphorus-Induced Delayed Neurotoxicity Following Phenyl Saligenin Phosphate Exposures in 2-, 5-, and 8-Week-Old Chickens

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Phenylmethylsulfonyl fluoride (PMSF), a nonneuropathic inhibitor of neurotoxic esterase (NTE), is a known potentiator of organophosphorus-induced delayed neurotoxicity (OPIDN). The ability of PMSF posttreatment (90 mg/kg, sc, 4 hr after the last PSP injection) to modify development of delayed neurotoxicity was examined in 2-, 5-, and 8-week-old White Leghorn chickens treated either one, two, or three times (doses separated by 24 hr) with the neuropathic OP compound phenyl saligenin phosphate (PSP, 5 mg/kg, sc). NTE activity was measured in the cervical spinal cord 4 hr after the last PSP treatment. Development of delayed neurotoxicity was measured over a 16-day postexposure period. All PSP-treated groups exhibited >97% NTE inhibition regardless of age or number of OP treatments. Two-week-old birds did not develop clinical signs of neurotoxicity in response to either single or repeated OP treatment regimens nor following subsequent treatment with PMSF. Five-week-old birds were resistant to the clinical effects of a single PSP exposure and were minimally affected by repeated doses. PMSF posttreatment, however, significantly amplified the clinical effects of one, two, or three doses of PSP. A single exposure to PSP induced slight to moderate signs of delayed neurotoxicity in 8-week-old birds with more extensive neurotoxicity being noted following repeated dosing. As with 5-week-old birds, PMSF exacerbated the clinical signs of neurotoxicity when given after one, two, or three doses of PSP in 8-week-old birds. Axonal degeneration studies supported the clinical findings: PMSF posttreatment did not influence the degree of degeneration in 2-week-old chickens but resulted in more severe degeneration (relative to PSP only exposure) in cervical cords from both 5- and 8-week-old birds. The results indicate that PMSF does not alter the progression of delayed neurotoxicity in very young (2 weeks of age) chickens but potentiates PSP-induced delayed neurotoxicity in the presence of 0–3% residual NTE activity in older animals. We conclude that posttreatment with neuropathic or nonneuropathic NTE inhibitors, following virtually complete NTE inhibition by either single or repeated doses of a neuropathic agent in sensitive age groups, can modify both the clinical and morphological indices of delayed neurotoxicity. This study further supports the hypothesis that potentiation of OPIDN occurs through a mechanism unrelated to NTE. © 1997 Society of Toxicology.
provides certain advantages over other commonly used neuropathic OPs. Specifically, PSP does not require metabolic activation nor does it cause significant inhibition of acetylcholinesterase (Jortner and Ehrich, 1987). In the present study, the ability of the nonneuropathic NTE inhibitor phenylmethylsulfonyl fluoride to modify expression of neuropathy following single or repeated exposures to PSP was examined in three age groups (2, 5, and 8 weeks old) of young chickens. We hypothesized that phenylmethylsulfonyl fluoride (PMSF) posttreatment would potentiate delayed neurotoxicity following either single or multiple doses of PSP sufficient to inhibit essentially all NTE activity.

MATERIALS AND METHODS

Materials. Mipafox (N, N'-diisopropyl-diamido-phosphoro fluoride, purity >98%) was purchased from Chemsyn (Lenexa, KS). Paraoxon (O, O'-diethyl-p-nitrophenyl phosphate) was purchased from Aldrich Chemical Company (Milwaukee, WI). Aliquots of paraoxon were further purified prior to assay (Johnson, 1977). Phenyl valerate was synthesized essentially as described by Johnson (1977) and distilled (bp = 70-70.5°C, 0.5 mm Hg). Phenylmethylsulfonyl fluoride (purity >99%) was purchased from Sigma Chemical Company (St. Louis, MO). Cyclic phenyl saligenin phosphate (purity, 97%, as determined by GC-MS) was a generous gift from Dr. Marion Ehrich and was synthesized as described by Jortner and Ehrich (1987). All other chemicals were reagent grade.

Animals and treatments. White Leghorn pullets (C.M. Estes Hatchery, Springfield, MO) were obtained at 1 day of age and maintained at 25°C under a 12-hr light:dark illumination cycle with food (Brood's Best Chick Starter/Grower Crumbles, Mountaire Feeds Inc., North Little Rock, AR) and water available ad libitum. Separate groups of 2-, 5-, and 8-week-old chickens were treated zero, one, two, or three times (treatments separated by 24 hr) with PSP (5 mg/kg, sc, 10 mg/ml in dimethyl sulfoxide, DMSO). Control birds received DMSO only (0.5 ml/kg, sc). Four hours after the last PSP injection the birds were either euthanized and their cervical cords were dissected for NTE determination or they were treated with either glycerol formal (GF, 1 ml/kg, sc) or PMSF (90 mg/kg, sc, 90 mg/ml in GF) and observed for clinical signs of OPIDN over a 16-day period. After observation on Day 16, birds were euthanized by decapitation and the cervical cords were collected in 10% neutral buffered formalin for morphologic analysis.

Neurotoxic esterase assay. Cervical cords were removed and stored at -70°C until time of assay. NTE activity was measured by the method of Johnson (1977) as modified by Pope and Padilla (1989). Briefly, tissues were thawed and weighed, and homogenates (1:10, w/v) were prepared in 50 mM tris(hydroxymethyl)aminomethane buffer (pH 8.0 @ 25°C) containing 0.2 mM EDTA (Tris-EDTA). Homogenates were centrifuged (9000 g for 15 min) and the resulting supernatants were used for NTE determination. Reaction volumes of paired samples contained 50 μl homogenate, 40 μl paraaxon, and 5 μl phenyl valerate (PV, 25 mg/ml in dimethyl formamide) with or without 50 μM mipafox. Tubes containing homogenate and OPS (or vehicle) were preincubated for 20 min at 37°C. Phenyl valerate was then added for a 20-min incubation (37°C). The reaction was stopped by addition of 500 μl of Tris-EDTA buffer containing 1% sodium dodecyl sulfate and 0.025% 4-aminocaproic acid. NTE activity was determined as the difference in hydrolysis of PV in the presence and absence of mipafox. Protein content was measured according to the method of Lowry and co-workers (1951) and NTE activity was reported as nanomoles of PV hydrolyzed/minute/milligram of protein.

Clinical signs of neurotoxicity. All birds were coded on Day 0 such that the observations were performed “blind.” Clinical signs of OPIDN (n = 4-5 birds/group) were measured at 6, 8, 10, 12, 14, and 16 days after PMSF according to the following scale (Roberts et al., 1983). In brief: 0, no signs; 1/2, slight, indefinite signs; 3/4, moderate degree of incoordination during movement; 5/6, severe impairment of ability to stand and walk; 7/8, virtual to complete hindlimb paralysis.

RESULTS

Inhibition of Neurotoxic Esterase Activity

Single doses of PSP (5 mg/kg, sc) caused >97% inhibition of cervical cord NTE 4 hr after treatment in all age groups (Table 1). No additional significant reduction was seen with multiple OP exposures. Extensive NTE inhibition (>95%) was also noted in birds treated with a single dose of PMSF (90 mg/kg, sc). The different treatment schedules caused similar reductions of enzyme activity across the different age groups.

Clinical Signs of OPIDN

No clinical signs of OPIDN were observed in any of the 2-week-old birds regardless of the number of PSP treatments or PMSF posttreatment (data not shown). Five-week-old birds (Figs. 1A-1D) were also resistant to OPIDN after receiving only PSP, but PMSF posttreatment was associated with moderate to severe clinical neurotoxicity in all three PSP-treated groups. Single doses of PSP caused minor signs
of clinical dysfunction in 8-week-old birds (Figs. 2A–2D) with multiple treatments causing more severe effects than a single dose. PMSF exacerbated delayed neurotoxicity in all 8-week-old birds receiving one, two, or three doses of PSP.

Morphology

Degeneration induced by exposure to PSP appeared consistently and selectively in the gracile fasciculus and dorsal spinocerebellar tracts of the cervical spinal cord. In both tracts the silver-impregnated degeneration appeared as fragmented, irregularly arranged, black silver-impregnated fibers on a light brown or yellow background. The density of the degeneration was dependent on the particular treatment to which the chick was exposed (Fig. 3). No axonal degeneration was found in the cervical cords from any control birds (n = 4–5 birds/age group; data not shown). Cords from 2-

### TABLE 1

Inhibition of Neurotoxic Esterase Activity in Cervical Cords of 2-, 5-, and 8-Week-Old Chickens Treated with Single Doses of PMSF (90 mg/kg, sc) or Single or Repeated Doses of PSP (5 mg/kg, sc)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2-week-old birds</th>
<th>5-week-old birds</th>
<th>8-week-old birds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min/mg protein</td>
<td>% control</td>
<td>nmol/min/mg protein</td>
</tr>
<tr>
<td>Control</td>
<td>4 5.00 ± 0.74</td>
<td>100.00 ± 14.87</td>
<td>6 9.14 ± 0.45</td>
</tr>
<tr>
<td>PMSF</td>
<td>4 0.13 ± 0.07</td>
<td>2.70 ± 1.35*</td>
<td>6 0.30 ± 0.04</td>
</tr>
<tr>
<td>PSP × 1</td>
<td>4 0.14 ± 0.02</td>
<td>2.88 ± 0.42*</td>
<td>6 0.15 ± 0.05</td>
</tr>
<tr>
<td>PSP × 2</td>
<td>4 0.04 ± 0.05</td>
<td>0.84 ± 0.95*</td>
<td>5 −0.07 ± 0.06</td>
</tr>
<tr>
<td>PSP × 3</td>
<td>3 0.10 ± 0.14</td>
<td>1.99 ± 2.73*</td>
<td>5 −0.15 ± 0.11</td>
</tr>
</tbody>
</table>

**Note.** Data are expressed as means ± SE.

*Statistically significant at p < 0.05 by one-way analysis of variance and Student–Newman–Keuls multiple comparison.
 FIG. 2. Development of clinical signs of neuropathy in 8-week-old chickens. (A) Ataxia in control birds and birds treated with PMSF (90 mg/kg, sc) only. (B) Compares a single dose of PSP (5 mg/kg, sc) to a single dose of PSP followed 4 hr later by PMSF. (C and D) Compare two or three doses of PSP only to two or three doses of PSP followed by PMSF. Data are shown as median ataxia scores ± interquartile ranges. Asterisks indicate significant effects of PMSF posttreatment. Pound signs indicate significant effects of repeated doses of PSP compared to a single dose.

In the gracile fasciculi of 8-week-old birds, degeneration scores ranged from moderate to heavy depending on the number of PSP exposures, with little difference between the PSP-only groups and their respective PMSF posttreated groups (Fig. 4C). In contrast, the spinocerebellar tracts from 8-week-old birds showed light to moderate degeneration in response to PSP only but moderate to heavy degeneration after PSP followed by PMSF.

Inhibition of NTE is the putative first step in initiation of OPIDN (for reviews see Abou-Donia and Lapadula, 1990; Johnson, 1990; Lotti, 1992; Richardson, 1992). In the present studies, single doses of phenyl saligenin phosphate inhibited NTE activity in cervical spinal cord by at least 97%. Subsequent doses of PSP did not significantly increase enzyme inhibition. In contrast to a report by Moretto and co-workers (1991), we found that spinal cord NTE activity in control birds increased with age (5.00 ± 0.74, 9.14 ± 0.45, and 9.86 ± 0.38 nmol/min/mg protein for 2-, 5-, and 8-week-old birds, respectively). Differences in tissue (cervical vs lumbosacral regions) or assay procedures could account for these contrasting results.

Two-week-old birds were completely resistant to clinical signs of OPIDN following either single or repeated doses of PSP and exhibited only slight degrees of axonal degeneration, even after PMSF posttreatment. Five-week-old birds were relatively resistant to clinical signs of OPIDN after one, two, or three doses of PSP but developed extensive deficits in locomotor function with PMSF posttreatment in all cases. In general, the degree of axonal degeneration correlated with clinical dysfunction. Similar to previous studies...
FIG. 3. Photomicrographs illustrating the different degeneration scores (0–3) noted in the dorsal spinocerebellar tract following administration of PSP and/or PMSF in 8-week-old chickens. (A) The absence of degeneration (degeneration score = 0) following an injection of PMSF only. (B) The light punctate degeneration (degeneration score = 1) present after a single injection of diisopropyl phosphorofluoridate (DFP) as the neuropathic agent (Pope et al., 1993), 8-week-old birds exhibited minimal degrees of clinical dysfunction following a single neuropathic OP exposure. Multiple PSP exposures induced more extensive clinical deficits, however. In addition, PMSF posttreatment amplified the delayed neurotoxicity in 8-week-old chickens initiated by one, two, or three doses of PSP.

In 5-week-old chickens, repeated PSP treatments had little effect on the development of clinical signs of neurotoxicity. These findings agree with a previous study (Pope et al., 1993) reporting minimal effects of repeated DFP exposures. In contrast, relatively similar ataxia scores were noted in 8-week-old birds treated with either (a) a single dose of PSP followed by PMSF or (b) two doses of PSP. These findings suggest that a subsequent exposure to PSP may be capable of potentiating the neurotoxic effects of a prior dose, but only in more mature animals. Collectively, these studies suggest that some neuropathic OPs may have multiple target sites of relevance to OPIDN and may be capable of both initiation and potentiation of delayed neurotoxicity. The resistance to clinical dysfunction following either repeated PSP or PMSF posttreatment in 2-week-old chickens, the resistance to potentiation by repeated OP exposures but sensitivity to potentiation of both clinical and morphological indicators of OPIDN by PMSF posttreatment in 5-week-old chickens, and finally, the greater sensitivity to both repeated OP exposures and PMSF posttreatment in 8-week-old chickens suggest that an age-related gradient exists in the degree of sensitivity to both initiators and potentiators of delayed neurotoxicity.

The results of this study indicate that both the gracile fasciculus and the dorsal spinocerebellar tracts undergo distal axonal degeneration following exposure to PSP. These data are consistent with previous reports indicating that both of these tracts are susceptible to a variety of organophosphorus compounds (Tanaka and Bursian, 1989; Tanaka et al., 1990; Carboni et al., 1992; Dyer et al., 1992). Although both dorsal spinocerebellar and ventral spinocerebellar tracts have been reported in birds (Whitlock, 1952; Karten and Hodos, 1967), the present results indicate that the larger dorsal tract undergoes more severe degeneration after exposure to PSP and PSP/PMSF, a finding similar to that reported in chickens after exposure to tri-ortho-tolyl phosphate (Tanaka and Bursian, 1989) and diisopropylphosphorofluoridate (Tanaka et al., 1990). Previous studies have hypothesized that degeneration of the dorsal spinocerebellar tract, which has been shown in chickens to terminate as mossy fiber afferents in the cerebellar cortex (Okado et al., 1987), may be closely linked to the onset of ataxia (Pope et al., 1993; Funk et al., 1994). The morphological data in the present study further support this hypothesis, as degeneration was more extensive in the larger dorsal tract following exposure to PSP and PSP/PMSF.
study add to the results from these previous investigations by showing that although PMSF posttreatment increases degeneration in both regions of the cervical cord, it appears to produce greater changes (relative to OP exposure only) in the dorsal spinocerebellar tract.

While the morphological data in the present study generally support the clinical findings, some interesting contrasts exist. For example, cords from 2-week-old birds had slight amounts of degeneration but the birds were completely resistant to clinical dysfunction. These results agree with a previous study (Funk et al., 1994) following repeated DFP exposures in 2-week-old chickens. Comparison of similarly treated groups of 5- and 8-week-old birds (Figs. 4B and 4C) indicated very similar degrees of axonal damage but the older birds exhibited more severe clinical signs of delayed neurotoxicity. These observations may indicate that younger animals are not just resistant to the initial neuropathic insult but, because of the developing state of their nervous system, are also more successful at compensating for the damage.

Although the putative target for initiation of OPIDN is neurotoxic esterase, Pope and Padilla (1990) proposed that potentiation of OPIDN by nonneuropathic NTE inhibitors did not involve NTE. The present study supports this hypothesis by demonstrating that PMSF can amplify clinical and morphological indicators of OPIDN in 5-week-old chickens when given after one, two, or three injections of PSP; dosing regimens which cause from 97 to 100% inhibition of NTE. In 8-week-old birds, PMSF posttreatment also exacerbated clinical dysfunction following similar dosing regimens. In addition, a second dose of PSP markedly increased the delayed neurotoxicity of an initial dose of the OP. More recently, Moretto and co-workers (1994) demonstrated potentiation of OPIDN by O-(2-chloro-2,3,3-trifluorocyclobutyl)-O-ethyl S-propyl ester (KBR-2822) at doses which did not significantly affect NTE activity. Collectively, these studies support the hypothesis that another target molecule is involved in potentiation of OPIDN.

In conclusion, we have demonstrated that PMSF can potentiate the delayed neurotoxic effects of PSP in the presence of less than 3% residual NTE activity. The results further support the hypothesis that potentiation of OPIDN involves a target molecule other than NTE.

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