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Protein levels of neurofilament subunits in the hen central nervous system following prevention and potentiation of diisopropyl phosphorofluoridate (DFP)-induced delayed neurotoxicity

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Abstract

Diisopropyl phosphorofluoridate (DFP) is an organophosphorus ester, which produces delayed neurotoxicity (OPIDN) in hens in 7–14 days. OPIDN is characterized by mild ataxia in its initial stages and severe ataxia or paralysis in about 3 weeks. It is marked by distal swollen axons, and exhibits aggregations of neurofilaments (NFs), microtubules, proliferated smooth endoplasmic reticulum, and multivesicular bodies. These aggregations subsequently undergo disintegration, leaving empty varicosities. Previous studies in this laboratory have shown an increased level of medium-molecular weight NF (NF-M) and decreased levels of high- and low-molecular weight NF (NF-H, NF-L) proteins in the spinal cord of DFP-treated hens. The main objective of this investigation was to study the effect of DFP administration on NF subunit levels when OPIDN is prevented or potentiated by pretreatment or post-treatment with phenylmethylsulfonyl fluoride (PMSF), respectively. Hens pretreated or post-treated with PMSF were killed 1, 5, 10, and 20 days after the last treatment. The alteration in NF subunit protein levels observed in DFP-treated hen spinal cords was not observed in protected hens. Estimation of NFs in the potentiation experiments, however, showed a different pattern of alteration in NF subunit levels. The results showed that an alteration in NF subunit levels in DFP-treated hens might be related to the development of OPIDN, since these changes were suppressed in PMSF-protected hens. However, results from PMSF post-treated hen spinal cords suggested that potentiation of OPIDN by PMSF was mediated by a mechanism different from that followed by DFP alone to produce OPIDN.

Keywords: DFP; Spinal cord; PMSF; Cerebrum; Chickens; OPIDN; NF-H; NF-M; NF-L

1. Introduction

DFP is an organophosphorus ester, which produces delayed neurotoxicity (OPIDN) in the hen and other susceptible species [1,2]. Organophosphorus esters are widely used in agriculture and industry as defoliants, fungicides, herbicides, insecticides, industrial fluids, flame retardants, nerve agents, and therapeutic agents [3]. OPIDN is characterized by distal axonopathy in the central and peripheral nervous system of susceptible species. The hen is an animal of choice to study the mechanisms of delayed neurotoxicity, since it is very sensitive to OPIDN. A single injection of DFP (1.7 mg/kg, s.c.) produces mild ataxia in 7–14 days, which progresses to severe ataxia or paralysis in 20 days. Ultrastructural studies show axonal swellings containing aggregations of NFs, microtubules, proliferation of smooth endoplasmic reticulum, and multivesicular vesicles soon after the appearance of clinical signs. This is followed by partial matting and disappearance of NFs from swollen axons [4,5]. PMSF inhibits serine proteinase activity as well as NTE activity, but does not produce OPIDN in treated animals. PMSF has been shown to protect hens from DFP-induced delayed neurotoxicity if injected before the administration of DFP and to potentiate delayed neurotoxicity if injected after the administration of DFP [6–9]. While it is generally thought that PMSF protects hens from DFP-in-

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Abbreviations: BN–PAGE, blue native urea gel electrophoresis; CC, cerebrum; DFP, diisopropyl phosphorofluoridate; ECL, enhanced chemiluminescence; NF, neurofilament; NF-H, high-molecular weight NF; NF-L, low-molecular weight NF; NF-M, medium-molecular weight NF; NTE, neuropathy target esterase; OPIDN, organophosphorus ester-induced delayed neurotoxicity; PMSF, phenylmethylsulfonyl fluoride; and SC, spinal cord.

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duced neurotoxicity by binding with the active site of NTE without undergoing aging, the mechanism of potentiation of OPIDN by PMSF is relatively unclear.

NFs are type IV intermediate filaments that are 10 nm in diameter and many microns in length [10]. NF proteins consist of three subunits: NF-H, NF-M, and NF-L, with molecular masses of 180–200, 130–170, and 60–70 kDa, respectively, as determined by SDS–PAGE. It is well established that neurofilaments are essential for the radial growth of large myelinated axons and that they contribute to the velocity of electrical signal conduction [11]. There is also growing evidence that NFs affect the function of other cytoskeletal proteins such as microtubules and actin by their interaction. Although NFs are primarily neuron-specific, NF-M and NF-L are also expressed in Schwann cells during Wallerian degeneration of axons in the sciatic nerve [12]. NF proteins are synthesized in the cell body and transported down the axons to nerve terminals where they are digested rapidly with proteinases. Recent studies using NFs tagged with green fluorescent protein in cultured nerve cells show that apparent slow axonal transport of NFs is derived from rapid transport of NF proteins interrupted by long pauses [13]. The dynamic nature of NFs is also supported by the presence of Triton X-100-soluble NFs in axons [11].

NFs are formed by the assembly of three subunits (NF-H, NF-M, NF-L) in a substoichiometric ratio. An overexpression or scarcity of any NF subunit results in the alteration of their axonal transport, accumulation in axons or perikarya, and/or degeneration of axons [10,11,14]. Overexpression of NF-M and NF-H also inhibits dendritic arborization, which is alleviated by increasing NF-L expression [15]. These effects of overexpression or scarcity are probably due to the disorganization of NF fibers, which otherwise run in parallel arrays in normal axons. This is supported by the fact that transgenic mice with knockout of all three NF subunits do not show any apparent developmental defect. Other factors such as mutation or abnormal post-translational modification of NF subunits may also lead to their disorganization in axons, which results in axonal degeneration [16]. Accumulation of NFs in the perikarya, proximal axons, or distal axons has been suggested to contribute towards the degeneration of neurons or axons in several diseases [amyotrophic lateral sclerosis (ALS), Lewy-body-type dementia, Parkinson's disease, Alzheimer's disease] [11,17]. Similarly, injury to an axon from a crush or cut [18] and chemically induced neurotoxicity [1,19] also lead to degeneration of axons. Studies on transgenic mice show that the absence of NF-L or NF-M has a more severe effect on radial growth, resulting in axonal atrophy, than does the absence of NF-H [11].

We recently observed enhanced expression of NF-M and decreased expression of NF-H and NF-L in the spinal cord of DFP-treated hens [20]. In the present investigation, we studied whether altered expression of NF subunits in the spinal cord is also observed in DFP-treated hens protected by pretreatment with PMSF. In addition, we determined NF expression in DFP-treated hens post-treated with PMSF. This study was planned to elucidate the role of altered expression of NF subunits in delayed neurotoxicity.

2. Materials and methods

2.1. Materials

DFP, atropine, eserine, PMSF, anti-NF-H (NE-14), anti-NF-M (NN-18), anti-NF-L (NR-4), and horseradish peroxidase (HRP)-conjugated anti-mouse IgG were purchased from the Sigma Chemical Co. Polyvinylidene fluoride (PVDF) and nitrocellulose membranes were purchased from Bio-Rad. Hyperfilms and western blotting detection reagents were purchased from Amersham Pharmacia Biotech. All other chemicals were of the highest quality commercially available.

2.2. Animal treatment

White Leghorn laying hens, 18-months-old and weighing 1.5 to 2.0 kg, were purchased from Featherdown Farm, kept in the animal facility of the Duke University Medical Center for 8 days for acclimatization, and randomly distributed in different groups. The hens were divided into three sets for this investigation, and each set contained 30 hens (6 control and 24 treated hens). All of the hens, control or treated, were injected with atropine (1 mg/kg, normal saline, s.c.) and eserine (1 mg/kg, DMSO, s.c.) 15 min before the administration of PMSF or DFP. The control hens were also administered the vehicle used to dissolve PMSF or DFP. One set was used to determine the protective effect of PMSF on DFP-induced delayed neurotoxicity in hens. Hens in this set were injected with PMSF (30 mg/kg, DMSO, s.c.) 24 hr before the administration of DFP (1.7 mg/kg, propylene glycol, s.c.). The control hens were treated with PMSF alone. The second set contained control hens and those treated with 0.5 mg/kg of DFP. The dose of DFP was reduced in the second set to examine the potentiation effect of PMSF post-treatment in the third set of hens. The control hens for this group were treated only with atropine, eserine, and the vehicle propylene glycol. The third set contained 6 control hens and 24 hens treated with PMSF (120 mg/kg) 24 hr after the administration of DFP (0.5 mg/kg). The control hens in the third set were treated with 120 mg/kg of PMSF alone. The hens were weighed twice a week and examined daily for the clinical signs of delayed neurotoxicity after the last injection of DFP or PMSF. OPIDN was assessed by an 8-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) [8]. DFP-treated and control hens were anesthetized with halothane and killed 1, 5, 10, and 20 days after the last injection. One or two control hens

were killed with each group of treated hens. The tissues, cerebrum, and spinal cord were dissected quickly and frozen in liquid nitrogen before being stored at -80^{o}.

2.3. Preparation of brain and spinal cord particulate fractions

The tissues were homogenized in 2 volumes of homogenizing buffer (10 mM HEPES, pH 6.8, 50 mM NaF, 1 mM EGTA, 1 mM EDTA, 2 mM levamisol, and 1 mM PMSF) in a Potter–Elvehjem homogenizer at 4^{o} and centrifuged at 29,000 *g* for 30 min at 4°. The whole cerebrum and spinal cord were used for this purpose. The pellets were again suspended in the original volume of homogenizing buffer and centrifuged as above. The pellet from the spinal cord was then suspended in the original volumes of the homogenizing buffer and that from the cerebrum in twice the original volume. The particulate fractions were stored at -80^{o}.

2.4. BN–PAGE and immunoblotting

The particulate fraction (50 μg protein) was mixed with half the volume of buffer A (150 mM BisTris buffer, pH 7.0, 6 M urea), left in ice for 30 min, and centrifuged at 29,000 *g* for 20 min at 4°. The final concentration of 2 M urea in solution resulted in the solubilization of NFs from the particulate fraction. This is probably due to the dissociation of intact NFs into the mixture of NF hetero-oligomers [21]. The supernatant was mixed with loading buffer containing 2 M urea and Coomassie Blue G [20,22], and immediately loaded onto 4–10% polyacrylamide gradient gels for electrophoresis. Both stacking (4% acrylamide) and resolving gels were prepared from identical ingredients and contained 2 M urea. Electrophoresis was performed in the cold room at 100 V, as described for BN–PAGE [22]. The separation of NF complexes by BN–PAGE is ascribed to the negative charge conferred upon it by Coomassie Blue G-250 dye. The protein bands were transferred electrophoretically to PVDF membrane and detected immunochemically using primary antibodies against NF subunits, secondary antibody (HRP-conjugated anti-mouse IgG), and ECL reagents (Amersham Pharmacia Biotech) [23]. Anti-NF-H, anti-NF-M, and anti-NF-L were diluted 1:500 and HRP-conjugated anti-mouse IgG was diluted 1:2000. The immunoreactive protein bands were visualized by exposing Hyperfilms to the blots. Autoradiograms were scanned by a Molecular Dynamics Personal Densitometer, and the bands were quantified by the IPLab Gel Analysis program. The values are measured in pixels and are proportional to the protein concentration when bands are compared on the same blot. The Hyperfilms were exposed to the blots for different periods to ensure the reproducibility of results. Each blot (total 20 lanes) contained samples in duplicate from five control and five DFP-treated hens. Thus, samples from each time period were compared with the control hen samples on separate blots.

2.5. SDS–PAGE and immunoblotting

The particulate fractions from spinal cord and cerebrum, without extraction with buffer containing 2 M urea, were subjected to SDS–PAGE on 4% stacking and 7.5% resolving gels [24]. The protein bands were transferred electrophoretically to PVDF or nitrocellulose membranes and quantified as described above. Each blot contained samples in duplicate from five control and five DFP-treated hens.

2.6. Statistical analysis

The results obtained by quantification of bands on autoradiograms were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test and by Student's unpaired two-tailed *t*-test. The value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Clinical signs

Hens pretreated with PMSF did not show any signs of delayed neurotoxicity following treatment with a single dose of DFP (1.7 mg/kg). Thus, pretreatment with 30 mg/kg of PMSF completely prevented hens from developing clinical signs of delayed neurotoxicity upon treating with a single dose of DFP (1.7 mg/kg). Another set of hens, treated with a lower dose of DFP (0.5 mg/kg), produced only mild ataxia (an ataxia score of ~3) by the end of 20 days [25]. In contrast, treatment of hens with this lower dose of DFP followed by PMSF (120 mg/kg) produced a significant increase (an ataxia score of 7 vs 3) in OPIDN [25].

3.2. Specificity of antibodies

Anti-NF-M (NN-18), anti-NF-H (NE-14), and anti-NF-L (NR-4) were used to study alterations in NF-M, NF-H, and NF-L concentrations, respectively, in the spinal cord and cerebrum of control and treated hens. Anti-NF-M, anti-NF-H, and anti-NF-L did not cross-react with each other. The specificity of these antibodies has been examined in our previous work [20].

3.3. NF-M subunit in spinal cord and cerebrum particulate fractions

Previous studies from this laboratory have shown an increased (36–68%) level of NF-M in the 2 M urea extract of the spinal cord particulate fraction in DFP-treated (1.7 mg/kg) hens [20]. The results from these experiments could show the role of enhanced NF-M level in delayed neuro-

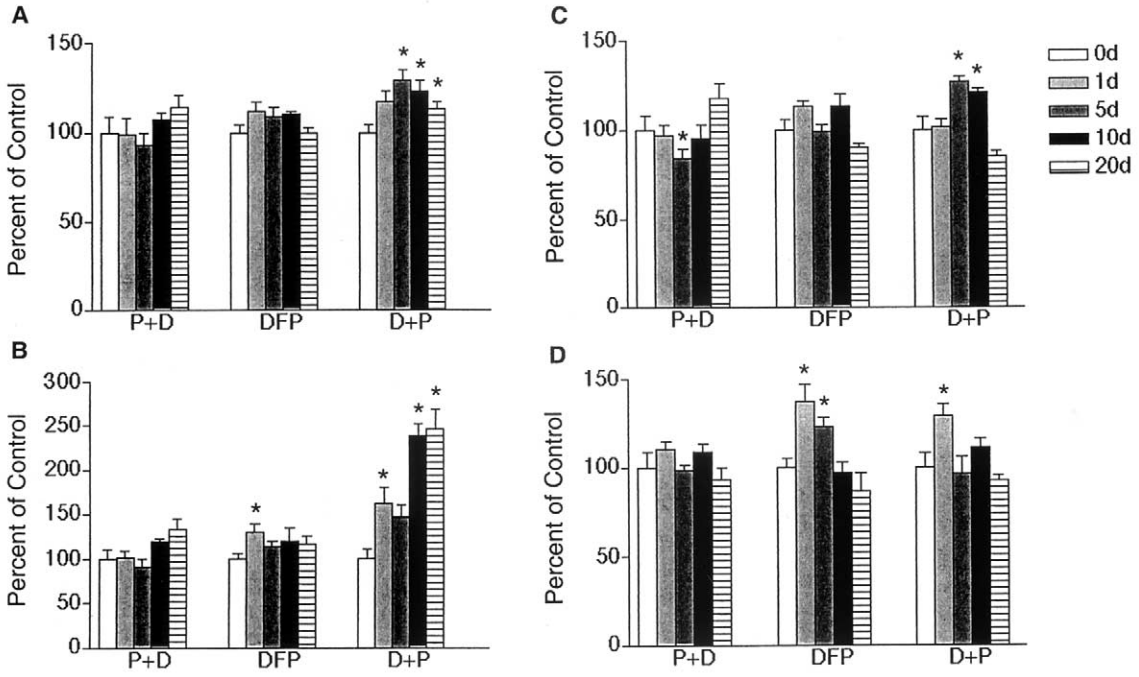

Fig. 1. Histograms of NF-M expression in spinal cord and cerebrum particulate fractions. PMSF was injected before DFP (P + D) to protect hens and after DFP (D + P) to potentiate DFP-induced (0.5 mg/kg) delayed neurotoxicity in hens, as described in "Materials and methods." The hens were treated with 0.5 mg/kg in the potentiation experiments and 1.7 mg/kg in the protection experiments. The hens were also treated with DFP (0.5 mg/kg) alone to compare the effect of PMSF post-treatment on DFP-induced delayed neurotoxicity. The hens were killed 1, 5, 10, and 20 days after the last treatment, and spinal cord (SC) (A, C) and cerebrum (CC) (B, D) were used for BN–PAGE (A, B) and SDS–PAGE (C, D) The separated NF-M complexes were estimated by immunostaining with anti-NF-M, secondary antibody, and ECL reagents. All of the immunoreactive bands against an antibody were scanned together to determine the concentration of that protein. The results are presented as a percentage of the control values, and represent means ± SEM from 4 to 5 hens. Asterisks indicate a significant difference ($P < 0.05$) from the control value of 100%. The blots were exposed for different times to ensure the reproducibility of results. There was an increase in NF-M expression at certain time points in SC and CC particulate fractions upon potentiation of delayed neurotoxicity and upon treatment with DFP alone.

toxicity. DFP-treated (1.7 mg/kg) hens pretreated with PMSF did not show any change in the level of the NF-M subunit in the spinal cord (Fig. 1A). The presence of several anti-NF-M immunoreactive protein bands [Fig. 2, SC (D + P)] has been observed previously in our laboratory [20] as well as by other investigators [22]. These multiple bands are derived from the interaction of NF-M with NF-L or some other cytoskeletal proteins [20,22]. We also carried out NF-M estimation in the cerebrum of protected hens. The cerebrum is resistant to axonal degeneration in DFP-induced OPIDN, whereas the spinal cord is very sensitive to axonal degeneration. The cerebrum also did not show any change in the NF-M level in protected hens (Fig. 1B).

The NF-M level was also estimated in the spinal cord particulate fraction of hens in which DFP-induced (0.5 mg/kg) delayed neurotoxicity was potentiated by post-treatment with PMSF. There was a moderate increase (13–29%) in the NF-M in the spinal cord particulate fractions after 5, 10, and 20 days of treatment [Figs. 1A and 2, SC (D + P)]. DFP (0.5 mg/kg) treatment alone did not show any alteration in NF-M expression in the spinal cord particulate fractions. NF-M estimation in the cerebrum particulate fractions from hens with potentiated delayed neurotoxicity showed an increase in NF-M after 1 day (162 ± 18% expression), 10 days (238 ± 14% expression), and 20 days (246 ± 23%) of treatment. The expression of NF-M after 5 days was 147 ± 14% of control samples, but it was not statistically significant. The increase after 5 days might become significant with a larger number of hens. DFP (0.5 mg/kg) treatment alone showed an increase in the NF-M in cerebrum fractions only after 1 day (130 ± 10% expression) (Figs. 1B and 2).

We also measured NF-M in particulate fractions solubilized in SDS solution (SDS–PAGE). NF-M was decreased after 5 days (84 ± 5% expression) in the spinal cord particulate fraction from protected hens [Figs. 1C and 3, SC (P + D)], but there was no change in the cerebrum particulate fraction from protected hens (Fig. 1C). In the case of PMSF post-treated hens, NF-M was increased after 5 days (127 ± 3% expression) and 10 days (121 ± 2% expression) of treatment in the spinal cord particulate fractions [Figs. 1C and 3, SC (D + P)] and after 1 day (129 ± 7% expression) in the cerebrum fractions. The cerebrum, however, also showed induction of NF-M after 1 day (137 ± 10% expression) and 5 days (123 ± 5% expression) in hens treated with DFP (0.5 mg/kg) alone [Figs. 1D and 3, CC (DFP)].

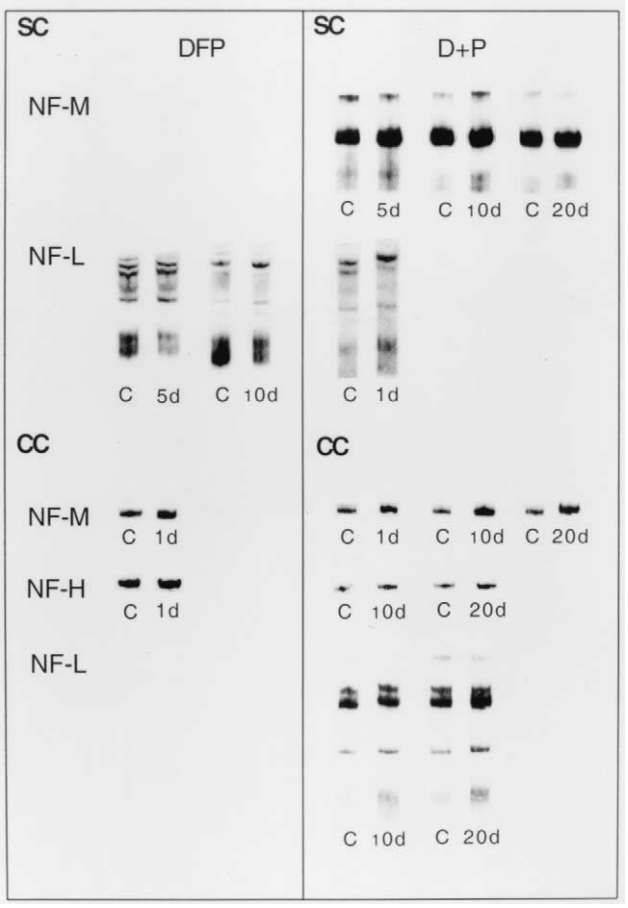

Fig. 2. Expression of NF subunits in 2 M urea extracts of spinal cord and cerebrum particulate fractions. Hens were treated as described in the legend to Fig. 1, and killed 1, 5, 10, and 20 days after the last treatment. Spinal cord (SC) and cerebrum (CC) particulate fractions were used for BN–PAGE. The separated NF complexes were estimated by immunostaining with primary antibody (anti-NF-M, anti-NF-H, or anti NF-L), secondary antibody, and ECL reagents. Each blot contained five samples (10 lanes) from control hens and five samples (10 lanes) from treated hens. Thus, the control samples were compared separately for each time point with samples from treated hens. The figure shows protein bands seen upon immunostaining with each antibody. DFP, treatment with DFP (0.5 mg/kg) alone; C, control sample; d, post-treatment days; NF-M, bands seen with anti-NF-M; NF-H, bands with anti-NF-H; NF-L, bands with anti-NF-L. The representative figure shows proteins, which exhibited alteration in expression under some conditions. Some blots were overexposed for this figure.

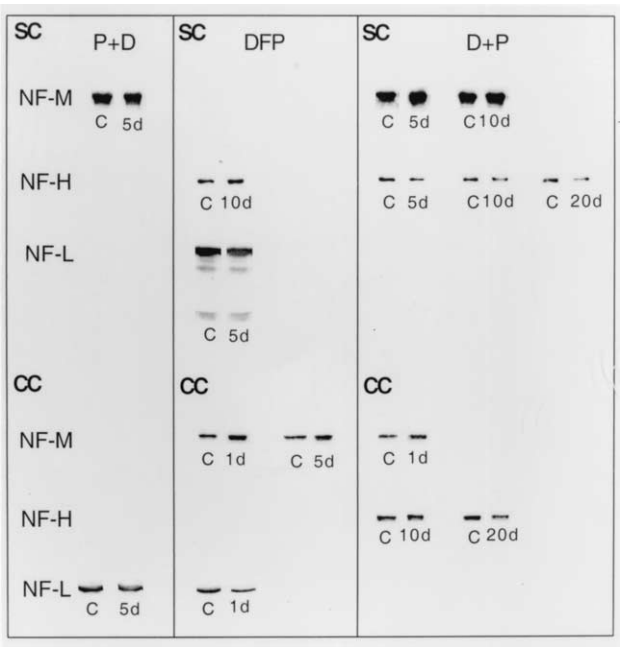

Fig. 3. Expression of NF subunits in SDS extracts of spinal cord and cerebrum particulate fractions. Hens were treated as described in the legend to Fig. 1, and killed 1, 5, 10, and 20 days after the last treatment. Spinal cord (SC) and cerebrum (CC) particulate fractions were used for SDS–PAGE. The NF subunits were estimated by immunostaining with primary antibody (anti-NF-M, anti-NF-H, or anti NF-L), secondary antibody, and ECL reagents. Each blot contained five samples (10 lanes) from control hens and five samples (10 lanes) from treated hens. Thus, control samples were compared separately for each time point with samples from treated hens. DFP, treatment with DFP (0.5 mg/kg) alone; C, control sample; d, post-treatment days; NF-M, bands with anti-NF-M; NF-H, bands with anti-NF-H; NF-L, bands with anti-NF-L. The representative figure shows proteins, which exhibited alteration in expression under some conditions. Some blots were overexposed for this figure.

3.4. NF-H subunit in spinal cord and cerebrum particulate fractions

We previously observed a 39–66% decrease in NF-H expression in spinal cord particulate fractions in DFP-treated (1.7 mg/kg) hens [20]. There was no change in the NF-H concentration in spinal cord or cerebrum particulate fractions from PMSF-protected hens [Fig. 4 (P + D)]. Similarly, there was no alteration in NF-H in the spinal cord upon potentiation of OPIDN by post-treatment with PMSF [Fig. 4A (P + D)]. In contrast, cerebrum from the PMSF post-treated hens showed enhanced expression of NF-H after 10 days (212 ± 26% expression) and 20 days (181 ± 28% expression) [Fig. 4B (D + P)]. In addition, cerebrum particulate fractions also showed increased expression of NF-H after 1 day (130 ± 8% expression) in hens treated with DFP alone (Figs. 2, CC (DFP), and 4B).

We also measured NF-H in particulate fractions solubilized in SDS solution (SDS–PAGE). There was no change in the NF-H concentration in spinal cord or cerebrum particulate fractions from PMSF-protected hens (Fig. 4, C and D). Potentiation of delayed neurotoxicity by PMSF showed decreased expression of NF-H after 5 days (51 ± 7% expression), 10 days (54 ± 10% expression), and 20 days (33 ± 7% expression) of treatment in the spinal cord particulate fraction (Figs. 3, SC (D + P), and 4C). In contrast, cerebrum from the PMSF post-treated hens showed decreased expression of NF-H after 10 days (78 ± 3% expression) and 20 days (64 ± 7% expression) of treatment [Figs. 3, CC (D + P), and 4D]. The spinal cord fraction from hens treated with DFP (0.5 mg/kg) alone showed enhanced expression of NF-H after 10 days (127 ± 9% expression) (Fig. 4C).

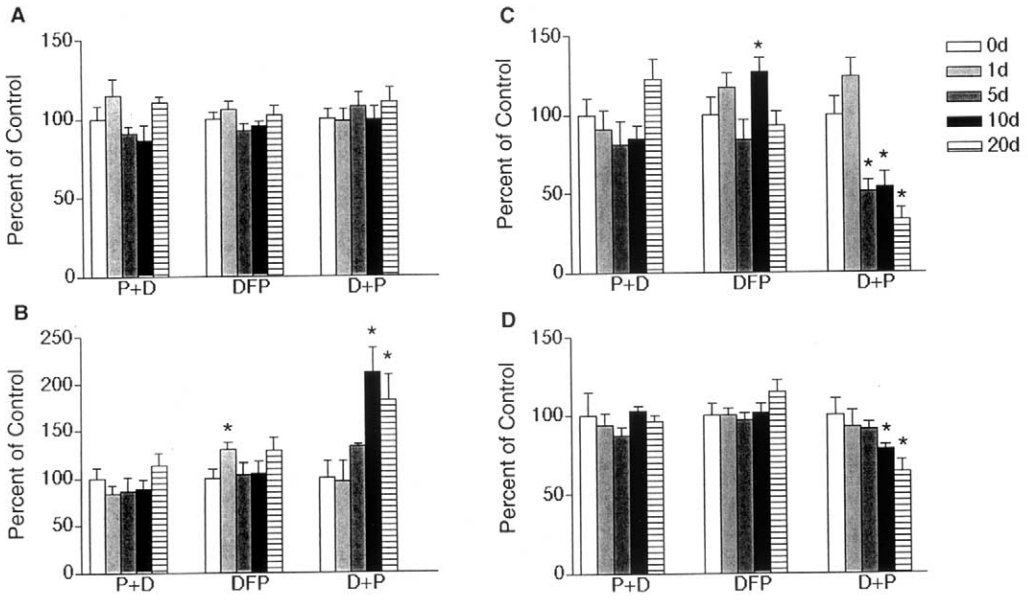

Fig. 4. Histograms of NF-H expression in spinal cord and cerebrum particulate fractions. Hens were treated as described in the legend to Fig. 1, and killed 1, 5, 10, and 20 days after the last treatment. Spinal cord (SC) (A, C) and cerebrum (CC) (B, D) particulate fractions were used for BN–PAGE (A, B) and SDS–PAGE (C, D). NF-H complexes were estimated by immunostaining with anti-NF-H, secondary antibody, and ECL reagents. The results are presented as a percentage of the control values and represent means ± SEM from 4 to 5 hens. Asterisks indicate a significant difference ($P < 0.05$) from the control value of 100%. The blots were exposed for different times to ensure the reproducibility of results. There was an increase in NF-H expression in CC (B) particulate fractions at certain time points upon potentiation of delayed neurotoxicity and treatment with DFP alone. In contrast, SDS–PAGE showed a decrease in NF-H expression in SC (C) and CC (D) at certain time points upon potentiation of delayed neurotoxicity. In addition, an increase was also observed in SC (C) after 10 days of treatment with DFP alone.

3.5. NF-L subunit in spinal cord and cerebrum particulate fractions

We previously observed 35–73% decreased expression of NF-L in spinal cord particulate fractions in DFP-treated (1.7 mg/kg) hens [20]. NF-L expression did not change in the spinal cord or cerebrum particulate fractions of hens protected by pretreatment with PMSF (Fig. 5, A and B). The presence of several anti-NF-L immunoreactive protein bands (Fig. 2) has been observed previously in our laboratory [20] and by other investigators [22]. These multiple bands are derived from the interaction of NF-L with the other two NF subunits or some other cytoskeletal proteins [20,22]. Delayed neurotoxicity potentiated by post-treatment with PMSF showed increased expression of NF-L after 1 day (134 ± 9% expression) of treatment (Fig. 5A). In contrast, treatment with DFP alone (0.5 mg/kg) decreased NF-L expression in the spinal cord fractions after 5 days (81 ± 5% expression) and 10 days (74 ± 8% expression) of treatment [Figs. 2, SC (DFP), and 5A]. The cerebrum particulate fraction from the PMSF-potentiated hens showed increased NF-L expression after 10 days (135 ± 6% expression) and 20 days (135 ± 10% expression) of treatment [Figs. 2, CC (D + P), and 5B].

NF-L was also measured in particulate fractions solubilized in SDS solution (SDS–PAGE). There was no change in the NF-L concentration in the spinal cord, but cerebrum particulate fractions exhibited a decrease in NF-L concentration after 5 days (73 ± 7% expression) in PMSF-protected hens (Figs. 3, CC (P + D), and 5D). Potentiation of delayed neurotoxicity by PMSF showed no change in NF-L concentration, but DFP treatment alone decreased NF-L concentration in the spinal cord particulate fraction after 5 days (78 ± 2% expression) [Figs. 3 SC (DFP), and 5C] and in the cerebrum fraction after 1 day (85 ± 3% expression) [Figs. 3, CC (DFP), and 5D].

4. Discussion

A single dose of DFP (1.7 mg/kg), which produces delayed neurotoxicity in hens, increased NF-M (36%) and decreased NF-L (55%) and NF-H (39%) protein levels in 24 hr in the 2 M urea extract of spinal cord particulate fractions [20]. This altered expression of NF subunits (36–68% NF-M, 35–73% NF-L, 39–66% NF-H) persisted for 10–20 days after DFP administration. In contrast, the cerebrum, which is resistant to axonal degeneration in DFP-treated hens, did not show a change in NF subunits immediately after DFP (1.7 mg/kg) treatment [20]. In the current study, we determined the effect of PMSF pretreatment and post-treatment on the expression of NF subunit protein levels. PMSF pretreatment protects hen from developing DFP-induced delayed neurotoxicity and post-treatment potentiates the development of delayed neurotoxicity [6,8]. We hypothesized that if alteration in the expression of NF sub-

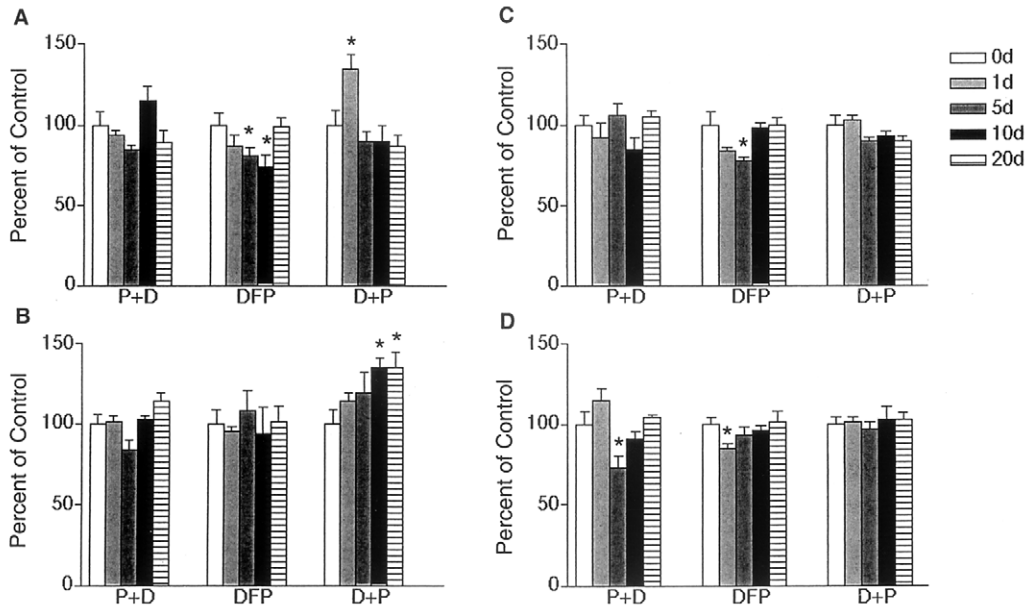

Fig. 5. Histograms of NF-L expression in spinal cord and cerebrum particulate fractions. Hens were treated as described in the legend to Fig. 1, and killed 1, 5, 10, and 20 days after the last treatment. Spinal cord (SC) (A, C) and cerebrum (CC) (B, D) particulate fractions were used for BN–PAGE (A, B) and SDS–PAGE (C, D). The separated NF-L complexes were estimated by immunostaining with anti-NF-L, secondary antibody, and ECL reagents. All of the immunoreactive bands against an antibody were scanned together to determine the concentration of that protein. The results are presented as a percentage of the control values and represent means ± SEM from 4 to 5 hens. Asterisks indicate a significant difference ($P < 0.05$) from the control value of 100%. The blots were exposed for different times to ensure the reproducibility of results. There was an increase in NF-L expression at certain time points in the SC (A) and CC (B) particulate fractions upon potentiation of delayed neurotoxicity, while a decrease was observed at certain time points in SC (A) upon treatment with DFP alone. SDS–PAGE showed a decrease in NF-L expression at one time point in SC (C) and CC (D) upon treatment with DFP alone and in CC (D) upon protection of delayed neurotoxicity by PMSF.

units in the spinal cord was related to axonal degeneration in DFP-treated hens, this alteration would not occur in the spinal cord of PMSF-protected hens.

A single dose of PMSF (30 mg/kg) injected 24 hr before DFP (1.7 mg/kg) administration completely prevented the development of delayed neurotoxicity in hens during the period of observation, 20 days. In contrast to the NF-M increase (36–68%) in DFP-treated (1.7 mg/kg) hens [20], the PMSF-protected hens did not show a change in the expression of NF-M in the 2 M urea extract of the spinal cord particulate fraction (Fig. 1A). In our previous studies, we also observed a decrease in NF-H (39–66%) and NF-L (35–73%) protein levels in 2 M urea extracts of DFP-treated hen spinal cords [20]. This decrease in NF-H and NF-L levels did not appear in the spinal cords when hens were protected from delayed neurotoxicity by PMSF pretreatment (Figs. 4A and 5A). In the case of 2 M urea extracts of the cerebrum, there was no alteration in the levels of NF-H or NF-L whether the hens were treated with DFP (1.7 mg/kg) [20] alone or pretreated with PMSF (Figs. 4B and 5B). The altered expression of the NF subunits in the spinal cords of DFP-treated (1.7 mg/kg) hens, but not in those of protected hens suggested that altered expression of NF subunits may be playing a role in the development of delayed neurotoxicity in hens at least by DFP. There was, however, an increase in cerebrum NF-M (130 ± 10% expression) and NF-H (130 ± 8% expression) 1 day after administration of a low dose of DFP, which was not seen upon treatment with 1.7 mg/kg [20]. At present, we are unable to explain why more changes are found in the expression of NFs in the cerebrum after administration of a low dose (0.5 mg/kg) but not a higher dose (1.7 mg/kg) of DFP [20].

Besides protecting DFP-treated hens by PMSF pretreatment, we also potentiated DFP-induced delayed neurotoxicity by PMSF post-treatment. In these experiments, we used a low dose of DFP (0.5 mg/kg), which produced very mild ataxia (2.8 score in an 8-point graded scale) [25] by the end of a 20-day period of observation. The hens treated with a low dose of DFP were subsequently treated with PMSF to potentiate delayed neurotoxicity. The low dose of DFP (0.5 mg/kg) did not change NF-M expression in the spinal cords of hens compared with those in the control hens (Fig. 1A). The potentiation of delayed neurotoxicity by PMSF produced only a moderate increase (13–29%) in the NF-M level from 5 to 20 days of PMSF post-treatment (Fig. 1A). These results were in contrast to the 36–68% increase in NF-M expression observed from 1 through 20 days of administration of DFP alone [20]. Furthermore, PMSF post-treatment induced NF-M expression not only in the spinal cord, but also in the cerebrum after 1 (162 ± 18%), 10 (238 ± 14%), and 20 (246 ± 23%) days of PMSF treatment. DFP (1.7 mg/kg) treatment alone, which produces delayed neurotoxicity in hens, did not show any alteration in the expression of NF-M or other NF subunits in the cere-

brum [20] in 2 M urea solution. The results on NF-M expression in the 2 M urea extracts of spinal cord and cerebrum in DFP-induced [20] and PMSF-potentiated delayed neurotoxicity suggested that DFP treatment and PMSF post-treatment produced delayed neurotoxicity in the hen by different mechanisms.

A discrepancy was also observed upon comparing NF-H expression in 2 M urea extracts of the spinal cord of DFP-treated and PMSF post-treated hens. There was no change in NF-H protein level in the spinal cords of PMSF post-treated hens compared with those of control hens (Fig. 4A). In contrast, the DFP (1.7 mg/kg) treatment alone showed a 39–66% decrease in the NF-H level from 1 to 10 days after DFP administration [20]. The results from NF-L expression in 2 M urea extracts of spinal cords from DFP-treated hens [20] and from hens with PMSF-potentiated delayed neurotoxicity also showed distinct differences. There was a 35–73% decrease in NF-L expression in 2 M urea extracts of spinal cords of DFP-treated hens from 1 though 20 days [20]. In contrast, PMSF post-treatment showed an increase in NF-L expression in 2 M urea extracts of spinal cords after 1 day (Fig. 5A). PMSF post-treatment, however, also showed an increase in NF-L expression in 2 M urea extracts of cerebrum after 10 and 20 days. Thus, results on the expression of all three NF subunits in 2 M urea extracts of spinal cords from DFP-treated hens and PMSF post-treated hens suggested that delayed neurotoxicity is probably produced by different mechanisms under these two conditions.

The total amount of NF subunits expressed in the spinal cord and cerebrum of PMSF pretreated and post-treated hens was also estimated. The pattern of expression of total NF subunits in spinal cord as well as cerebrum was different from that of NFs soluble in 2 M urea solution at some time points. Similar differences were also noted in these two methods of estimating NF subunits while determining NF subunit expression in samples from DFP-treated hen spinal cord and cerebrum [20]. These differences in results from a 2 M urea solution of NF subunits and their total amount in particulate fractions may be ascribed to two pools of NFs in axons. One pool of NFs is suggested to be moving along the slow axonal transport, while the other pool is almost stationary in axons [26]. However, the conclusions drawn from the estimation of NF subunits in 2 M urea extracts of spinal cord and cerebrum particulate fractions were also supported by estimating total NF subunits in particulate fractions.

The potentiation of DFP- or other organophosphorus compound-induced delayed neurotoxicity by PMSF has been studied by several investigators [7,8]. *In vitro* studies showed that PMSF and *n*-butanesulfonyl fluoride (BuSF), which also potentiates delayed neurotoxicity, inhibited most of the carboxylesterase (PVase) activity that was resistant to 40 μM paraoxon and 250 μM mipafox [27,28]. On the basis of these experiments, it was suggested that the target for the promotional effect of PMSF was probably not the NTE enzyme [27]. NTE activity is defined as the PVase activity resistant to 40 μM paraoxon and sensitive to 250 μM mipafox. NTE enzyme has been suggested to be the target enzyme for DFP-induced delayed neurotoxicity, although its biological functions and role in development of OPIDN are still unclear. Milatovic *et al.* [28] also suggested that NTE was not the target for the potentiation effect of PMSF. This was based upon the finding that d-(+)-methamidophos potentiated delayed neurotoxicity like PMSF and BuSF, but did not inhibit PVase activity, which was resistant to 250 μM mipafox. Studies in both of these laboratories suggested that PMSF potentiated delayed neurotoxicity by a mechanism different from that followed by DFP.

It is reasonable to accept on the basis of the findings in this study and those carried out in other laboratories [27–29] that PMSF potentiates DFP-induced delayed neurotoxicity by a mechanism different from that followed by DFP alone for the induction of delayed neurotoxicity. On the other hand, alteration in the expression of NF subunits in DFP-treated hens [20] and not in protected hens suggested the possible involvement of NF subunits in the development of neurotoxicity. However, it is also feasible that there is some other axonal cytoskeletal protein involved in the development of delayed neurotoxicity by DFP in hens, whose expression is altered by DFP treatment and PMSF post-treatment, but not by PMSF pretreatment. It should be noted that axonal degeneration has also been observed in transgenic species that did not have NFs in their axons [30].

In this study, we examined the effect of PMSF pretreatment and post-treatment on DFP-induced delayed neurotoxicity. The pattern of expression of NF subunits in protected hens suggested the involvement of NFs in delayed neurotoxicity, whereas that in PMSF post-treated hens indicated the involvement of some other pathway in the potentiation of DFP-induced delayed neurotoxicity by PMSF in hens.

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