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Original Article

Delayed Neurotoxicity of Diisopropylfluorophosphate (DFP): Autoradiographic Localization of High-affinity [^{3}H]DFP Binding Sites in the Chicken Spinal Cord.

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Abstract

The delayed neurotoxic organophosphate, diisopropylfluorophosphate (DFP) binds with high affinity to membrane-bound proteins from chicken nerve tissues. The autoradiographic distribution of [^{3}H]DFP binding sites in spinal cord sections of chicken showed higher concentrations of binding sites in gray matter than in white matter. In the cervical region, fairly high densities of [^{3}H]DFP binding sites were found in laminae X and to a lesser extent, in the ventral horn gray matter. To identify the membrane-associated DFP-binding proteins, detergent-solubilized membranes were labeled with 5-10nM [^{3}H]DFP (10pmol/mg protein) for 70 min at 37 ℃. Gel-exclusion chromatography of the [^{3}H]DFP-radiolabeled membranes indicated at least two major radioactive proteins with apparent molecular weights of 150-670 kDa and 40-129 kDa. Although we could not identify the high affinity DFP binding proteins, the autoradiographic experiments clearly demonstrated that the DFP binding proteins localized on gray matter of chicken spinal cord.

Key words: OPIDN, diisopropylfluorophosphate, organophosphate, autoradiographs of [^{3}H]DFP. chicken

Introduction

Several organophosphorus compounds (OPs), i.e., tri-o-cresyl phosphate (TOCP), leptophos, diisopropylfluorophosphate (DFP) and triphenyl phosphite, induce delayed neuropathy (OP-induced DN,OPIDN) in humans, hens, cats and other sensitive species[1,2,3]. In hens, the neuropathic signs appear 1-2 weeks after acute exposure to leptophos, TOCP and DFP. It has been proposed that an inhibition of neuropathy target esterase (NTE) is involved in the initiating stage of OPIDN[4]. It has been also suggested that Ca^{2+}/calmodulin-dependent kinase II phosphorylation plays a role in the pathogenesis of OPIDN[1,5,6]. It is clear that inhibition of NTE's esterase activity alone is not a sufficient signal to initiate OPIDN. As we previously reported, DFP binds to the chicken spinal cord membrane proteins at very low concentrations (in the nanomolar range) *in vitro,* and the binding sites apparently differ from the active site of acetylcholinesterase, butyrylcholinesterase, and NTE, as well as from muscarinic acetylcholine receptors[7]. Although the involvement of the "high-affinity" DFP binding proteins with OPIDN is still unclear, the elucidation of the structure and subcellar distribution of the proteins are of interest for neurotoxicology.

The aim of the present study is to clarify the autoradiographic distribution of high affinity [^{3}H]DFP binding sites in spinal cord sections and to find a biochemical purification method for identifying the [^{3}H]DFP-radiolabeled membrane protiens.

Materials and Methods

Chemicals. [1,3-^{3}H] diisopropylfluorophosphate (specific activity: 310.8Gbq/mmol, 8.4 Ci/mmol) was purchased from DuPont/NEN Research Products (Boston, MA, USA). Unlabeled DFP was obtained from Wako Pure Chemical Industries (Osaka, Japan). Eserine was obtained from Sigma Chemical Co. (St.Louis, MO, USA).

3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was from Nacalai Tesque Inc. (Kyoto, Japan). Sephadex G-25 and Ampholines were purchased from Pharmacia (Uppsala, Sweden). Electrophoresis standards and markers for size exclusion chromatography were obtained from Bio-Rad Laboratories (Hercules,CA, USA). All other chemicals were obtained from commercial sources.

Animals and tissues. Chicken nerve tissues for the binding

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assay and autradiography were obtained from laying hens (*Gallus gallus domesticus*), aged 23 months and weighing 1.4 to 1.8kg. The chicken spinal cords for biochemical purification experiments were obtained from a local slaughterhouse.

Preparation of nerve tissue membranes. Nerve tissue membranes from four control chickens were prepared as described[7]. Briefly, nerve tissues including the cerebrum, cerebellum, brain stem, spinal cord and sciatic nerve were homogenized in 10 vol of HEPES buffer (50mM, pH 7.4). The homogenates were then clarified by centrifugation at 1,000g for 10 min at 4 ℃. The supernatants were combined and centrifuged again at 50,000g for 10 min at 4 ℃. The pellet was resuspended in HEPES buffer, and sedimented by centrifugation once again at 50,000g for 10 min. The washed pellet was suspended in HEPES buffer and dispersed with a Polytron homogenizer. These membrane preparations were stored at -80 ℃ until use .

[^{3}H]DFP binding assays. To measure the high-affinity binding of [^{3}H]DFP, 50 μL of [^{3}H]DFP (final concentration of 0.5nM, 9,320dpm/ml) and 100 μL of 10 μM unlabeled DFP or 100 μL of 10mM phosphate buffer, pH 7.4 were incubated with 100 μL (50-300 μg protein/100 μL) of membrane preparation at 37 ℃ for 60 min. The incubation was terminated by rapid vacuum filtration through a glass-fiber filter (Whatman GF/B) followed by three 3-mL washes with ice-cold buffer (140 mM NaCl, 10mM Tris-HCl, pH 7.4). Radioactivity trapped on the filter was measured in 5mL of Aquasol-2 (DuPont, NEN, USA) by liquid scintillation counting (model 3500, Aloka, Tokyo, Japan). High-affinity binding was defined as the amount of radioactivity bound after subtraction of the non-specific binding assayed in the presence of unlabeled DFP (10 μM).

Autoradiography of spinal cord sections. Chickens were sacrificed by a 200V electric shock. The spinal cords were removed, rapidly snap-frozen in 2-methyl-butane at -40 ℃, mounted on cryostat chucks and cut into 20-μm thick sections at -18 ℃. Sections were thaw-mounted on precleaned gelatincoated slides, dried overnight in a desiccator at 4 ℃, then stored at -80 ℃ until use. Spinal cord sections were incubated for 90 min at 25 ℃ in 50mM HEPES, pH 7.4 containing 15 nM [^{3}H]DFP only or 15 nM [^{3}H]DFP and 100nM eserine. Non-specific labeling was determined in the presence of 10 μM DFP. These incubation conditions have been demonstrated to be optimal for the characterization of the high-affinity [^{3}H]DFP binding sites in nerve tissues[7]. It was also previously confirmed that more than 70% of total binding should correspond to high-affinity binding at the concentration used in this study[8]. At the end of the incubation period, slides were transferred through 4 rinses (1 min each) of 10mM Tris-HCl, pH 7.4 at 4 ℃ followed by a rapid dip in cold deionized water. Incubated slides were rapidly dried under a stream of cold air, juxtaposed tightly against tritium-sensitive film (Hyperfilm, Amersham, Buckinghamshire, England) and stored at room temperature for 20 weeks. At the end of the exposure period, the film was developed using a Fuji Film (Tokyo, Japan) HI-RENDOL I developer for 5 min, rinsed in a stop bath for 1 min and fixed with a Fuji Film HI-RENFIX I fixer for another 5 min. After a 60 min rinse in water, the film was air-dried.

Solubilization of membrane proteins. Solubilization of spinal cord membrane proteins was also achieved according to procedures previously described in detail[7]. Briefly, the membrane preparation was diluted with 10mM sodium phosphate buffer (pH 7.4) containing 1% CHAPS.The protein-detergent suspension was mixed gently by means of a magnetic stirrer at 4 ℃ for 60 min, followed by centrifugation at 100,000g for 60 min. The supernatant was used as solubilized membrane protein for the [^{3}H]DFP labeling and purification experiments.

[^{3}H]DFP labeling of solubilized proteins and ammonium sulfate precipitation.

Following pre-incubation at 37 ℃ for 15 min, [^{3}H]DFP (10pmol DFP /mg protein, 5-10nM final concentration) was added to the above soluble protein preparation. The incubation was continued for 70 min at 37 ℃. The labeling reaction was terminated by the addition of ammonium sulfate (65% final concentration). After it had been left standing at 4 ℃ overnight, the solution was centrifuged at 11,000g for 15 min, and the protein pellet was dissolved in 10mM phosphate buffer, pH 7.4. The suspension was loaded on columns packed with SephadexG-25 for desalting and to eliminate free radioactive compounds. The columns were centrifuged at 1,500g for 3 min, and the protein-containing eluates were pooled.

Partial purification by high performance liquid chromatography (HPLC).

Chromatography was performed using a Hitachi (Tokyo, Japan) model L-4000H liquid chromatograph equipped with an ultraviolet monitor. Eluent fractions were collected using a Hitachi model L-5200 fraction collector. The radioactivity in the fractions was measured using an Aloka model 3500 liquid scintillation counter. The solubilized proteins labeled with [^{3}H]DFP were loaded onto a HiPrep 16/60 Sephacryl S-200HR gel exclusion column (1.6 x 60cm Pharmacia) that was pre-equilibrated with buffer containing 50mM phosphate, pH 7.0, 0.3M NaCl, 0.3% CHAPS and 0.02% NaN_3. The labeled proteins were eluted with the same buffer medium over 120 min at the flow rate of 1ml/min. The elution of proteins was monitored by the detection of absorbance at 280nm. One ml fractions of the eluates were collected and 20 μl aliquots were taken for scintillation counting (Fig. 2).

One-dimensional gel electrophoresis. For one-dimensional (1D) gels, the concentrated fractions containing labeled proteins were diluted with 2x sample buffer to obtain a final concentration of 63 mM Tris (pH 6.7), 3% SDS, 5% glycerol and 2% mercaptoethanol and then boiled for 5 min prior to loading. Samples were loaded onto vertical conventional (90mm (W) x 73mm (H)) slab gels containing 7.5% polyacrylamide and run at 15-17mA/gel in the buffer system of Laemmli[9]. Proteins were visualized by staining with Coomassie brilliant blue or by fluorography.

Fluorography of gel after 1D gel electrophoresis. After being destained, the gels were immersed in En^{3}Hance (DuPont, NEN, USA) for 15-30 min and dried on filter paper. The dried gels were exposed to X-ray film (Hyperfilm-MP, Amersham, Buckinghamshire, England)) to determine the location of radioactive proteins for 3-6 weeks at -80 ℃. The film was then processed as described above.

Statistics. For comparisons involving several groups, one-way analysis of variance followed by Duncan's multiple range test was performed.

Results

Regional distribution of high-affinity [^{3}H]DFP binding sites in chicken nerve tissues.

The regional distribution of high-affinity [^{3}H]DFP binding

sites in chicken nerve tissues was examined using membrane preparations. The density of the binding sites was highest in the spinal cord and brain stem and lowest in the sciatic nerve. The results demonstrated variation in the quantity of binding sites in different nerve tissues (Table 1).

Autoradiographic localization of [³H]DFP binding sites. The autoradiographic distribution of [³H]DFP binding sites in spinal cord is shown in Fig. 1. As mentioned in our previous paper[8], when membrane fractions (suspension of 50,000g pellet) were incubated in 15nM [³H]DFP, more than 80% of the total binding activity belonged to high-affinity (or specific) binding sites. Thus, the density of autoradiographic grains (Fig. 1A, D) was mostly associated with high-affinity binding sites. The photomicrographs of autoradiograms of [³H]DFP binding sites in spinal cord slices showed significantly higher concentrations of binding sites in gray matter than in white matter. In the cervical region, fairly high densities of [³H]DFP binding sites were found in laminae X and to a lesser extent, in the ventral horn gray matter (Fig. 1A). The lumbar region exhibited extensive [³H]DFP binding in the dosal horn and laminae X. High concentrations of binding sites were also visible in a portion of the ventral horn. Labelling was weak along the anterior median fissure (Fig. 1D). An effect of eserine (a specific blocker of AChE active sites) on the distribution of [³H]DFP binding sites was observed in the lamina X and ventral horn in the lumbar region (Fig. 1E). The effect was lesser in the cervical region (Fig. 1B) and the dorsal horn in the lumbar region (Fig. 1E).

Table 1 Quantitative analysis of [³H]DFP binding sites in nerve tissue membranes of chickens.

Tissue	high-affinity binding sites (fmol/mg protein)
Cerebrum	22.4 ± 4.6[a]
Cerebellum	33.0 ± 3.7[b]
Brain stem	38.4 ± 10.3[b]
Spinal cord	42.4 ± 7.1[b]
Sciatic nerve	4.1 ± 0.6[c]

Individual regions were dissected on ice and membranes were prepared as described in Methods. The high-affinity binding (total binding minus non-specific binding) of 0.5nM [³H]DFP was determined using membrane fractions stored at -80℃ as described in Methods. The results are the ,mean ± SD for triplicate assays of total and nonspecific binding in four separate experiments (4 birds).

a,b,c Statistical comparisons between tissues were achieved by one-way analysis of variance followed by Duncan's multiple range test. Numbers not followed by the same superscript are significantly different ($p<0.05$ or 0.01).

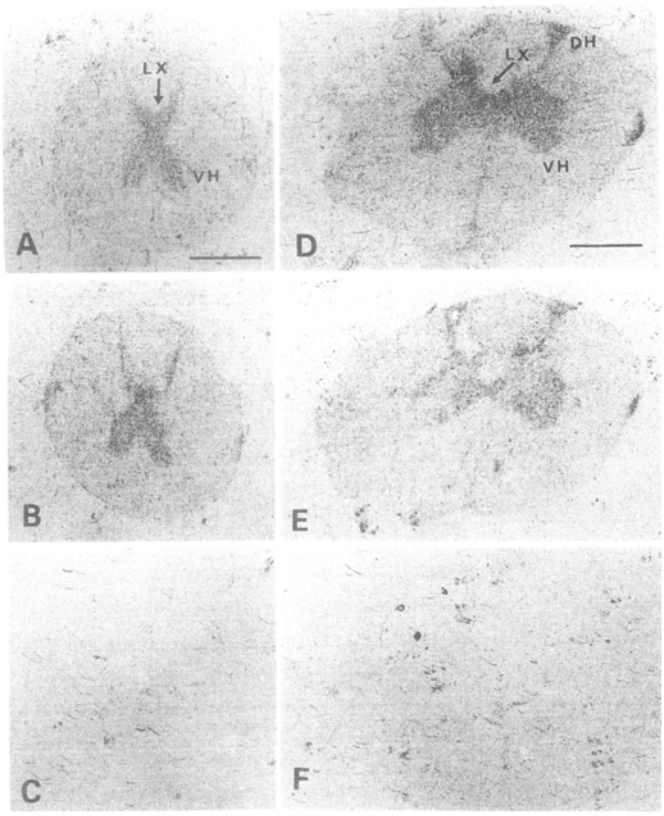

Fig. 1 Photomicrographs of the autoradiographic distribution of [³H]DFP binding sites in the chicken spinal cord.
(A), (B) and (C), cervical ; (D), (E) and (F), lumbar portion of the spinal cord. LX, lamina X; VH, ventral horn; DH, dorsal horn. Section A and D depict the total binding while sections B and E show binding remaining in the presence of eserine (100nM) used to selectively block acetylcholinesterase active sites. (C) and (F), sections incubated in the presence of 10μM unlabeled DFP. Bar = 1000 microns.

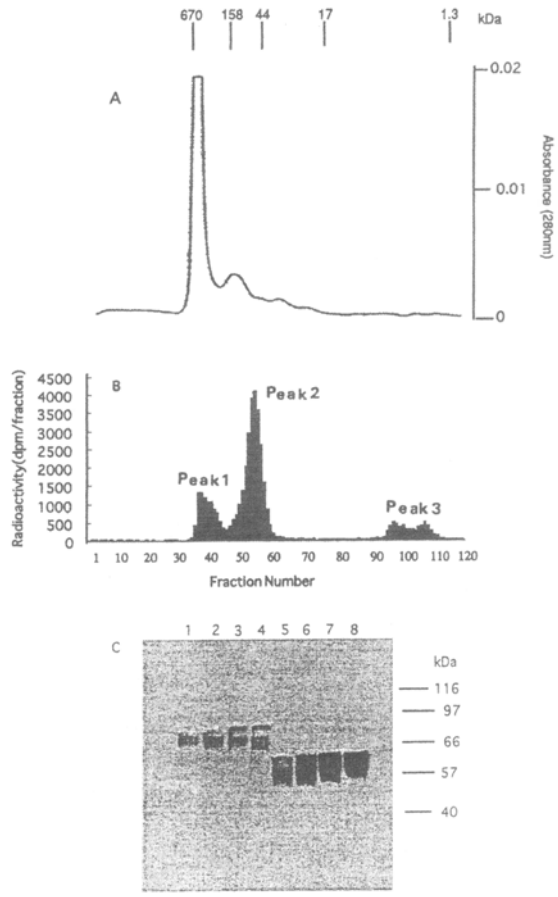

Fig. 2 Gel exclusion chromatography of detergent-solubilized [³H]DFP-labeled proteins.
The protein profile monitored at 280nm is shown in A, and the solid bars show molecular weight markers. Total radioactivity (dpm) in each fraction is shown in B. A fluorograph of SDS-PAGE of [³H]DFP-labeled proteins in peaks 1 and 2 is shown in C. The dried gel was exposed to Amersham Hyperfilm-MP for 3 weeks. Lane 1 corresponds to pooled fractions 36 and 37; Lane 2, pooled fractions 38 and 39; Lane 3, pooled fractions 40 and 41; Lane 4, pooled fractions 42 and 43; Lane 5, pooled fractions 51 and 52; Lane 6, pooled fractions 55 and 56; Lane 8, pooled fractions 57 and 58.

Partial purification of [3H]DFP binding proteins by gel exclusion chromatography. When crude detergent-solubilized [3H]DFP-labeled proteins were subjected to gel exclusion chromatography (HiPrep 16/60 Sephacryl S-200HR), the radioactive proteins were distributed into peak 1, peak 2 and peak 3 (Fig. 2 A,B). The apparent molecular masses of the two major and one minor peaks were 150-670kDa, 40-120kDa and less than 2 kDa, respectively. A recovery of 81% was obtained against the total applied radioactivity. A fluorograph of one-dimensional SDS-PAGE gel of proteins from peaks 1 and 2 is shown in Fig. 2C. Lanes 1 to 4 corresponding to peak 1 were observed to contain two or three protein bands between 66 and 97 kDa. Lanes 5 to 8 corresponding to peak 2 showed a protein band between 55 and 63 kDa, but these proteins did not separate well on this gel under the present exposure conditions. Though the fraction 96-111 corresponding to peak 3 contained radioactive macromolecules, the molecular mass was too low (under 10kDa). Thus, fluorography of the SDS-PAGE gel of these macromolecules was not carried out in the present study.

Discussion

The results of the present study show that the high-affinity [3H]DFP binding sites were differentially localized throughout the chicken nerve tissues, being most prevalent in the spinal cord and less so in the cerebrum (Table 1). The distribution profile of the binding sites is striking in view of the histopathological lesions which were evident 7 days or more after the administration of DFP and TOCP[10,11]. According to the present autoradiographic findings, the [3H]DFP binding sites are more highly concentrated in the dorsal horn at the lumbar level than at the cervical level. Thus the binding sites might be distributed in a gradient manner along the spinal cord. Axonal degeneration in the posterior and anterior columns of the spinal cord is often detected by the traditional hematoxylin-eosin method in chicken treated with DFP and TOCP[12]. However, these stains are less useful for identifying small diameter unmyelinated degenerating axons and terminal fields spread over wide areas of the brain and spinal cord. Tanaka *et al* detected moderate amounts of terminal degeneration in the medial part of the ventral horn at lumber cord levels in chicken treated with 1mg/kg body weight DFP by using Fink-Heimer silver impregnation method[13]. Moreover, exposing the European ferret to DFP results in axonal degeneration at the lumbar levels and through laminae III-VIII [14]. The presence of high densities of [3H]DFP binding sites around the laminae X region in cervical and lumber sections is thus also of interest in connection with the pathological abnormalities. Although in the present model the density of the binding sites was reduced in lamina X of the lumbar region by co-incubation with eserine which is a specific acetylcholinesterase inhibitor, the laminae X of the cervical region was insensitive to eserine. Further autoradiographic experiments are needed to determine whether the eserine-insensitive [3H]DFP binding sites are differentially distributed in the spinal cord.

Delayed neurotoxic OPs interact with Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II), an enzyme responsible for the endogenous phosphorylation of cytoskeletal proteins, i.e. microtubules, neurofilaments, and microtubule associated protein-2 (MAP-2). This leads to an increased activity of CaM kinase II and enhanced phosphorylation of cytoskeletal elements, and eventually to the disassembly of cytoskeletal proteins[6, 15-17]. Although increased CaM kinase II activity might be involved in the development of OPIDN, CaM kinase II exhibits broad substrate specificities and is likely to mediate many of the second-messenger actions of Ca^{2+}. The second-messenger hypothesis states that many types of first messengers in the brain, through the activation of specific plasma membrane receptors and G proteins, stimulate the formation of intracellular second-messengers[18]. A possibility exists that DFP, a direct acting neurotoxicant, interacts with some membrane receptors and the adverse effects occur on the second-messenger systems. Therefore the identification of the DFP binding proteins in membranes is important for elucidating the mechanism of OPIDN.

The radioactive membrane-associated proteins were eluted into two large peaks, peak 1 (150-670kDa) and peak 2 (40-120kDa) in the gel exclusion chromatography. This result supports a previous labeling experiment using the eluate after gel exclusion chromatography[7]. However, the DFP-labeled protein(s) in peak 1 yielded two or three bands between 66-97 kDa by SDS-PAGE (Fig.2C). The high-molecular-weight materials were possibly resolved into two or three low-molecular-weight species by proteolysis. The protein band(s) from peak 2 labeled by [3H]DFP were somewhat diffuse, which would indicate that [3H]DFP interacted with different molecular size proteins between 55-63 kDa. These electrophoretic patterns of labeled proteins might be due to an overexposure of x-ray-sensitive film or an overload of protein sample on the well of the 1D gel.

It is well known that DFP is a serine protease inhibitor[19]. Thus it is possible that the DFP-binding sites (proteins) which are heavily distributed over the gray matter are the serine proteases. A recent work[20], which determined the N-terminal amino acid sequences of proteolytic fragments of NTE, suggests hypotheses for the involvment of NTE in pathogenesis. Furthermore, the immunohistochemical study defined that NTE is expressed in neurons throughout the chicken nervous system[21]. The number of DFP binding sites in membrane preparations from the spinal cord of chicken treated with a neuropathic dose of TOCP is markedly decreased, and it is thus apparent that the TOCP metabolite(s) interact with the DFP binding sites *in vivo*[7]. To elucidate exactly how the high-affinity DFP binding sites (proteins) interact with OPIDN, NTE and serine proteases, further purification and concentration of these proteins is required along with amino acid sequence analysis and database matching.

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