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Carbon disulfide inhalation increases Ca^{2+}/calmodulin-dependent kinase phosphorylation of cytoskeletal proteins in the rat central nervous system

Kenneth R. Wilmarth, Michael E. Viana, Mohamed B. Abou-Donia *

Department of Pharmacology, Duke University Medical Center, Durham, NC 27710, USA

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The Ca^{2+}/calmodulin-dependent phosphorylation of neuronal cytoskeletal proteins was studied in brain supernatants prepared from rats exposed via inhalation to 600 or 800 ppm carbon disulfide (CS_2) for 14 days. Exposure to CS_2 resulted in increased phosphorylation of endogenous MAP-2 and exogenously added neurofilament triplet proteins. There also was an observed increase in the autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II). Slight increases in the binding of a monoclonal antibody to the α subunit of CaM kinase II were seen, while large increases in the binding of [^{125}I]calmodulin to the α subunit of CaM kinase II also were observed. The finding of large increases in the autophosphorylation and calmodulin-binding to CaM kinase II with only slight increases in the amount of antibody-binding suggests that CS_2 exposure results in increased Ca^{2+}/calmodulin-dependent phosphorylation of proteins by inducing an increase in kinase activity.

INTRODUCTION

Carbon disulfide (CS_2) has been utilized as a solvent in a variety of industrial processes for > 100 years. Currently, the principle use for CS_2 is in the manufacture of viscose rayon fibers[11]. CS_2 is a systemic toxicant shown to affect the cardiovascular, endocrine, gastrointestinal, renal, reproductive and nervous systems[1,13,17,38,48,59]. In humans, acute high-level exposure to CS_2 has been associated with psychogenic dysfunctions[58]. Chronic low-level exposure of workers produces changes in cognitive and psychomotor function[1,29] as well as development of a polyneuropathy[33,49]. Chronic CS_2 exposure in animals produces morphological changes in the distal regions of long myelinated axons characterized by the perinodal accumulation of neurofilaments in axonal swellings[25,27,28]. The morphology and distribution of this lesion are similar to that seen with other neurotoxicants, such as *n*-hexane, methyl *n*-butyl ketone (M*n*BK), 2,5-hexanedione (2,5-HD) and acrylamide, all of which produce distal neurofilamentous axonopathies in experimental animals[47]. In addition, β,β'-iminodipropionitrile (IDPN[26]) and 3,4-dimethyl-2,5-HD have been shown to produce neurofilamentous axonal swellings initially distributed in the proximal region of the axon[10].

The mechanism of action involved in the accumulation of neurofilaments and the development of neuropathy induced by these compounds has not been identified. A number of studies suggest that binding of the toxicant, or a reactive biotransformation product, to neurofilament proteins may alter the physico–chemical properties or conformational state of the proteins, which leads to alterations in the normal organization and function of the cytoskeletal matrix and neurofilament accumulation[3,16,19,37,47]. Binding of CS_2 to axonal protein fractions[46] and neurofilament proteins[45] in the spinal cord of rats has been reported. In vitro binding studies have shown stable adduct formation of CS_2 with the ϵ-amino group of lysine residues in BSA and intramolecular cross-linkage of the protein[20]. Specific binding to the ϵ-amino group on lysine residues in neurofilaments also has been proposed as a mechanism of action for other neurofilamentous axonopathy-producing compounds, including acrylamide[36] and 2,5-HD[19]. It has been suggested that CS_2-binding to proteins and the formation of isothiocyanate adducts lead to formation of covalent cross-linkages and may be

* Corresponding author. Fax: (1) (919) 681–8224.

responsible for the production of neurotoxicity[9]. To date, however, there have been no reports which demonstrate the cross-linking of neurofilament proteins by CS_2 in vivo or in vitro; DeCaprio et al.[20] found no evidence of intermolecular cross-linking in their study.

Aberrant phosphorylation of neurofilament proteins consequent to neurotoxicant-binding has been suggested as one possible mechanism involved in the development of neurofilamentous axonopathies[31,37,47,57]. The phosphorylation state of neurofilament and microtubule proteins is an important factor in regulating the normal interaction of these proteins[21,30]. Alterations in the phosphorylation state of the neurofilament proteins could lead to dissociation of the cytoskeletal matrix and neurofilament accumulation. Changes in the phosphorylation state of neurofilament proteins have been reported after exposure of animals to 2,5-HD[37,57], acrylamide[31,43] and IDPN[56]. The phosphorylation state of neurofilament proteins may be determined by factors other than direct binding to the toxicant, including alterations in protease, phosphatase and kinase activities[37,47]. Acrylamide has been shown to enhance the in vitro Ca^{2+}/calmodulin-dependent[43] and -independent[31] phosphorylation of neurofilament proteins isolated from treated rats. Organophosphorus esters (OPs) are a class of compounds that produce neurotoxicity characterized by the abnormal axonal accumulation of phosphorylated neurofilaments[3,5,32]. A consistent finding in OP-induced neuropathy is a dramatic increase in the activity of Ca^{2+}/calmodulin protein kinase II (CaM kinase II)[4,5], a kinase shown to phosphorylate both neurofilament and microtubule proteins[3]. Aberrant phosphorylation of neurofilament proteins appears to be a common feature in neurofilamentous axonopathies. It is possible that CS_2 also produces changes in the phosphorylation state of neurofilament proteins and that these changes represent a potential mechanism of action involved in CS_2-induced neuropathy.

The goal of this study was to determine the potential role of Ca^{2+}/calmodulin-dependent phosphorylation of neuronal cytoskeletal proteins in the pathogenesis of CS_2-induced neurotoxicity. Soluble protein fractions prepared from the brains of rats exposed subacutely to high concentrations of CS_2 were assayed for CaM kinase II autophosphorylation, the Ca^{2+}/calmodulin-dependent phosphorylation of endogenous MAP-2 and the Ca^{2+}/calmodulin-dependent phosphorylation of exogenously added neurofilament triplet proteins. In addition to activity, the amount of CaM kinase II present in the isolates was determined by probing with a monoclonal antibody to the α subunit of the enzyme. Calmodulin-binding was also determined because it has been implicated in the regulation of CaM kinase II activity[52].

MATERIALS AND METHODS

Materials

[γ^{32}P]ATP (3000 Ci/mmol), [^{125}I]protein A (361.62 Ci/mmol) and [^{125}I]calmodulin (1564.8 Ci/mmol) were obtained from Dupont NEN Research Products (Boston, MA). Electrophoresis-grade reagents and molecular-mass standards for SDS–PAGE were purchased from BioRad Laboratories (Richmond, CA); CS_2 (HPLC grade) from Aldrich Chemical Company (Milwaukee, WI); calmodulin and trifluoperazine (TFP) from Sigma Chemical Company (St Louis, MO); TEMED, Tris base, monoclonal anti-MAP-2 and monoclonal anti-α-CaM kinase II from Boehringer Mannheim (Indianapolis, IN); rabbit anti-mouse IgG from Dako (Carpinteria, CA); and BCA protein assay from Pierce (Rockford, IL). Kodak X-OMAT RP-1 (8×10-in) film was procured from Eastman Kodak Company (Rochester, NY). Nitrocellulose was purchased from Schleicher and Schuell (Keene, NH) in 3×33-cm rolls and hand cut to 14×16-cm sheets. All other incidental materials for SDS–PAGE were purchased from BioRad Laboratories.

Animals

Male Sprague–Dawley rats (225–240 g; Charles River Laboratories, Raleigh, NC), at the start of the experiment, were housed three/cage, maintained on a 12/12-h light/dark cycle and given food and water ad libitum. They were divided into groups of six and allowed to acclimate to the exposure chambers (see below) for 3 days before the start of the experiment. Animals were weighed and clinical signs were monitored daily inside and when allowed to move freely outside the cage.

Inhalation chambers

Animals were exposed in 11.4 ft^3 (0.32 m^3) stainless steel inhalation chambers (Young and Bertke, Cincinnati, OH) with controlled temperature and humidity (21–24°C, 40–60% relative humidity), as described previously[2,3]. In-chamber concentrations of CS_2 were monitored daily using a Miran Model 1A-CVF IR gas analyser (The Foxboro, Foxboro, MA). CS_2 concentrations varied by $<10\%$.

Inhalation exposure

Exposures to CS_2 were for 10 h each day, with 14 h allowed for recovery. One group of animals received 800 ppm while another received 600 ppm for 14 consecutive days. A third group of rats was exposed to air and served as a control.

Preparation of brain supernatant fractions

Brain supernatants were prepared using a modification of the subcellular fractionation procedure previously described[4,8]. Briefly, brains were excised immediately after decapitation and homogenized in 20 ml ice-cold 0.32 M sucrose, 0.3 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin, using 10 strokes of a Teflon glass homogenizing unit. These homogenates were then spun at 4°C for 25 min at 10,000×g; the resulting supernatants were spun at 4°C for 60 min at 100,000×g. An aliquot of each was saved for determination of protein concentration, using the bicinchoninic acid method[51], and the remainders were frozen at −70°C.

In vitro phosphorylation assays

The Ca^{2+}/calmodulin-dependent phosphorylation of endogenous MAP-2 and exogenous neurofilament triplet proteins, as well as autophosphorylation of CaM kinase II, were determined using a modification of the method of Patton et al.[42] Supernatants (38.2 μg protein) were preincubated for 1 min at 30°C in a buffer containing 0.05 M Tris, pH 7.5, 0.01 M $MgCl_2$, 5 mM 2-mercaptoethanol plus either 0.4 mM EGTA or 0.2 mM $CaCl_2$ and 5 μg calmodulin. Buffer volume was adjusted to a final volume of 100 μl and all tubes were

run in duplicate. The reaction was initiated by the addition of 2 μCi [γ^{32}P]ATP (0.05 M final concentration, 0.4 nCi/pmol), incubated for 1 min at 30°C and terminated by the addition of 50 μl SDS–PAGE "stop" buffer (0.125 M Tris, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue). Each of the above reactions was also carried out in the presence of 0.1 mM TFP. These samples were boiled for 2 min, stored at 4°C overnight and separated by SDS–PAGE. Phosphorylation of exogenous neurofilaments involved the addition of neurofilament triplet proteins (10 μg) isolated from bovine spinal cord by the method of Dahl et al.[18] to each sample, adjusting the buffer volume accordingly. The phosphorylation reaction was then run as above.

SDS–PAGE

Supernatant samples were electrophoretically resolved on 7.5/4% discontinuous gels, according to the method of Laemmli[35]. Each sample of 19.1 μg (75 μl) was run on duplicate gels, along with molecular-mass standards for determination of apparent electrophoretic mobilities. Immediately after the runs, gels were fixed/stained in 0.1% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid; then, they were destained in 50% methanol and 10% acetic acid, dried between cellophane sheets and autoradiographed. Films were exposed for four different time periods to determine a linear response range and quantified using an LKB 2202 Ultroscan laser densitometer interfaced with an LKB 2220 recording integrator (Pharmacia LKB Biotechnology, Piscataway, NJ). Immunoblotting with monoclonal antibodies specific to MAP-2 and the α subunit of CaM kinase II were used to identify these proteins. Apparent molecular masses were assigned based on their relative position to molecular-mass standards run on each gel and blot. Indirect identification of the β subunit of CaM kinase II was based on reported molecular-mass values for rat brain isolates of the kinase[23], the fact that it bound calmodulin with a high specificity and, when compared with anti-α-CaM kinase-binding data, showed an appropriate difference in apparent molecular mass (10 kDa).

[^{125}I]Calmodulin-binding

Supernatants (100 μg) were separated by SDS–PAGE, fixed overnight at room temperature in 100 ml 25% (v/v) 2-propanol and 10% (v/v) acetic acid and radiolabeled by the method of Carlin et al.[15] Briefly, the gel was washed in 100 ml deionized water for 10 min (all washes were 100 ml), followed by four 1.5-h and one overnight wash in 0.05 M Tris, pH 7.1, 0.2 M NaCl, 1 mM $CaCl_2$ (Tris buffer). The following day, the gel was washed for 2 h in fresh Tris buffer, 2 h in Tris buffer with 1 mg/ml BSA and overnight in Tris buffer with 2 μCi [^{125}I]calmodulin/100 ml. The gel was washed thoroughly (eight times over a 48-h period, with two overnight washes) and stained, destained, dried, autoradiographed and quantitated, as previously described.

Immunoblotting

Supernatants (100 μg) were separated by SDS–PAGE, electrophoretically transferred to nitrocellulose by the method of Towbin et al.[53] and the blot allowed to air-dry overnight at room temperature (all steps were performed at room temperature, all washes were 100 ml). The blot was then washed 1 h in Tris-buffered saline (TBS; 0.05 M Tris, pH 7.4, 0.2 M NaCl, 0.02% (w/v) NaN_3) with 0.5% (w/v) gelatin (blocking solution), followed by a 2-h incubation in blocking solution plus 0.1% (v/v) Triton X-100 (antibody solution) containing monoclonal anti-α-CaM kinase II diluted 1/2000 or monoclonal anti-MAP-2 diluted 1/1000. The blot was then washed 5×10 min with TBS, washed 30 min in blocking solution and incubated 1 h in antibody solution containing polyclonal rabbit anti-mouse IgG diluted 1/1000. The blot was again washed 5×10 min in TBS, blocked 30 min and incubated 1 h in antibody solution containing 0.2 mCi/ml [^{125}I]protein A. The blot was washed in TBS with 0.1% Triton X-100 4×10 min, followed by a 150-ml wash overnight. The following day, the blot was briefly rinsed in deionized water (~10 min), allowed to thoroughly air-dry and exposed to film. We have found that covering the blot with a sheet of plastic wrap prevents it from adhering to the film without degradation of exposure. The autoradiograph was quantitated, as previously described. Specificity of the antibodies was determined by exactly following the above-described procedure with the exception of omitting incubation with the primary antibody.

RESULTS

General observations

Animals exposed to CS_2 displayed progressive weight loss over the course of the experiment resulting in 14

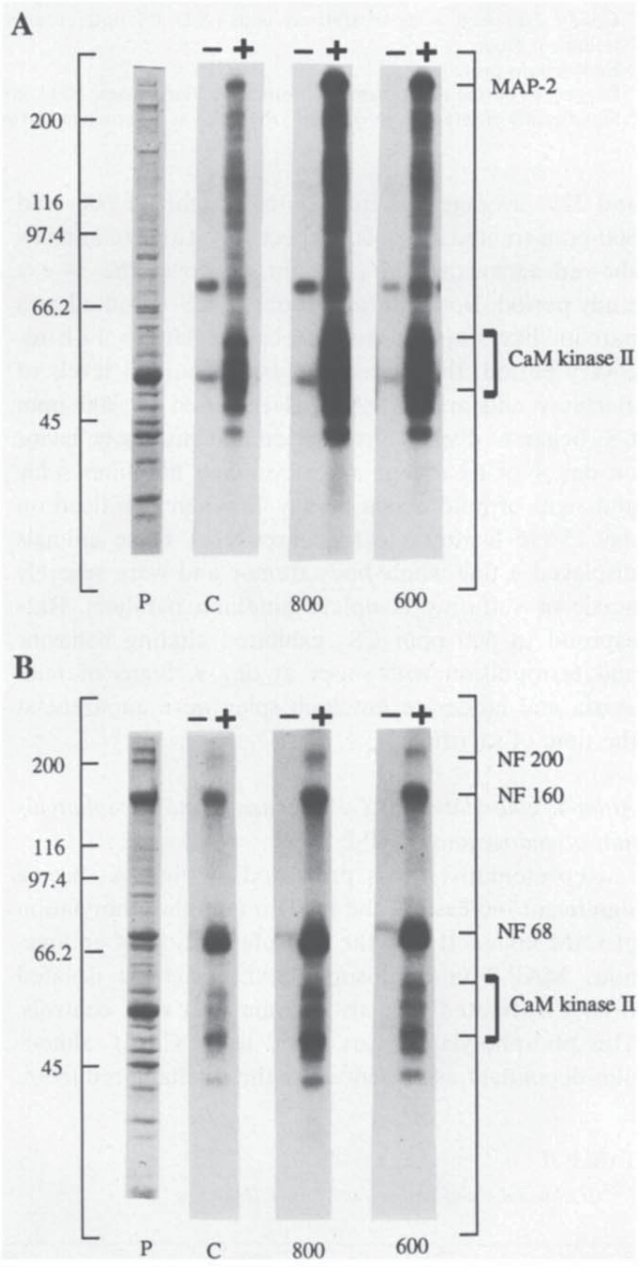

Fig. 1. In vitro phosphorylation of brain-supernatant proteins (A) or supernatants with exogenously added neurofilament triplet proteins (B) from control (C) and animals exposed to 800 ppm CS_2 (800) or 600 ppm CS_2 (600) after 14 days of treatment. A representative protein-staining lane also is included (P). Autoradiography shows phosphorylation in absence of Ca^{2+}/calmodulin with EGTA (−) or in absence of EGTA and with Ca^{2+}/calmodulin present (+). Increases were seen in autophosphorylation of kinase, phosphorylation of endogenous MAP-2 and all three neurofilament proteins.

TABLE I

Ca^{2+}/calmodulin-dependent phosphorylation of endogenous and exogenous substrates in brain of CS_2-treated and control rats

Parameter	*Control*	*600 ppm*	*800 ppm*
Phosphorylation			
α- and β-CaM kinase II	5.21 ± 2.13 [a]	16.64 ± 4.61 (219.4) [d]	20.43 ± 6.39 (292.1) [d]
MAP-2 [b]	0.81 ± 0.25	2.24 ± 0.52 (176.5) [d]	3.16 ± 0.69 (290.1) [d]
NFH [c]	0.25 ± 0.13	0.45 ± 0.12 (80.0)	0.60 ± 0.25 (140.0) [d]
NFM [c]	0.87 ± 0.51	2.31 ± 0.35 (165.5) [d]	2.61 ± 0.87 (200.0) [d]
NFL [c]	1.96 ± 0.89	3.41 ± 0.69 (74.0) [d]	4.53 ± 1.79 (131.1) [d]

[a] Results expressed as mean arbitrary units ± S.D. (% difference from control). Duplicate samples from six animals were analysed for each treatment group.
[b] Endogenous protein.
[c] Exogenous bovine neurofilaments, with molecular masses: NFH, 200 kDa; NFM, 160 kDa; and NFL, 68 kDa.
[d] Significantly different from controls ($P \leq 0.05$) as determined by two-tailed Student's *t*-test.

and 32% average loss from initial weight for 600- and 800-ppm-treated animals, respectively. Control animals showed an average 37% weight gain over the 14-day study period. Both concentrations of CS_2 resulted in a narcotic-like stupor during exposure. After a 14-h recovery period, there was a return to normal levels of alertness and activity. Animals exposed to 800 ppm CS_2 began to display retropulsion and circling behavior on day 4 of treatment and developed hindlimb splay and signs of mild ataxia by day 7. When sacrificed on day 15 (16 h after the final exposure), these animals displayed a fine whole-body tremor and were severely ataxic or suffering complete hindlimb paralysis. Rats exposed to 600 ppm CS_2 exhibited circling behavior and retropulsion with onset at day 9. Signs of mild ataxia and moderate hindlimb splay were apparent at the time of sacrifice.

Autophosphorylation of CaM kinase II and phosphorylation of endogenous MAP-2

Representative lanes presented in Fig. 1A show a significant increase in the in vitro autophosphorylation of CaM kinase II and the phosphorylation of endogenous MAP-2 in brain-supernatant fractions isolated from CS_2-treated animals as compared with controls. This phosphorylation was found to be Ca^{2+}/calmodulin-dependent as evidenced by the dramatic reduction in phosphorylation seen in the presence of EGTA without added Ca^{2+}/calmodulin. The addition of 0.1 mM TFP to the reaction mixture resulted in a > 95% inhibition in phosphorylation of all detectable cytosolic proteins. Data presented in Table I show that in the presence of Ca^{2+}/calmodulin there was a 219 and 292% increase in the autophosphorylation of the kinase for animals treated with 600 or 800 ppm CS_2, respectively. Increases of 177 and 290% were seen for the phosphorylation of MAP-2 in 600- and 800-ppm-exposed animals.

Phosphorylation of exogenous neurofilament proteins

The addition of neurofilament triplet proteins to the brain supernatant fractions from animals treated with CS_2 resulted in increased Ca^{2+}/calmodulin-dependent phosphorylation of all of these proteins (Fig. 1B). The increase in phosphorylation of all three neurofilament proteins was greatest when incubated with the supernatants isolated from animals treated with 800 ppm CS_2. There was also a significant increase in the phosphorylation of these proteins when incubated with supernatants from animals exposed to 600 ppm CS_2 (Table II). The relative increase in phosphorylation observed in both treatment groups was NFM > NFH > NFL.

TABLE II

[^{125}I]Calmodulin and anti-α-CaM kinase II-binding

Parameter	*Control*	*600 ppm*	*800 ppm*
[^{125}I]Calmodulin			
α-CaM kinase II	1.92 ± 0.24 [a]	6.79 ± 0.95 (253.6) [b]	8.20 ± 1.12 (327.1) [b]
β-CaM kinase II	10.47 ± 0.87	11.81 ± 1.03 (12.8)	14.11 ± 2.14 (34.8) [b]
Anti-α-CaM kinase II			
α-CaM kinase II	3.07 ± 0.40	3.46 ± 0.31 (12.7)	3.55 ± 0.29 (15.6) [b]

[a] Results expressed as mean arbitrary units ± S.D. (% difference from control). Duplicate samples from six animals were analysed for each treatment group.
[b] Significantly different from controls ($P \leq 0.05$) as determined by two-tailed Student's *t*-test.

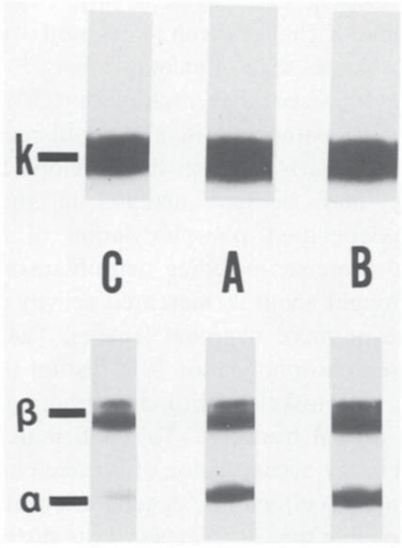

Fig. 2. Representative immunoblots of anti-α-CaM kinase II-binding (k) and [^{125}I]calmodulin-binding to α and β subunits of enzyme in brain-supernatant fractions isolated from control (C), 600-ppm CS_2- (A) and 800-ppm CS_2-treated animals (B) after 14-day exposure.

[^{125}I]Calmodulin-binding and immunoassay with anti-CaM kinase II

Autoradiographs presented in Fig. 2 show that there was an increase in the binding of [^{125}I]calmodulin in both treatment groups as compared with controls which is consistent with the pattern of increased phosphorylation presented in Fig. 1. Calmodulin-binding to the α subunit of CaM kinase II was increased 327% in animals exposed to 800 ppm CS_2 while calmodulin-binding to the β subunit in these animals was increased only 35% (Table II). For animals exposed to 600 ppm CS_2, there was a 254% increased binding to the α subunit and a statistically insignificant increase in binding to the β subunit. Results of probing Western blots with monoclonal anti-α-CaM kinase II presented in Fig. 2 show a slight increase in the amount of binding to the α subunit as a function of treatment. There was a statistically significant increase of 15% in the binding of the antibody in the 800-ppm treatment group.

DISCUSSION

The normal association of neurofilaments with microtubules is dependent on the phosphorylation state of these proteins[40]. Neurofilament phosphorylation inhibits proteolysis of the filament[24], prevents the assembly of NFL into filaments and induces filament disassembly[30]. Enhanced aberrant phosphorylation of neurofilament proteins has also been shown to decrease their transport rate in the axon[14,40]. Phosphorylation of MAP-2 results in microtubule dissociation[12]. Our results indicate that exposure of rats to CS_2 vapor at high concentration produced signs of neurotoxicity concurrent with significant increases in calmodulin-binding to CaM kinase II, autophosphorylation of CaM kinase II and Ca^{2+}/calmodulin-dependent phosphorylation of endogenous MAP-2 and exogenous neurofilament triplet proteins. It is possible that increased Ca^{2+}/calmodulin-dependent kinase activity results in aberrant phosphorylation of cytoskeletal proteins and subsequent alteration in association and transport in the axon.

Increased phosphorylation of neurofilaments in the axon and perikarya of neurons has been reported after acrylamide intoxication in rats[22,43]. Similar patterns of increased immunoreactivity are seen after axotomy[44]. It appears that this increase in phosphorylation is a common response of the neuron after both traumatic or chemical lesioning. While the increased presence of phosphorylated neurofilament protein has been demonstrated as a response to cell injury, the mechanism involved in this response has not been determined. It is unclear which enzymes are responsible for this increased phosphorylation, and whether the response represents a regulatory increase in the activity of an existing enzyme pool or the increased expression of one or more neuronal kinases.

In this study, CS_2-induced increases in protein phosphorylation were found to be both C^{2+}- and calmodulin-dependent as evidenced by the lack of γ^{32}P incorporation into both endogenous and exogenous proteins when incubated in the absence of C^{2+} or the presence of 0.1 mM TFP, an inhibitor of calmodulin[23]. This demonstrated C^{2+}/calmodulin dependence as well as the broad specificity of substrate proteins showing increased phosphorylation suggest an increase in CaM kinase II activity resulting from CS_2-induced chemical lesion to the neuron.

There was an overall increase observed in the binding of calmodulin to both the α and β subunits of the enzyme as a function of CS_2 treatment. Calmodulin-binding is thought to produce conformational changes in the enzyme, transforming it from an inactive to an active form, allowing for phosphorylation of substrates[52]. This is consistent with the finding of increased autophosphorylation of the enzyme and phosphorylation of substrate proteins. The levels and relative binding pattern of calmodulin to the α and β subunits of CaM kinase II which we observed in the brainsupernatant preparations from control animals in this study are similar to calmodulin-binding patterns we have reported previously in hen[7] and rat brain (manuscript submitted) supernatants prepared from naive animals using the same method of sample preparation.

Data presented in Table II show that there was an order of magnitude difference in the increase of CaM-binding to the α subunit as compared with the β subunit after exposure to CS_2. Probing with a monoclonal antibody to the α subunit showed only a slight increase in antibody-binding as a function of CS_2 treatment. This information indicates that CS_2 exposure results in a small increase in the amount of CaM kinase II protein and much greater increases in CaM-binding to the α subunit. However, since only one antibody was used in this study, it is possible that CS_2 modification of the epitome recognized by the antibody resulted in decreased antibody-binding and an underestimation of the amount of α subunit protein present in the treated animals. It is, therefore, not possible to state with certainty whether the increased protein phosphorylation observed in this study resulted from an increase in the amount of enzyme present or an alteration in the specific activity of the enzyme, possibly linked to changes in the level of CaM-binding.

The observed increase in autophosphorylation of the enzyme and the large increase in calmodulin-binding to the a subunit seen in this study are similar to changes reported to occur in organophosphorus compound-induced neuropathy[3]. We have shown that organophosphorus compounds capable of inducing delayed neurotoxicity produce axonal swellings containing phosphorylated neurofilaments[32]. Phosphorylated neurofilament protein has also been identified in the axonal swellings induced by CS_2[41].

The clinical signs displayed by animals exposed to 600-ppm concentrations of CS_2 were much less severe than those seen in animals exposed to 800 ppm. However, there was still a significant increase in kinase activity and substrate phosphorylation in supernatant isolated from these animals. Therefore, it appears that alterations in phosphorylation may be an early event in the pathogenesis of CS_2-induced neuropathy and represents a possible mechanism involved in the process of other neurofilamentous axonopathies.

The α subunit of CaM kinase II is a major protein component in the postsynaptic density of central neurons[34]. Our data showing an increased in vitro activity of CaM kinase II and increased phosphorylation of endogenous MAP-2 after exposure of animals to CS_2 suggest the possibility for these events to occur in vivo. This potentially represents an effect of CS_2 on the cell body and dendrites. MAP-2 participates in both dendritic transport[39,55] and mediation of second-messenger systems in the postsynaptic density[50,54]. Disruption of synaptic function and cell-signaling resulting from increased kinase activity and aberrant phosphorylation of MAP-2 could be involved in the psychogenic and psychomotor changes seen in exposed workers and behavioral changes seen in animals.

It is hypothesized that multiple mechanisms are involved in the pathogenesis of neurofilamentous axonopathies. An early event in the development of the neuropathy may be an increase in the Ca^{2+}/calmodulin-dependent phosphorylation of important cytoskeletal proteins, including neurofilaments, MAPs and Tau brought about by increased activity or expression of one or more neuronal kinases. The effect of this increased phosphorylation is to disrupt the normal association of cytoskeletal proteins resulting in a disruption of axonal transport. This deficit in transport would lead to the accumulation of cytoskeletal proteins in the axon and, ultimately, degeneration of the neuron. It is possible that differences in the distribution of lesions and time course in the development of the neuropathies seen with different compounds may be attributable to compound-specific differences in reactivity with individual cytoskeletal proteins or regional binding patterns to specific target proteins. These specific patterns of binding may differentially alter the availability of phosphorylatable binding sites, protease and phosphatase activity and, possibly, have different potentials for the activation of specific kinases. Ongoing work in this laboratory is directed at elucidating the relative contribution of these mechanisms to the overall pattern of neurotoxicity observed with neurofilamentous axonopathy producing compounds.

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REFERENCES

1 Aaserud, O., Hommeren, O.J., Tvedt, B., Nakstad, P., Mowe, G., Efskind, J., Russell, D., Jorgensen, E.B., Nyberg-Hansen, R., Rootwelt, K. and Gjersted, L., Carbon disulfide exposure and neurotoxic sequelae among viscose rayon workers, *Am. J. Ind. Med.*, 18 (1990) 25–37.

2 Abdo, K.M., Graham, D.G., Timmons, P.R. and Abou-Donia, M.B., Neurotoxicity of continuous (90 days) inhalation of technical grade methyl butyl ketone in hens, *J. Toxicol. Environ. Health*, 9 (1982) 199–215.

3 Abou-Donia, M.B., Lapadula, D.M., Campbell, G. and Timmons, P.R., The synergism of *n*-hexane-induced neurotoxicity by methyl isobutyl ketone following subchronic (90 days) inhalation in hens: induction of hepatic microsomal cytochrome P-450, *Toxicol. Appl. Pharmacol.*, 81 (1985) 1–16.

4 Abou-Donia, M.B., Lapadula, D.M. and Carrington, C.D., Biochemical methods for assessment of neourtoxicity. In B. Ballantyne (Ed.), *Perspectives in Basic and Applied Toxicology.*, John Wright and Sons, Bristol, UK, 1988, pp. 1–30.

5 Abou-Donia, M.B. and Lapadula, D.M., Studies on the molecular pathogenesis of organophosphorus compound-induced delayed neurotoxicity (OPIDN). In T. Naharashi and J.E. Chambers (Eds.), *Insecticide Action*, Plenum Publishing, New York, NY, 1989, pp. 205–216.

6 Abou-Donia, M.B. and Lapadula, D.M., Mechanism of organophosphorus ester induced delayed neurotoxicity: type I and type II, *Annu. Rev. Pharmacol. Toxicol.*, 30 (1990) 405–440.

7 Abou-Donia, M.B., Viana, M.E., Gupta, R.P. and Anderson, J.K., Enhanced calmodulin binding concurrent with increased kinase-dependent phosphorylation of cytoskeletal proteins following a single subcutaneous injection of diisopropyl phosphorofluoridate in hens, *Neurochem. Int.*, 22 (1993) 165–173.

8 Abou-Donia, M.B., The cytoskeleton as a target for organophosphorus ester-induced delayed neurotoxicity (OPIDN), *Chemic. Biol. Interact.*, 87 (1993) 383–393.

9 Amarnath, V., Anthony, D.C., Valentine, W.M. and Graham, D.G., The molecular mechanism of the carbon disulfide mediated cross-linking of proteins, *Chem. Res. Toxicol.*, 4 (1991) 148–150.

10 Anthony, D.C., Boekelheide, K. and Graham, D.G., The effect of 3,4-dimethyl substitution on the neurotoxicity of 2,5-hexanedione. I. Accelerated clinical neuropathy is accompanied by more proximal axonal swellings, *Toxicol. Appl. Pharmacol.*, 71 (1983) 362–371.

11 Beauchamp, R.D., Bus, J.S., Popp, J.A., Boreiko, C.J. and Golberg, L., A critical review of the literature on carbon disulfide toxicity, *CRC Crit. Rev. Toxicol.*, 11 (1983) 169–278.

12 Burns, R.G., Islam, K. and Chapman, R., The multiple phosphorylation of the microtubule-associated protein MAP-2 controls the MAP-2 tubulin interaction, *Eur. J. Biochem.*, 141 (1984) 609–615.

13 Butler, W., Hampsall, V. and Magos, C., Histochemical studies of hydropic degeneration of the rat liver induced by carbon disulfide, *Pathology*, 113 (1974) 79–84.

14 Carden, M.J., Goldstein, M.E., Bruce, J., Cooper, H.S. and Schlaepfer, W.W., Studies of neurofilaments that accumulate in proximal axons of rats intoxicated with β,β'-iminodipropionitrile (IDPN), *Neurochem. Pathol.*, 7 (1987) 189–205.

15 Carlin, R.K., Grab, D.J. and Siekevitz, P., Function of calmodulin in postsynaptic densities. III. Calmodulin-binding proteins of the postsynaptic density, *J. Cell Biol.*, 89 (1981) 449–455.

16 Carrington, C.D., Lapadula, D.M., Dulak, L., Friedman, M. and Abou-Donia, M.B., In vivo binding of [^{14}C]acrylamide to proteins in the mouse nervous system, *Neurochem. Int.*, 18 (1991) 191–197.

17 Chengelis, C.P., Changes in hepatic glutathione concentrations during carbon disulfide-induced hepatotoxicity in the rat, *Res. Commun. Chem. Pathol. Pharmacol.*, 61 (1988) 97–109.

18 Dahl, D., Crosby, C.J., Gardner, E.E. and Bignami, A., Purification of the glial fibrillary acidic protein by anion-exchange chromatography, *Anal. Biochem.*, 126 (1982) 165–169.

19 DeCaprio, A.P. and Fowke, J.H., Limited and selective adduction of carboxyl-terminal lysines in the high molecular weight neurofilament proteins by 2,5-hexanedione in vitro, *Br. Res.*, 586 (1992) 219–228.

20 DeCaprio, A.P., Spink, D.C., Chen, X., Fowke, J.H., Zhu, M. and Bank, S., Characterization of isothiocyanates, thioureas, and other lysine adduction products in carbon disulfide-treated peptides and protein, *Chem. Res. Toxicol.*, 5 (1992) 496–504.

21 Eyer, J. and Leterrier, J.F., Influence of the phosphorylation state of neurofilament proteins on the interactions between purified filaments in vitro, *Biochem J.*, 252 (1988) 655–660.

22 Gold, B.G., Price, D.L., Griffin, J.W., Rosenfeld, J., Hoffman, P.N., Sternberger, N.H. and Sternberger, L.A., Neurofilament antigens in acrylamide neuropathy, *J. Neuropathol. Exp. Neurol.*, 47 (1988) 145–157.

23 Goldenring, J.R., Gonzalez, B., McGuire, Jr., J.S. and DeLorenzo, R.J., Purification and characterization of Ca^{2+}- and calmodulin-dependent protein kinase from rat brain, *J. Biol. Chem.*, 258 (1983) 12632–12640.

24 Goldstein, M.E., Sternberger, N.H. and Sternberger, L.A., Phosphorylation protects neurofilaments against proteolysis, *J. Neuroimmunol.*, 14 (1987) 149–160.

25 Gottfried, M.R., Graham, D.G., Morgan, M., Casey, H.W. and Bus, J.S., The morphology of carbon disulfide neurotoxicity, *Neurotoxicology*, 6 (1985) 89–96.

26 Griffin, J.W., Cork, L.C., Tronscoso, J.C. and Price, D.L., Experimental neurotoxic disorders of motorneurons: neurofibrillary pathology, *Adv. Neurol.*, 36 (1982) 419–431.

27 Haltia, M. and Linnoila, I., Distal axonopathy induced by carbon disulfide (CS_2), *J. Neuropathol. Exp. Neurol.*, 37 (1978) 621.

28 Haltia, M. and Linnoila, I., The neuropathy of carbon disulfide intoxication. Experimental studies, *Acta Neurol. Scand. Suppl.*, 67 (1978) 255.

29 Hänninen, H., Psychological picture of manifest and latent carbon disulfide poisoning, *Br. J. Ind. Med.*, 28 (1971) 374–381.

30 Hisanaga, S., Gonda, Y., Inagaki, M., Ikai, A. and Hirokawa, N., Effects of phosphorylation of the neurofilament L protein on filamentous structures, *Cell Reg.*, 1 (1990) 237–248.

31 Howland, R.D. and Alli, P., Altered phosphorylation of rat neuronal cytoskeletal proteins in acrylamide induced neuropathy, *Brain Res.*, 363 (1986) 333–339.

32 Jensen, K.F., Lapadula, D.M., Knoth-Anderson, J., Haykal-Coates, N. and Abou-Donia, M.B., Anomalous phosphorylated neurofilament aggregations in central and peripheral axons of hens treated with tri-*ortho*-cresyl phosphate (TOCP), *J. Neurosci. Res.*, 33 (1992) 455–460.

33 Juntunen, J. and Haltia, M., Polyneuropathies in occupational neurology: pathogenetic and clinical aspects, *Acta Neurol. Scand. Suppl.*, 92,(1982) 59–73.

34 Kennedy, M.B., Bennett, M.K. and Erondu, N.E., Biochemical and immunochemcal evidence that the "major postsynaptic density protein" is a subunit of calmodulin-dependent protein kinase, *Proc. Natl. Acad. Sci. USA*, 80 (1983) 7357–7361.

35 Laemmli, U.K., Clevage of structural proteins during the assembly of the head of bacteriophage T4, *Nature (London)*, 227 (1970) 680–685.

36 Lapadula, D.M., Suwita, E. and Abou-Donia, M.B., Evidence for multiple mechanisms responsible for 2,5-hexanedione-induced neuropathy, *Br. Res.*, 458 (1988) 123–131.

37 Lapadula, D.M., Bowe, M., Carrington, C.D., Dulak, L., Friedman, M. and Abou-Donia, M.B., In vitro binding of ^{14}C acrylamide to neurofilament and microtubule proteins of rats, *Br. Res.*, 481 (1989) 157–161.

38 MacMahon, B. and Monson, R.R., Mortality in the US rayon industry, *J. Occup. Med.*, 30 (1988) 698–705.

39 Matus, A., Microtubule-associated proteins: their potential role in determining neuronal morphology, *Annu. Rev. Neurosci.*, 11 (1988) 29–44.

40 Nixon, R.A., Axonal transport of cytoskeletal proteins. In R.D. Burgoyne (Ed.), *The Neuronal Cytoskeleton*, Wiley-Liss, New York, NY, 1991, pp. 283–307.

41 Papolla, M., Penton, R., Weiss, H., Miller, C., Sahenk, Z., Autilio-Gambetti, L. and Gambetti, P., Carbon disulfide axonopathy. Another experimental model characterized by acceleration of neurofilament transport and distinct changes of axonal size, *Br. Res.*, 424 (1987) 272–281.

42 Patton, S.E., O'Callaghan, J.P., Miller D.B. and Abou-Donia, M.B., The effect of oral administration of tri-*o*-cresyl phosphate on in vitro phosphorylation of membrane and cytosolic proteins from chicken brain, *J. Neurochem.*, 41 (1983) 897–901.

43 Reagan, K.E., Wilmarth, K.R. and Abou-Donia, M.B., Acrylamide and glycidamide treatment increased calcium/calmodulin-dependent phosphorylation of cytoskeletal proteins in rat brain and spinal cord, *Toxicologist*, 13 (1993) 128.

44 Rosenfeld, J., Dorman, M.E., Griffin, J.W., Gold, B.G., Sternberger, L.A., Sternberger, N.H. and Price, D.L., Distribution of neurofilament antigens after axonal injury, *J. Neuropathol. Exp. Neurol.*, 46 (1987) 269–282.

45 Savolainen, H. and Vainio, H., High binding of CS_2 sulphur in spinal cord axonal fraction, *Acta Neuropathol.*, 36 (1976) 251–257.

46 Savolainen, H., Lehtonen, E. and Vainio, H., CS_2 binding to rat spinal neurofilaments, *Acta Neuropathol.*, 37 (1977) 219–223.

47 Sayre, L.M., Autilio-Gambetti, L. and Gambetti, P., Pathogenesis of experimental giant neurofilamentous axonopathies: a unified hypothesis based on chemical modification of neurofilaments, *Brain Res. Rev.*, 10 (1985) 69–83.

48 Seppäläinen, A.H. and Tolonen, M., Neurotoxicity of long-term exposure to carbon disulfide in the viscose rayon industry. A neurophysiological study, *Work Environ. Health*, 11 (1974) 145–153.

49 Seppäläinen, A.H. and Italtia, M., Carbon disulfide. In P.S. Spencer and H.H. Schaumburg (Eds.), *Experimental and Clinical Neurotoxicology,* Williams and Williams, Baltimore, MD, 1980, pp. 356–373.
50 Sloboda, R.D., Rudolf, S.A., Rosenbaum, J.L. and Greengard, P., Cyclic-AMP mediated endogenous phosphorylation of a microtubule-associated protein, *Proc. Natl. Acad. Sci. USA,* 72 (1975) 177–181.
51 Smith, P.R., Krohn, R.I., Hermanson, G.T., Mallea, A.K., Gartner, L.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C., Measurement of protein using bicinchonic acid, *Anal. Biochem.,* 150 (1985) 76–85.
52 Soderling, T.R., Fukunaga, K., Rich, D.P., Fong, Y.L., Smith, K. and Colbran, R.J., Regulation of brain Ca^{2+}/calmodulin-dependent protein kinase II, *Biology Med. Signal Transduction,* 24 (1990) 206–211.
53 Towbin, H., Staehelin, T. and Gordon, J., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci., USA,* 76 (1979) 4350–4354.
54 Vallee, R.B., Structure and phosphorylation of microtubule-associated protein 2 (MAP 2), *Proc. Natl. Acad. Sci. USA,* 77 (1980) 3206–3210.
55 Vallee, R.B. and Bloom, G.S., Mechanisms of fast and slow axonal transport, *Annu. Rev. Neurosci.,* 14 (1991) 59–92.
56 Watson, D.F., Fittro, K.P., Griffin, J.W. and Hoffman, P.N., Phosphorylation-dependent immunoreactivity of neurofilaments increases during axonal maturation and IDPN intoxication, *J. Neurochem.,* 53 (1989) 1818–1829.
57 Watson, D.F., Fittro, K.P., Hoffman, P.N. and Griffin, J.W., Phosphorylation-related immunoreactivity and the rate of transport of neurofilaments in chronic 2,5-hexanedione intoxication, *Br. Res.,* 539 (1991) 103–109.
58 World Health Organization, *Environmental Health Criteria, 10: Carbon Disulfide,* Geneva, Switzerland, 1979.
59 Zenick, H., Blackburn, K., Hope, E. and Baldwin, D., An evaluation of the copulatory, endocrinologic, and spermatotoxic effects of carbon disulfide in the rat, *Toxicol. Appl. Pharmacol.,* 73 (1984) 275–283.