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Effect of carbamate esters on neurite outgrowth in differentiating human SK-N-SH neuroblastoma cells

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Abstract

Carbamate esters are widely used as pesticides and can cause neurotoxicity in humans and animals; the exact mechanism is still unclear. In the present investigation, the effects of carbamates at sublethal concentration on neurite outgrowth and cytoskeleton as well as activities of acetylcholinesterase (AChE) and neuropathy target esterase (NTE) in differentiating human SK-N-SH neuroblastoma cells were studied. The results showed that 50 μM of either aldicarb or carbaryl significantly decreased neurite length in the retinoic acid-induced differentiation of the neuroblastoma cells, compared to cells treated with vehicle. Western blot analyses revealed that neither carbamate had significant effects on the levels of actin, or total neurofilament high molecular proteins (NF-H). However, increased NF-H phosphorylation was observed following carbamate treatment. These changes may represent a useful in vitro marker of carbamate neurotoxicity within a simple model of neuronal cell differentiation. Furthermore, activity of AChE, but not NTE, was significantly inhibited by aldicarb and carbaryl in differentiating cells, which suggested that cytoskeletal protein changes induced by carbamate esters in differentiating cells was associated with inhibition of AChE but not NTE.

Keywords: Carbamate; Neurite outgrowth; Neurofilaments; Acetylcholinesterase; Neuropathy target esterase; Neuroblastoma SK-N-SH cells

1. Introduction

Carbamates, which are all derived from the basic structure of carbamic acid, represent a broad variety of compounds. Many carbamates are potential neurotoxicants, particularly following occupational, accidental or intentional exposure. Acute toxic symptoms of carbamate poisoning are generally caused by inhibition of the enzyme acetylcholinesterase (AChE), which leads to accumulation of acetylcholine (ACh) [1]. However, studies on chronic exposure to carbamate insecticides and case reports of long-term exposure provide conflicting data, wherein some show no effects after chronic exposure [2], and others describe memory impairment [1,3,4], degenerative polyneuropathy [5], neurobehavioral effects [6] and other neurological disorders [3,7]. Overall, it appears that at least some carbamate esters may initiate neurological and behavioral changes at dose levels that produce few overt signs of acute toxicity or significant reduction in nervous tissue AChE activity [1]. However, the mechanisms involved in these changes remain to be elucidated.

Aldicarb is a commonly used and very potent carbamate pesticide. Aldicarb has appeared in some foods

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at toxic levels [8,9] and its toxicity and exposure hazards have been widely studied [2,10]. Carbaryl, another carbamate shown to cause acute neurotoxicity through inhibition of cholinesterase, is used extensively in agriculture and veterinary medicine. Additionally, subacute neurotoxicity and "delayed neurotoxicity"-like toxicity in man and animals after exposure to carbaryl have also been reported [7,11]. An extensive survey of carbaryl toxicology reports a variety of reversible neurobehavioral and neurotoxic effects in vertebrates, all associated with acute poisoning symptoms [12]. However, there is a paucity of data on the biochemical changes underlying neurodegenerative effects of aldicarb and carbaryl.

Potential models for studying morphological and biochemical damage from neurotoxic compounds include cultured cells with neuronal properties and stable cell lines. Differentiating mouse N2a neuroblastoma cells represent an appropriate in vitro cell system for conducting mechanistic studies. Studies in these cells with organophosphorus compounds (OPs) and carbaryl suggest these pesticides act through different cytotoxic mechanisms [13,14]. Human neuroblastoma SK-N-SH cells, the parental cells of SH-SY5Y, have been used to test the inhibitory effects of OPs on neural differentiation [15]. SK-N-SH cell is a useful in vitro cell model for studying potential mechanisms of neurotoxicity since they extend processes following retinoic acid treatment and may maintain many properties inherent to neuroblastoma cells [16]. In this investigation, aldicarb and carbaryl were chosen as representatives of carbamates because of their wide use and differing structures as well as the potential of causing different neurotoxicity in differentiating cells and were used at the same concentration as those OPs that was reported to have effect on cells differentiation and neurofilament heavy subunit protein (NF-H) at 50 μM [15,17]. As axon outgrowth proceeds, NF-H levels may increase and undergo post-translational events (e.g., phosphorylation) linked to axon stabilization in differentiating cells, additional phosphorylated cytoskeletal components were investigated and several relative target proteins were assayed in differentiating SK-N-SH cells for the possible target of different carbamates during this process.

2. Material and methods

2.1. Materials

The human neuroblastoma SK-N-SH cell line was purchased from the Cell Center of Chinese Academy of Medical Sciences (Beijing, China). Cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA). All-*trans* retinoic acid (ATRA), aldicarb [2-methyl-2-(methylthio) propionaldehyde O-(methylcarbamoyl)oxime], carbaryl (1-naphthalenol methylcarbamate) and paraoxon [O,O-dimethyl-O-(4-nitrophenylmethyl) phosphate] were purchased from Sigma (St. Louis, MO, USA). Mipafox and phenyl valerate (PV) were synthesized in our laboratory as described by USA patent no. 2678334 and by Johnson [18], respectively. Monoclonal anti-neurofilament 200 (phosphorylated and non-phosphorylated specific) antibody (clone N52), monoclonal anti-neurofilament 200 (phosphorylated specific) antibody (clone NE14), monoclonal anti-β-actin antibody (AC-15) and anti-mouse IgG (Fc specific) peroxidase conjugate were purchased from Sigma (St. Louis, MO, USA). Enhanced chemiluminescence (ECL) reagents were obtained from Pierce Biotechnology (Rockford, IL, USA).

2.2. Cell culture and maintenance

Cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and streptomycin. Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in the logarithmic phase of growth and subcultured at 3–4 days intervals.

2.3. Differentiation of SK-N-SH cells

Cells were plated at 1×10^4 cells/well in 24-well plates and cultured for 24 h before inducing differentiation. The cells were then induced to differentiate in DMEM medium containing 20 μM ATRA in the dark. Differentiating cells were treated with 50 μM of either aldicarb or carbaryl in 0.1% DMSO, with 0.1% DMSO treatment as vehicle control, the conditioned media was replaced every 48 h. After exposure of 7 days, cell cultures were washed in Tris-buffered saline (TBS; 50 mM Tris–HCl and 150 mM NaCl, pH 7.4) before fixation with ice-cold 90% (v/v) methanol in TBS at −20 °C for 20 min. Cells were stained using Coomassie Brilliant Blue for 2 min at room temperature, and then viewed using an inverted phase-contrast microscope (DMRBE, Leica). Cells were considered to be differentiated if they had at least one process longer than the cell body, which could be regarded as a neurite [19]. The length of the longest neurite was measured in at least 100 cells in randomly chosen fields with an inverted microscope. At least three independent experiments were conducted and the results are expressed as mean ± standard error (S.E.).

2.4. Cytotoxicity assessment

Differentiating SK-N-SH cells were treated as described above and cell viability evaluated. At the end of each experiment, cell medium containing 0.5 mg/ml MTT was added to each well and incubated at 37 °C in 95% air/5% CO_2 for 4 h. The insoluble formazan formed was dissolved in DMSO, and the absorbance was measured in a spectrophotometer at 570 nm with a background reading of 660 nm.

2.5. Western blot analyses

The levels of cytoskeletal components in differentiating cells treated with carbamates were evaluated by Western blot analyses. Cells were washed twice with cold PBS, harvested in lysis buffer (125 mM Tris–HCl, pH 6.8, 1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 μg/ml final concentration of each of aprotinin, leupeptin, and pepstatin), and then boiled for 2 min before removal of aliquots for protein content estimation according to the method of Lowry et al. [20]. Lysates (40 μg) were electrophoresed on 10% SDS-polyacrylamide gels, and then transferred to nitrocellulose membrane and probed with primary antibodies. Membranes were washed, and then incubated with horseradish peroxidase-conjugated secondary antibody. The Western blots were developed using standard ECL method.

2.6. Densitometric analyses

Densitometry was performed using a Beckman DU-650 spectrophotometer. Blots were digitalized, and the cytoskeletal protein levels were quantified by directly relating antibody reactivity to specific protein level. Using the features of the Quantiscan software program, individual lanes were superimposed over the region of interest, from which a peak profile could be constructed. An arbitrary value of the peak area was then calculated and corrected for the background profile. At least three independent experiments were conducted and data are presented as mean ± S.E.

2.7. AChE and NTE activity assays

Cells (2×10^5) were plated on 100-mm Petri dishes and differentiated by ATRA as described above. Cells were then treated with aldicarb and carbaryl, respectively, for 7 days (see Section 2.3 for induction of the cell differentiation). The cells were harvested by trypsinization. The cell pellet was resuspended in TE buffer (50 mM Tris–HCl/1 mM EDTA, pH 8.0) after centrifugation, homogenized with 10 passages through a 25-gauge hypodermic needle, and centrifuged at 100 × *g* at 4 °C for 2 min. The supernatant fraction was used as cell homogenates for assays of activities of NTE and AChE. Cellular NTE activity was calculated as the difference in phenol liberated from the paraoxon-resistant and mipafox-sensitive hydrolysis of PV according to Johnson [18] with modification for reduced volume microassay. Cell homogenates (150 μl) with 40 μM paraoxon and 0 or 50 μM mipafox was incubated at 37 °C for 30 min, then 150 μl substrate solution (mixture of 1 volume of 15 mg/ml PV and 30 volume of 0.03% Triton X-100 in TE buffer) was introduced. After incubation for 30 min, the reaction was stopped with 150 μl of 1% SDS/0.025% 4-aminoantipyrine in water. Adding 75 μl of 0.4% potassium ferricyanide in water and waiting 5 min allowed colorimetric determination of NTE activity at 510 nm. AChE activity was assayed as described by Gorun et al. [21]. Concentration of protein was measured by the method Lowry et al. with bovine serum albumin as the standard [20].

2.8. Statistical analysis

Data were generally expressed as mean ± standard error values. Groups of data were compared by ANOVA and by post hoc analysis using Student–Keuls method. A difference between means was considered significant at $P < 0.05$.

3. Results

3.1. Effect of aldicarb and carbaryl on neurite length in SK-N-SH cells

Cell differentiation was induced in SK-N-SH cells by addition of 20 μM ATRA, which restricted cell division and produced profound morphological alterations characterized by the elaboration of axonal-like processes. To quantify any proposed changes in cell morphology, cells induced to differentiate in the absence or presence of 50 μM aldicarb or carbaryl were fixed, stained with Coomassie Brilliant Blue, and examined by light microscopy (Fig. 1). Measurements of morphological changes revealed a reduction in the average length of axon-like processes after a 7 day incubation with non-cytotoxic doses of the carbamates. As shown in Fig. 2, the average length of neurites of differentiating cell treated with either aldicarb or carbaryl was 44.8 ± 1.7 and 54.3 ± 2.6 μm, respectively. In contrast, the average

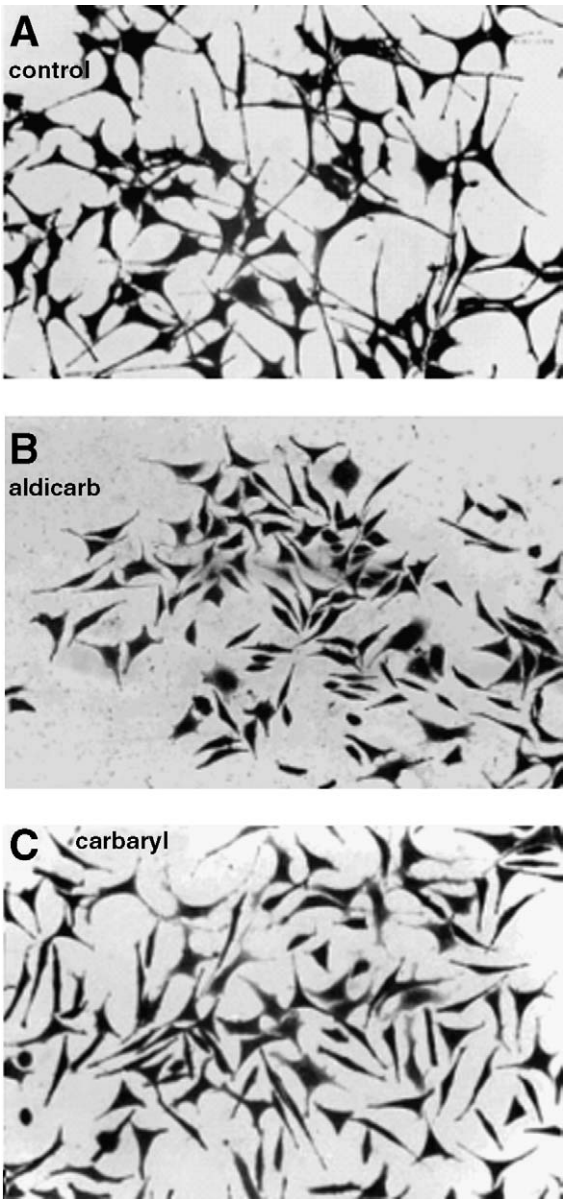

Fig. 1. Phase-contrast photomicrographs demonstrate the effects of carbamates on the morphology of differentiating human SK-N-SH neuroblastoma cells induced by retinoic acid. Control cells exhibit extensive neurites with multiple branches (A). In contrast, aldicarb, and carbaryl-treated cells exhibit the cell bodies with multiple protrusions and some minor neurites (B and C, respectively). All photographs were obtained at the same magnification.

length of neurites in control cells was 114.4 ± 6.9 μm. However, in undifferentiated cells, there was no significant difference in neurite length between control and carbamates-treated cells, suggesting that aldicarb and carbaryl did not affect average neurite length in undifferentiated SK-N-SH cells.

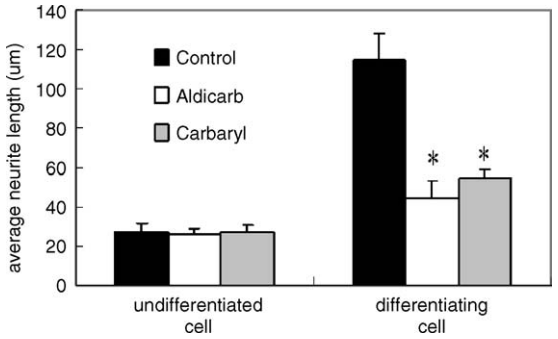

Fig. 2. Effect of carbamates on axon outgrowth in undifferentiated and differentiating SK-N-SH cells. Undifferentiated cells were treated with carbamates indicated for 7 days; SK-N-SH cells were induced to differentiate in the absence or presence of carbamates for 7 days before morphological assessment. Cells were fixed in methanol and stained with Coomassie Blue. Axon length was quantified using an inverted light microscope. Statistical analysis of control vs. neurotoxicant-treated cells was carried out by ANOVA. $^{*}P < 0.05$, compared with control, $n = 3$.

3.2. Cytotoxicity assessment of aldicarb and carbaryl on differentiating SK-N-SH cells

As shown in Fig. 2, aldicarb or carbaryl induced changes in cell morphology in terms of a lack of axon-like processes in the chemicals-treated cells compared with the controls. To confirm the lack of cell death, the MTT reduction assay was used to check cell viability. As shown in Table 1, neither aldicarb nor carbaryl induced cell death. Together, these data suggest that aldicarb and carbaryl induce changes in cell morphology without affecting cell viability.

3.3. Western blotting analysis of cytoskeletal components in differentiating SK-N-SH cells

To understand the molecular basis of the morphological changes observed during neuronal differentiation,

Table 1
Effects of 50 μM carbamates on MTT reduction in SK-N-SH cells

Treatment	MTT reduction (A570)
Control	1.15 ± 0.07
Aldicarb	1.08 ± 0.08
Carbaryl	1.07 ± 0.07

SK-N-SH cells were cultured in the absence or presence of 50 μM aldicarb or carbaryl for 7 day exposure before assessment of the level of MTT reduction. Data shown are the mean absorbance ± S.E. from four separate cultures. These experiments were performed in triplicate. Statistical analysis of control vs. carbamates-treated cells was carried out by ANOVA. All P values of treated cells were >0.05 vs. control, $n = 3$.

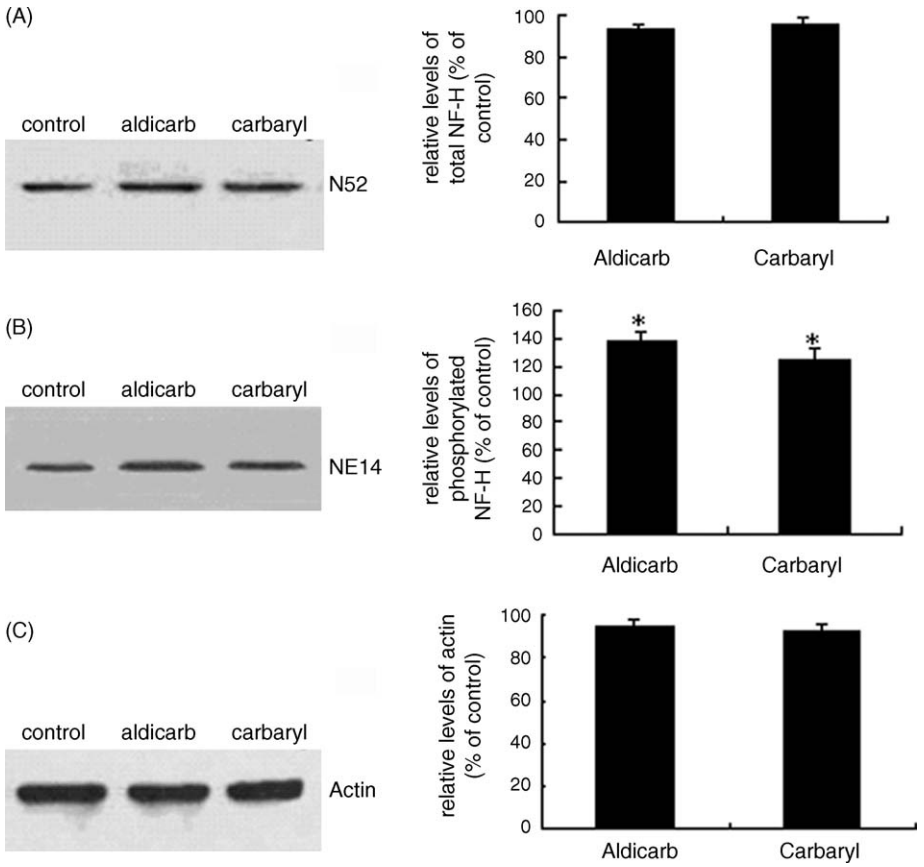

Fig. 3. Western blot analyses of cytoskeletal components from differentiating SK-N-SH cells exposed to aldicarb and carbaryl. Blots were probed with antibodies that recognize total NF-H protein (N52) (A), a phosphorylation-dependent epitope of NF-H (NE14) (B), or actin (C). Blots were then digitalized, and changes in antibody reaction were densitometrically analyzed. Data are mean ± S.E. (bars) of peak area values (*y*-axes) from three separate experiments. The values shown represent change in densitometry of cells exposed to different carbamates relative to control (defined as the 100% value). Shown are control cells and cells exposed to 50 μM aldicarb or carbaryl. $^{*}P < 0.05$, compared with the controls, $n = 3$.

we measured the levels of specific cytoskeletal component. We analyzed the status of the NF-H subunit protein and measured actin protein levels using Western blot analyses of total cell extracts from differentiating SK-N-SH cells exposed to aldicarb or carbaryl for 7 days. To monitor NF-H levels, two distinct antibodies that preferentially recognize a phosphorylation-dependent epitope of NF-H (NE14), and an epitope of NF-H that is independent of phosphorylation state (N52) were used. As shown in Fig. 3A, neither aldicarb nor carbaryl changed the total levels of NF-H protein as compared with control cells. Conversely, either aldicarb or carbaryl produced an increase in the level of phosphorylated NF-H protein (Fig. 3B). The results of densitometric analyses are presented to the right of each and indicate an increase to 147.5 ± 7.6 and 124.3 ± 8.7%, respectively, compared with controls. As shown in Fig. 3C, there was no difference in the levels of actin (a microfilament component) between differentiating SK-N-SH cells exposed to aldicarb and carbaryl compared with controls (Fig. 3C).

3.4. Effect of carbamates on activities of AChE and NTE in differentiating SK-N-SH cells

In order to further reveal the targets of carbamate-induced neurotoxicity in the differentiating cells, we measured changes in relative esterase activities. AChE, the target of these carbamates for acute toxicity, and NTE whose phosphorylation and aging were proposed to be the initiating event in organophosphate-induced delayed neuropathy (OPIDN), were assayed after exposure to aldicarb or carbaryl in differentiating SK-N-SH cells. The basal NTE activity in control cells was 18.9 ± 1.4 nmol phenol/(min mg protein). The basal AChE activity in control cells was 22.8 ± 1.8 nmol acetylthiocholine hydrolyzed/(min mg protein). As indi-

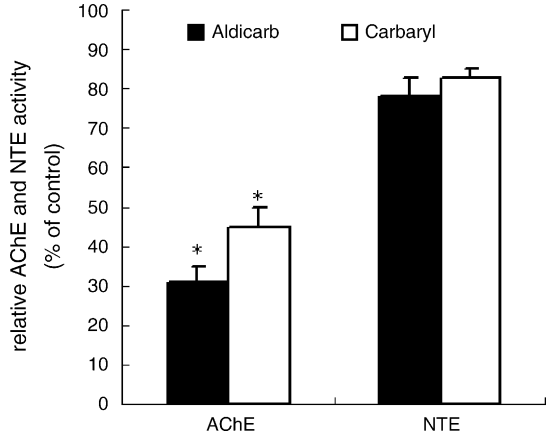

Fig. 4. Effect of carbamates on AChE and NTE activity in differentiating SK-N-SH cells. SK-N-SH cells were induced to differentiate in the absence or presence of carbamates for 7 days and then the activity of AChE and NTE was quantified. The basal activity of NTE and AChE in control cells was 18.9 ± 1.4 nmol phenol/(min mg protein) and 22.8 ± 1.8 nmol acetylthiocholine hydrolyzed/(min mg protein), respectively. Data are mean ± S.E. (bars) from three independent tests. Statistical analysis of control vs. neurotoxicant-treated cells was carried out by ANOVA. $^{*}P < 0.05$, compared with controls, $n = 3$.

cated in Fig. 4, the activity of AChE was significantly reduced to $30.8 \pm 3.9\%$ and $44.6 \pm 5.0\%$ of control cells by aldicarb and carbaryl, respectively. However, the activity of NTE was not affected by treatment with either aldicarb or carbaryl (Fig. 4).

4. Discussion

Cultured neuronal cell lines, including mouse and human neuroblastoma cell, such as N2a and SH-SY5Y, possess the ability of extending and maintaining axon-like processes, and they are suitable for determining induction of axonal degeneration in vitro [22]. Moreover, the inhibition of the formation of extensions by cultured neuroblastoma cells is a valid indicator of neurotoxicity and has been used to discriminate neuropathic from non-neuropathic OPs in vitro [15,23,24]. In differentiating neuroblastoma SH-SY5Y cells, the effects of paraoxon and mipafox (each 50 μM) on neural differentiation were different, suggesting that SH-SY5Y cell was a useful in vitro model to differentiate neurotoxic actions of selected OPs [15]. Studies using clonal neuroblastoma N2a cells, which express a neuronal phenotype, have indicated that carbaryl can alter neuronal morphology following short or extended exposure [13,14]. Mouse neuroblastoma cells (e.g., N2a) are consistently more sensitive than the human neuroblastoma cell line to equimolar doses of various OPs [18]. Therefore, in light of the concentrations used in previous studies [15,17], we chose 50 μM as the test concentration in the carbamates study. The results obtained in this study indicate that 50 μM aldicarb or carbaryl inhibited the development of axon-like processes from differentiating neuroblastoma SK-N-SH cells, but not in undifferentiated cells, without affecting cell viability after 7 day exposure. However, use of different concentrations of aldicarb and carbaryl was not investigated and may enable a better correlation between the effects witnessed and carbamates.

Changes in cell morphology have often been associated with alterations to the cytoskeleton. Indeed, carbaryl toxicity has been correlated with cytoskeletal changes in cultured neuroblastoma cells [13,14]. Neuronal differentiation in vitro is accompanied by profound morphological alterations, including the formation of neurites. This event is accompanied by specific distribution of cytoskeletal components, including neurofilament (NF) subunits, which are associated with aspects of axon outgrowth and axon caliber [25]. Initial axon outgrowth is associated with the assembly of NF subunits and the expression of NF-H is a marker for axonal damage. NF-H contains numerous serine residues that present themselves in a recurring lysine-serine-proline motif, these serine residues acts as a substrate for post-translational phosphorylation [26]. As axon outgrowth continues, the levels of NF protein may increase and the proteins may undergo post-translational phosphorylation, a process linked to axon stabilization. In this study, we did not observe any change in total NF-H protein in cells treated with carbaryl (50 μM), which is consistent with the effect of carbaryl at lower concentration (3 μM) on differentiating N2a cells [14]. Exposure of differentiating SK-N-SH cells to aldicarb and carbaryl produced an altered NF-H reactivity with the phospho-specific antibody NE14. The increase in NE14 reactivity suggests that differentiating SK-N-SH cells exposed to subcytotoxic levels of carbamates exhibit a net increase in NF-H phosphorylation, and that this may be associated with the observed inhibition of neurite elongation. These changes may represent a useful in vitro marker of carbamate-induced neurotoxicity within a simple differentiating neuronal cell model system.

It should also be noted that the average length of neurite was inhibited in SH-SY5Y cells by mipafox [15], an OP with known potential for eliciting delayed neurotoxicity, and NF-H has been an indicator during this process [17]. However, it is evident that different neurotoxicants may cause similar changes in the NF network of differentiating cells, possibly through other pathways. Indeed, leptophos and carbaryl inhibited the formation of axons

in vitro through different early biochemical changes and mechanisms [14]. The change in NF-H phosphorylation characterized in our studies could signify the involvement of protein kinase signaling cascade. Some protein kinases, for example cyclin dependent kinase-5 (CDK-5), are involved in NF-H phosphorylation in SH-SY5Y cells, a cell line derived from SK-N-SH cells [27]. Work is therefore underway to establish the possible involvement of this protein kinase pathway in carbamate-induced changes in NF-H phosphorylation.

Because some carbamates can inhibit both AChE and NTE, carbamates-induced inhibition of neurite-like outgrowth might be related to the cholinergic signaling or NTE-associated pathways. The activity of AChE and NTE are present in SH-SY5Y cells [28]. Our studies for the first time reveal the effect of aldicarb and carbaryl on AChE and NTE in differentiating SH-N-SH cells. These results showed that AChE, but not NTE, was significantly decreased by either aldicarb or carbaryl in differentiating SK-N-SH cells. The sensitivity of AChE to aldicarb or carbaryl is higher than the sensitivity of NTE, and may reflect a variety of reversible neurobehavioral and neurotoxic effects in vertebrates induced by the common insecticide carbaryl, since carbaryl is associated with acute poisoning symptoms via inhibition of AChE [12]. The primary function of AChE is hydrolysis of ACh and expression of AChE during early neuronal development may be closely related to the regulation of neurite outgrowth [29,30]. Together, these results suggest that inhibition of neurite outgrowth by carbamates was associated with inhibition of AChE. However, in differentiated SY5Y cells, AChE activity appears to be unrelated to neurite extension, which was suggested by effect of different OPs on neural differentiation [15,17]. These results suggested that carbamates and OPs blocked axon-like processes by different target in differentiating cells.

In summary, aldicarb and carbaryl blocked neurite length in differentiating SK-N-SH cells at a sublethal concentration (50 μM), which may reflect axonal degeneration induced by carbamates in vitro. Moreover, no change in the total levels of NF-H or increases of NF-H phosphorylation were observed. These changes may represent a useful in vitro marker of carbamates neurotoxicity within a simple differentiating neuronal cell model system and the expression profiles of these proteins are compelling targets for investigation of carbamates neurotoxicity. Furthermore, activity of AChE, but not NTE, was significantly inhibited by aldicarb and carbaryl in the cells, which suggested that inhibition of AChE was involved in the process of neurotoxicity induced by carbamates in differentiating SK-N-SH cells.

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