

available at www.sciencedirect.comjournal homepage: www.elsevier.com/locate/biochempharm

Inhibition of neurite outgrowth in differentiating mouse N2a neuroblastoma cells by phenyl saligenin phosphate: Effects on MAP kinase (ERK 1/2) activation, neurofilament heavy chain phosphorylation and neuropathy target esterase activity

Alan J. Hargreaves^{a,*}, Maxine J. Fowler^{a,1}, Magdalini Sachana^b, John Flaskos^c, Mary Bountouri^a, Ian C. Coutts^a, Paul Glynn^d, Wayne Harris^a, W. Graham McLean^e

^aSchool of Biomedical and Natural Sciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK

^bDepartment of Veterinary Pathology, Faculty of Veterinary Science, University of Liverpool, Leahurst CH64 7TE, UK

^cLaboratory of Biochemistry and Toxicology, Faculty of Veterinary Medicine, Aristotelian University of Thessaloniki, 54124 Thessaloniki, Greece

^dMRC Toxicology Research Unit, University of Leicester, Leicester LE11 5RH, UK

^eDepartment of Pharmacology and Therapeutics, University of Liverpool, Ashton Street, Liverpool L69 3GE, UK

ARTICLE INFO

Article history:

Received 25 October 2005

Accepted 6 January 2006

Keywords:

Phenyl saligenin phosphate

Mouse N2a neuroblastoma

Axon outgrowth

Neurofilament heavy chain

Neuropathy target esterase

MAP kinase (ERK1/2)

Abbreviations:

DFP, diisopropyl

phosphorofluoridate

ERK, extracellular signal-regulated kinase

MAP kinase, mitogen-activated protein kinase

ABSTRACT

Sub-lethal concentrations of the organophosphate phenyl saligenin phosphate (PSP) inhibited the outgrowth of axon-like processes in differentiating mouse N2a neuroblastoma cells (IC₅₀ 2.5 μM). A transient rise in the phosphorylation state of neurofilament heavy chain (NFH) was detected on Western blots of cell extracts treated with 2.5 μM PSP for 4 h compared to untreated controls, as determined by a relative increase in reactivity with monoclonal antibody Ta51 (anti-phosphorylated NFH) compared to N52 (anti-total NFH). However, cross-reactivity of PSP-treated cell extracts was lower than that of untreated controls after 24 h exposure, as indicated by decreased reactivity with both antibodies. Indirect immunofluorescence analysis with these antibodies revealed the appearance of neurofilament aggregates in the cell bodies of treated cells and reduced axonal staining compared to controls. By contrast, there was no significant change in reactivity with anti-α-tubulin antibody B512 at either time point. The activation state of the MAP kinase ERK 1/2 increased significantly after PSP treatment compared to controls, particularly at 4 h, as indicated by increased reactivity with monoclonal antibody E-4 (anti-phosphorylated MAP kinase) but not with polyclonal antibody K-23 (anti-total MAP kinase). The observed early changes were concomitant with almost complete inhibition of the activity of neuropathy target esterase (NTE), one of the proposed early molecular targets in organophosphate-induced delayed neuropathy (OPIDN).

© 2006 Elsevier Inc. All rights reserved.

* Corresponding author. Tel.: +44 1158483378; fax: +44 1158486616.

E-mail address: alan.hargreaves@ntu.ac.uk (A.J. Hargreaves).

¹ Present address: School of Biomedical Sciences, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK. 0006-2952/\$ – see front matter © 2006 Elsevier Inc. All rights reserved. doi:10.1016/j.bcp.2006.01.008

NFH, neurofilament heavy chain
NTE, neuropathy target esterase
OP, organophosphate
OPIDN, OP-induced delayed
neuropathy
PSP, phenyl saligenin phosphate
TOCP, triorthocresyl phosphate
SCOTP, saligenin cyclic-O-tolyl
phosphate
TPCP, triparacresyl phosphate

1. Introduction

Certain organophosphate compounds (OPs) are capable of inducing a delayed neurodegenerative condition in central and peripheral nervous system of man, birds and livestock [1–6]. This condition, which may arise up to 3 weeks after exposure to neuropathic OPs, is known as organophosphate-induced delayed neuropathy (OPIDN). Studies on animal models have shown that the onset of clinical signs of the disease is preceded by inhibition of neuropathy target esterase (NTE) and also by cytoskeletal disruption [4,5,7–10], suggesting that these lesions may represent key molecular events in the development of this delayed neurodegenerative condition. The ability to block the neurodegenerative effects of neuropathic OPs by Ca^{2+} channel blockers and calpain inhibitors also suggests that increased intracellular Ca^{2+} levels and calpain activation may be involved [11]. However, the precise molecular basis of OPIDN is poorly understood.

For many years NTE has been regarded as the primary target for neuropathic OPs, although its inhibition in non-neuronal cells and tissues that are not damaged has raised doubts about a direct role for its esterase activity in the pathogenesis of OPIDN. However, it has been shown that the ability to inhibit NTE alone is not sufficient to induce the chain of events leading to OPIDN. Indeed, a modification that involves an increase in negative charge at the active site is proposed to occur before the induction of OPIDN, a phenomenon known as ageing [5,12,13]. Furthermore, recent work suggests that NTE may have important non-esterase functions, the alteration of which may in some way trigger a series of molecular events leading to Ca^{2+} release and calpain activation [13].

A number of other studies have shown that there is a major rearrangement of the axonal cytoskeleton and substantial changes in the phosphorylation states of key cytoskeletal proteins in microtubule and neurofilament networks within 24 h of exposure to neuropathic OPs [1,7–10]. However, the ability of OPs to disrupt specific cell signalling pathways has not yet been thoroughly investigated. Moreover, there is disagreement as to the exact nature of the changes [8,9] and the relationship between NTE inhibition and cytoskeletal alterations remains to be established.

Detailed in vivo studies of the molecular basis of OPIDN are hampered by the costly and time-consuming nature of experiments on large numbers of laboratory animals. This has led to the development of in vitro alternatives to animal studies and, in particular, the use of cultured nerve cells [14].

One approach has been to measure the effects of neuropathic and non-neuropathic OPs on NTE activity in neuroblastoma cell lines [15–18], which is potentially very useful in the screening of OPs for their potential to cause OPIDN. A second approach has been to study the effects of neuropathic and non-neuropathic OPs on the outgrowth of neurites by neuronal and glial cells induced to differentiate in vitro [19–23]. Interestingly, the neuropathic properties of protoxicants can be enhanced in cultured cells either by bromination [15,16] or by co-incubation with NADPH-activated rat liver microsomes [15,16,20], suggesting that in vitro cellular models are able to simulate at least some of the neurodegenerative effects of OPs in vivo. Furthermore, in a number of the above mentioned studies the authors have reported selective inhibition of neurite outgrowth by neuropathic OPs, although the involvement of NTE in the inhibition of neurite outgrowth remains to be established.

Our work has focused on the sub-lethal effects of OPs on the outgrowth of axon-like processes by differentiating N2a cells. Our previous findings show that the neuropathic *ortho* isomer of tricresyl phosphate (TOCP) inhibits axon outgrowth to a greater extent than the non-neuropathic isomer *triparacresyl* phosphate [20]. This effect, which is most evident following shorter exposure times, is enhanced by incubation in the presence of NADPH-activated microsomes. Since TOCP is thought to be metabolised into active (in terms of ability to induce OPIDN) congeners such as saligenin cyclic-O-tolyl phosphate (SCOTP) [24–27], our findings suggest that differentiating N2a neuroblastoma cells represent a useful model for mechanistic studies of the neurodegenerative effects of OPIDN-inducing OPs. As the exact nature of the microsomal activation products in vitro is not known, it is important to show that known in vivo metabolites are able to induce neuropathic changes in this cell model. The work presented here focuses on the effects of phenyl saligenin phosphate (PSP; a neuropathic homologue of SCOTP) on neurite outgrowth, cytoskeletal proteins, NTE activity and MAP kinase activation in vitro.

2. Materials and methods

2.1. Cell lines and reagents

Mouse N2a neuroblastoma cells were purchased from ICN (Thane, UK). Cell culture plastics were supplied by SLS Laboratory Supplies (Nottingham, UK). Cell culture reagents and monoclonal antibodies against α -tubulin (clone B512) and

NFH (clone N52) were obtained from Sigma-Aldrich Co. Ltd. (Poole, UK). Mouse anti-phospho ERK 1/2 (E-4) and rabbit anti-total ERK 1/2 (K-23) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Fluorescein isothiocyanate (FITC)-conjugated and alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin secondary antibodies were obtained from DakoCytomation (Ely, UK). Monoclonal antibody Ta51 hybridoma culture supernatant [28] was a gift from Dr. M. Carden (Biological Laboratory, University of Kent).

Phenyl saligenin phosphate (PSP) synthesis was based on the method of SCOTP synthesis by Nomeir and Abou-Donia [29] using phenyl phosphorodichloridate in place of *o*-cresyl phosphorodichloridate. Chemical purity was confirmed by NMR and mass spectrometry.

2.2. Maintenance of cultured cells

The cells were grown in T-25 flasks at 37 °C in a humidified atmosphere of 5% CO₂–95% air, in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 1 mM glutamine, penicillin G (100 units/ml) and streptomycin (100 µg/ml). They were maintained in the logarithmic phase of growth and passaged at 4–5 day intervals.

2.3. Induction and assessment of N2a cell differentiation

Cells were taken at 60–80% confluence, seeded in 24-well culture dishes at a density of 50,000 cells/ml in 0.5 ml growth medium, and induced to differentiate by serum withdrawal and the addition of dibutyryl cAMP, as described previously [19,20,30]. They were then incubated for up to 24 h in the presence and absence of PSP to yield various final concentrations, as indicated in Section 3. Cell growth and viability were assessed under all experimental conditions employed by the reduction of methyl blue tetrazolium using the method of Denizot and Lang [31].

For morphological assessment, at the end of the incubation period the cells were fixed and stained with Coomassie brilliant blue and the outgrowth of axon-like processes was determined using an inverted light microscope, as described previously [19,20,30]. This involved the quantification of axon-like processes, defined according to Keilbaugh et al. [32] as neurites greater than 2 cell body diameters in length. Small extensions were defined as outgrowths between 0.5 and 2 cell body diameters in length and the numbers of cells with round or flattened morphology were also determined.

2.4. Western blot analysis

For immunoblot analysis, N2a cells were induced to differentiate in the presence and absence of PSP as described above, except that they were seeded in to 10 ml of growth medium in T-25 flasks. Cell monolayers were then solubilised by boiling in electrophoresis sample buffer comprising 62.5 mM Tris, 2% w/v sodium dodecyl sulphate (SDS), 10% w/v glycerol, 5% w/v β-mercaptoethanol and 0.002% w/v bromophenol blue or in 0.5% w/v SDS. Protein was estimated on acetone-precipitated cell extracts by the method of Lowry et al. [33] with minor modifications, using bovine serum albumin (BSA) as the

standard. Equal amounts of cell protein were subjected to gel electrophoresis in the presence of SDS (SDS-PAGE) using a 7.5% polyacrylamide resolving gel overlaid with a 4% stacking gel [34].

Separated proteins were then electrophoretically transferred onto nitrocellulose membrane filters [35]. The resultant Western blots were blocked with 3% w/v BSA in Tris-buffered saline (BSA/TBS) and then probed overnight at 4 °C with appropriate dilutions of primary antibodies in BSA/TBS [20]. These included mouse monoclonal anti-α-tubulin (B512), mouse monoclonal anti-total neurofilament heavy chain (N52) or rat monoclonal anti-phosphorylated neurofilament heavy chain (Ta51). Alternatively, they were probed with mouse monoclonal anti-phosphorylated ERK1/2 MAP kinase (clone E-4) or rabbit polyclonal anti-total ERK 1/2 (K-23). After washing extensively with TBS/Tween-20, blots were probed with appropriate dilutions of secondary antibodies and enzyme substrates, as described previously [20]. Antibody reactivity on developed blots was quantified densitometrically using Quantiscan software (BioSoft) and values expressed as a percentage of their corresponding controls. The phosphorylation status of ERK 1/2 was also determined as the ratio between the levels of reactivity with anti-phosphorylated ERK and anti-total ERK compared to controls.

2.5. Indirect immunofluorescence staining

N2a cells were induced to differentiate for 24 h in the presence and absence of 2.5 µM PSP. They were then fixed with methanol and stained with antibodies that recognise total neurofilament heavy chain (NFH) independent of its phosphorylation state (clone N52) or NFH phosphorylated in its KSP domain (clone Ta51) followed by FITC-labelled secondary antibodies, as described previously [19].

2.6. Measurement of neuropathy target esterase activity

For assays of cellular NTE activity, cells were induced to differentiate as above, except that they were seeded into 40 ml of growth medium in T-75 cell culture flasks. At the end of the incubation period, cells were harvested by centrifugation at 1200 × *g* for 10 min, and then washed by resuspension and centrifugation in PBS. Pellets were stored at –20 °C until required. NTE activity was determined in homogenates of control (untreated) and PSP-treated cells by the absorbance difference for phenyl valerate hydrolysis between samples exposed to paraoxon only and those exposed to both paraoxon and mipafox, as previously described [36].

2.7. Statistical analysis

Unless indicated otherwise, axon outgrowth, NTE activity and densitometric peak areas of bands revealed on probed Western blots of cells/cell extracts following exposure to OPs were expressed as a percentage of control values ± S.E.M. When appropriate, the statistical significance of differences from corresponding controls was established using the Mann–Whitney *U*-test for non-parametric distributions or by ANOVA, with 95% confidence limits.

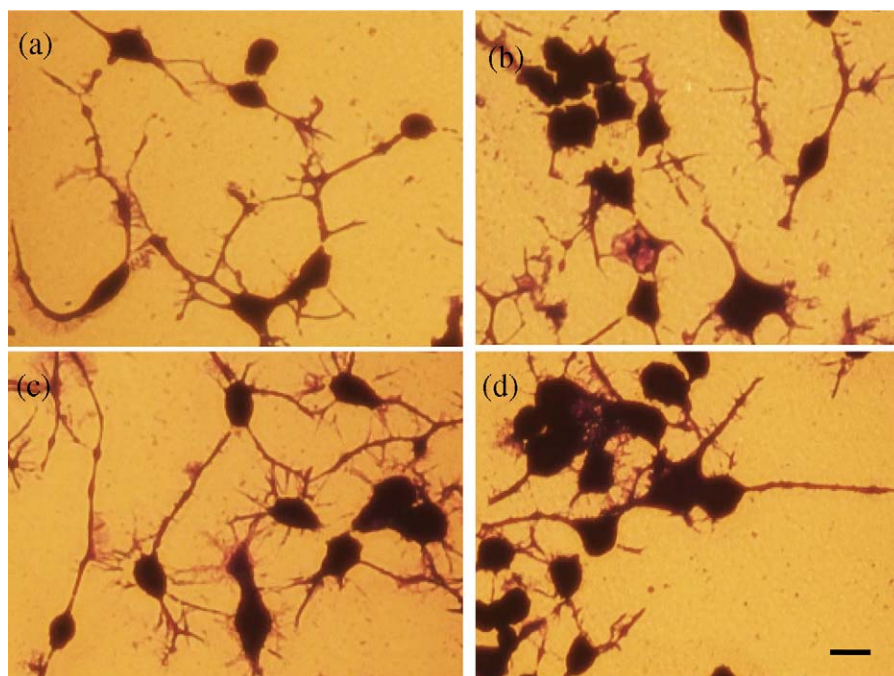


Fig. 1 – Effects of PSP on the morphology of differentiating N2a cells. N2a cells were induced to differentiate for 4 h (a and b) or 24 h (c and d) in the presence (b and d) and absence (a and c) of 2.5 μ M PSP, as described in Section 2. Shown are images of typical fields of cells viewed by phase contrast microscopy after 4 h (a and b) or 24 h (c and d) differentiation, using an inverted light microscope following methanol fixation and staining with Coomassie blue. Bar represents 15 μ m.

3. Results

Since our previous work suggested that metabolic activation of TOCP could enhance its ability to inhibit the outgrowth of axon-like processes [20], it was of interest to determine the direct effects of active congeners of TOCP on neurite outgrowth in the present work. Experiments were therefore carried out with PSP, a structural analogue of SCOTP that has the ability to cause OPIDN *in vivo* without the need for metabolic activation [37]. Preliminary work (not shown) indicated that PSP inhibited the outgrowth of axon-like processes at various exposure times between 4 and 48 h, with IC_{50} values all within the range of 2.2–3.0 μ M. Accordingly, a concentration of 2.5 μ M was adopted for subsequent experiments. As indicated in Fig. 1, there was an apparent decrease

in the outgrowth of neurites compared to controls in cells induced to differentiate for 4 and 24 h in the presence of 2.5 μ M PSP. Similar effects were observed at 8 h and 48 h time points (not shown). Quantitative assessment of cell morphology following treatment with this concentration of PSP confirmed that there was a statistically significant reduction in the outgrowth of axon-like processes compared to controls (Table 1). By contrast, no significant effect was observed on the number of smaller neurites (extensions) or on the proportion of round and flat cell (Table 1).

In order to study the molecular basis of these effects in more detail, Western blots of extracts from cells induced to differentiate in the presence and absence of 2.5 μ M PSP were probed with antibodies that recognise key proteins involved in the process of neurite outgrowth. These included antibodies

Table 1 – Effects of PSP on neurite outgrowth in differentiating N2a cells

Incubation time	Treatment	Axons	Extensions	Round cells	Flat cells
4 h	CONTROL	32.35 \pm 2.2	29 \pm 1.1	16 \pm 0.5	84 \pm 0.5
	2.5 μ M PSP	13.2 \pm 1.4*	29 \pm 1.1	15 \pm 0.6	85 \pm 0.6
24 h	CONTROL	35.9 \pm 6.5	24 \pm 0.8	19 \pm 0.8	81 \pm 0.8
	2.5 μ M PSP	18.5 \pm 1.1*	28 \pm 1.5	25 \pm 2.0	75 \pm 2.0

N2a cells were induced to differentiate for 4 or 24 h in the presence and absence of 2.5 μ M PSP, stained with Coomassie blue and assessed for axon outgrowth, outgrowth of small extensions and round/flat cell morphologies, as described in Section 2. Data are expressed as number per 100 cells \pm S.E.M. from four independent experiments. Significance of differences from controls was determined by ANOVA.

* $p < 0.05$.

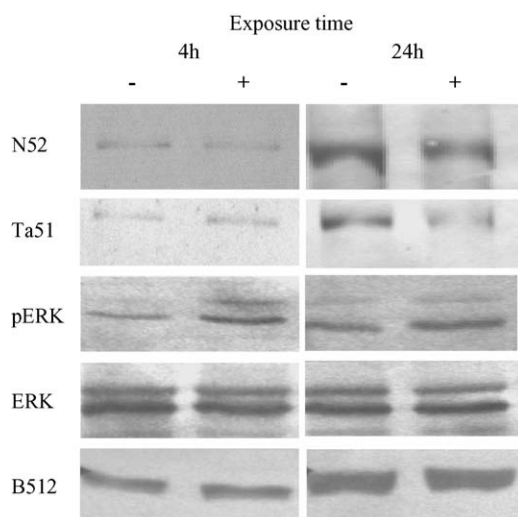


Fig. 2 – Western blotting analysis of extracts from N2a cells induced to differentiate in the presence and absence of PSP. N2a cells were induced to differentiate for 4 or 24 h in the presence (+) and absence (–) of 2.5 μ M PSP, as indicated, after which extracts were solubilised, separated by SDS-PAGE and transferred onto nitrocellulose membrane filters as described in Section 2. Shown are Western blots of equal amounts of control and PSP-treated cell protein probed with antibodies that recognise total NFH (N52), phosphorylated NFH (Ta51), activated ERK (pERK), total ERK (ERK) and total α -tubulin (B512), followed by an appropriate alkaline phosphatase-conjugated secondary antibody.

against NFH (clones N52 and Ta51, which recognise total NFH and phosphorylated NFH, respectively), α -tubulin (clone B512) and MAP kinase (ERK1/2; monoclonal antibody E-4 and polyclonal antibody K-23, which recognise activated and total ERK 1/2, respectively). As shown in Fig. 2 and Table 2, cross-reactivity of an approximately 200 kDa polypeptide with N52 (which recognises NFH independently of its phosphorylation state) shows a slight but significant decrease compared to controls at 4 h and after 24 h exposure. By contrast, reactivity with Ta51 (which recognises a phosphorylated epitope in the KSP domain of NFH) was significantly higher than control values at 4 h, followed by a decline compared to control values at 24 h exposure. By contrast, reactivity of cell extracts with monoclonal anti-tubulin antibody B512 showed no significant difference from controls at either time point.

In order to determine whether the observed relative increase in reactivity with Ta51 compared to N52 in PSP-treated cell extracts reflected altered activation of MAP kinase (ERK 1/2), of which NFH is a known substrate during neuronal cell differentiation [38,39], blots were also probed with antibodies against activated and total ERK. As shown in Fig. 2 and Table 2, total ERK levels were slightly lower than controls at 4 h exposure but showed no significant change from controls at 24 h. By contrast, reactivity of cell extracts with anti-phospho ERK antibody was 240% of control values at 4 h and 157% of control values at 24 h, corresponding to phosphorylation ratios of 3.6 and 1.6, respectively.

Table 2 – Effects of PSP on the levels of cytoskeletal proteins and ERK in N2a cells induced to differentiate in the presence and absence of PSP

Antibody	Exposure time	
	4 h	24 h
	Densitometric peak area (% control)	
N52	75.9 \pm 10.4*	74.2 \pm 15.9
Ta51	168.2 \pm 24.1*	66.5 \pm 24.3
pERK	301.0 \pm 27.4*	168.6 \pm 7.6*
ERK	82.9 \pm 6.8*	102.8 \pm 6.9
B512	83.8 \pm 11.2	104.7 \pm 11.3

N2a cells were induced to differentiate in the presence (+) and absence (–) of 2.5 μ M PSP for 4 or 24 h (as indicated), solubilised, separated by SDS-PAGE and subjected to Western blotting as described in Section 2. They were then probed with antibodies against total NFH (N52), phosphorylated NFH (Ta51), activated ERK (pERK), total ERK (ERK) and total α -tubulin (B512), as described in Section 2. Densitometric analysis was performed using Quantiscan software. All values were normalised to the levels of α -tubulin reactivity and are expressed as mean % control values \pm S.E.M. for a minimum of three independent experiments.

* Indicates a significance of $p < 0.05$ compared to the corresponding control.

The possibility that the observed change in NFH phosphorylation in PSP-treated cells might cause neurofilament disruption was further investigated by indirect immunofluorescence staining. As shown in Fig. 3, differentiated cells exhibited relatively strong axonal staining (vertical arrows) with both monoclonal antibodies N52 and Ta51. By contrast, PSP-treated cells showed relatively strong aggregate staining (horizontal arrows) in the cell body with both antibodies and staining of axon-like processes was relatively weak.

Since NTE inhibition is thought to be an early lesion occurring within hours of exposure to PSP both in vivo and in vitro, but its relationship to cytoskeletal changes or cell signalling is not yet known, it was of interest to measure NTE activity at early time points in differentiating N2a cells. Indeed, the activity of NTE in extracts from differentiating N2a cells exposed for 4 or 8 h to 2.5 μ M PSP was greatly reduced to 2.6 \pm 1.4% and 6.3 \pm 4.5% of the corresponding control values, respectively (four independent experiments).

4. Discussion

The work presented here shows that PSP is able to inhibit the outgrowth of axon-like processes by differentiating N2a cells with no significant effect on the outgrowth of smaller processes or cell shape. Since PSP is an analogue of one of the main neuropathic metabolites of TOCP [37], the observed inhibition of neurite outgrowth is in good agreement with our previous work, in which the neuropathic effect of TOCP in vitro was enhanced by metabolic activation with NADPH activated rat liver microsomes [20]. It is also consistent with the findings from in vivo studies of the pathogenesis of OPIDN, in which PSP and other metabolites of TOCP are more effective than TOCP in the induction of OPIDN [1,2,20,21,23,32]. The fact that the longer (i.e. “axon-like”) processes are more sensitive to

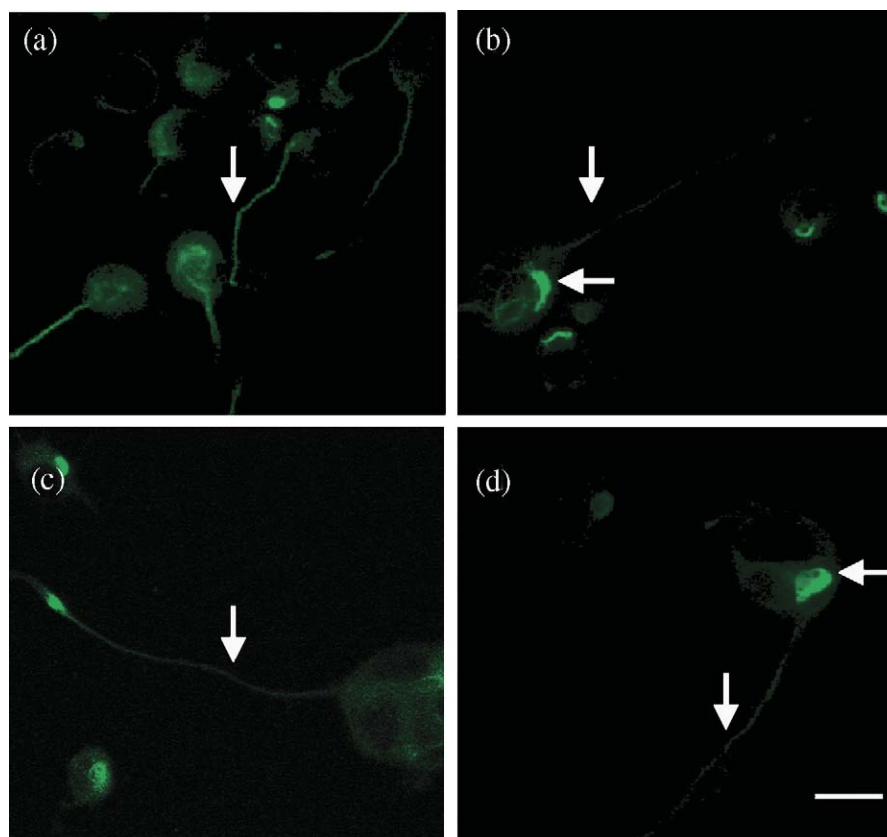


Fig. 3 – Effects of PSP on neurofilament heavy chain distribution in differentiating N2a cells. N2a cells were induced to differentiate for 24 h in the presence (b and d) and absence (a and c) of 3 μ M PSP; they were then stained by indirect immunofluorescence using monoclonal antibodies N52 and Ta51, which recognise total NFH (a and b) and NFH phosphorylated in its KSP domain (c and d), respectively. Downward arrows indicate the position of axon-like processes, horizontal arrows indicate cell body accumulation of NFH staining and the bar represents 10 μ m.

PSP treatment *in vitro* agrees with previous studies using TCP (mixed isomers) and TOCP, in which the outgrowth of smaller processes was also unaffected [19,20]. These observations are also consistent with the finding that axons of longer neurons tend to be more vulnerable to neuropathic OPs *in vivo* [1].

Interestingly, the observation of decreased reactivity of PSP-treated cell extracts with N52 is in agreement with our previous findings for TOCP [20]. The fact that it is also in agreement with a more recent *in vivo* study using sciatic nerves of hens treated with TOCP [40] indicates that this cellular model is capable of predicting the pattern of *in vivo* delayed toxicity of OPs. Since the epitope recognised by N52 is independent of its phosphorylation state, this suggests that there may be a decrease in the levels of NFH at this time point. This could be attributed to increased degradation of NFH by proteases such as cathepsin D or calpain [41,42]. Indeed, activation of calpain has been shown to occur in response to OP treatment *in vivo* [11]. Alternatively, the reduced reactivity could be caused by decreased synthesis of NFH. In this respect, it has been shown that the OP pesticide chlorpyrifos is able to reduce the levels of DNA and protein synthesis *in vivo* [43]. However, the OP compound diisopropyl phosphorofluoridate (DFP) causes an increase in levels of neurofilament mRNA [44], suggesting that some OPs are able to induce expression of

NFH, although the levels of protein were not measured in that work. It may therefore be that structurally different groups of OPs are able to inhibit neurite outgrowth through a variety of mechanisms.

Quantitative analysis of Western blots probed with Ta51, indicated that PSP induced a significant increase in reactivity concomitant with reduced levels of N52 reactivity (total NFH) at the 4-h time point. Since Ta51 recognises NFH in a phosphorylation-dependent manner and the binding of N52 is independent of phosphorylation state, this result indicates an increase in the phosphorylation state of NFH at this time point. The subsequent decrease in Ta51 reactivity following 24 h exposure suggests that the increase in NFH phosphorylation at 4 h is transient. Furthermore, the levels of reactivity at 24 h exposure are similar to those found in TOCP-treated cells [20] indicating that sustained inhibition of neurite outgrowth is associated with reduced levels of NFH.

The fact that PSP but not TOCP, TCP or TPCP causes a transient increase in NFH phosphorylation suggests that PSP may have some distinct molecular targets compared to the other compounds and that increased NFH phosphorylation may require metabolic activation of TOCP. As mentioned in our previous work, smaller increases in the phosphorylation state of NFH may be masked by proteolytic degradation.

Interestingly, Suwita et al. [10] demonstrated an increased level of in vitro Ca^{2+} /calmodulin activated protein kinase-dependent phosphorylation of NFH in spinal cord neurofilaments isolated from TOCP-treated hens, suggesting that altered phosphorylation of NFH and other cytoskeletal proteins was an early event in the pathogenesis of OPIDN. This suggests that increased phosphorylation of cytoskeletal proteins is a key early event following exposure to TOCP and, presumably, its activated metabolites in vivo. However, work by Jortner et al. [9] suggested that reactivity of the phosphorylated NFH specific antibody SMI31 with PSP-treated hen spinal cord neurofilaments was lower than controls between 4 days exposure and the onset of clinical OPIDN. This suggests that, if it occurs in vivo, the OP-induced increase in phosphorylation of NFH observed by Suwita et al. [10] is not sustained for more than a few days.

The changes in NFH phosphorylation suggested that disruption of cell signalling pathways might occur following exposure to PSP. It was of particular interest to study the effects of this OP on the activation of the MAP kinase ERK 1/2, since this protein kinase is a convergence point of cell signalling events involved in neurite outgrowth and it is known to phosphorylate NFH [38,39]. The increase in reactivity of anti-activated (phosphorylated) ERK but not anti-total ERK antibody with cell extracts following 4 h exposure to PSP suggests that PSP treatment leads to increased ERK activation, which may account for the increased level of NFH phosphorylation at this time point. Reactivity with probed blots of extracts exposed to PSP for 24 h indicates a smaller increase in activation over control levels at this time point than at 4 h, possibly due to phosphatase activity or negative feedback phosphorylation of MAP kinase kinases such as MEK [45]. Thus, it may be that the decreased levels of NFH phosphorylation at 24 h occurs as a combination of both down regulation of ERK activation and the proteolytic degradation suggested earlier.

A recognised early target for OPIDN-inducing OPs is NTE, although a link between NTE inhibition and cytoskeletal disruption had not been previously established in the differentiating N2a model. The almost complete loss of NTE activity observed at the 4 h time point in PSP-treated differentiating N2a cells is similar to that observed by Ehrich et al. [46] who used mitotic human neuroblastoma cells treated with 10 μM PSP. This result confirms that NTE inhibition is another key early event following exposure of differentiating N2a cells to PSP. The precise way in which NTE inhibition and ageing are related to the observed changes in MAP kinase activation and NFH levels and phosphorylation is not yet known. It has been suggested that endoplasmic reticulum-associated NTE may have the ability to hydrolyse lipids or act as an ion channel [47,48]. It may therefore be that the disruption of these properties is involved in the response to OP exposure in this cellular system.

In conclusion, the work presented in this paper shows that exposure of differentiating N2a cells to PSP causes selective inhibition of the outgrowth of axon-like processes. This is the first demonstration that inhibition of neurite outgrowth in a differentiating neuronal cell system is associated with reduced NTE activity (i.e. a proposed early molecular target of OPs in vivo). In addition, it is the first report of transiently enhanced phosphorylation of KSP domains on NFH and increased MAP kinase activation in situ. Further work on differentiating N2a

cells will help to unravel the critical early molecular events following intoxication with neuropathic OP compounds.

Acknowledgements

The authors would like to thank Mr. Michael Wood for technical assistance. The early stages of the work were funded by the Wellcome Trust (grant no. 044388). M.B. received a grant from the European Social Fund.

REFERENCES

- [1] Abou-Donia MB, Lapadula DM. Mechanisms of organophosphorus ester-induced delayed neurotoxicity: Type I and Type II. *Ann Rev Pharmacol Toxicol* 1990;30: 405–40.
- [2] Chambers H. Organophosphate compounds: an overview. In: *Organophosphates: chemistry, fate and effects*. New York: Academic Press; 1992. p. 3–17.
- [3] Hatch RC. Poisons causing nervous stimulation or depression. In: Booth NH, McDonald LE, editors. *Veterinary pharmacology and therapeutics*. Ames: Iowa State University Press; 1988. p. 1053–100.
- [4] Johnson MK. Organophosphorous esters causing delayed neurotoxic effects. Mechanism of action and structure/activity studies. *Arch Toxicol* 1975;34:259–88.
- [5] Johnson MK. Organophosphates and delayed neuropathy – Is NTE alive and well? *Toxicol Appl Pharmacol* 1990;102:385–9.
- [6] Rosenstock L, Keifer M, Daniell WE, McConnell R, Claypoole K. The Pesticide Health Effects Study Group. Chronic central nervous system effects of acute organophosphate pesticide intoxication. *Lancet* 1991;338:223–7.
- [7] Abou-Donia MB. The cytoskeleton as a target for organophosphorus ester-induced delayed neurotoxicity (OPIDN). *Chem Biol Interact* 1993;87:383–93.
- [8] Jensen KF, Lapadula DM, Knott Anderson J, Haykal-Coates N. Anomalous phosphorylated neurofilament aggregations in central and peripheral axons of hens treated with tri-*ortho*-cresyl phosphate (TOCP). *J Neurosci Res* 1992;33:455–60.
- [9] Jortner BS, Perkins SK, Ehrich M. Immunohistochemical study of phosphorylated neurofilaments during the evolution of organophosphorus ester-induced delayed neuropathy. *NeuroToxicology* 1999;20:971–6.
- [10] Suwita E, Lapadula DM, Abou-Donia MB. Calcium and calmodulin enhanced in vitro phosphorylation of hen brain cold stable microtubules and spinal cord neurofilament proteins following a single oral dose of tri-*o*-cresyl phosphate. *Proc Natl Acad Sci U S A* 1986;83:6174–8.
- [11] El-Fawal HA, Ehrich MF. Calpain activity in organophosphorus-induced delayed neuropathy (OPIDN): Effects of a phenylalkylamine calcium channel blocker. *Ann N Y Acad Sci* 1993;679:325–9.
- [12] Glynn P. Neuropathy target esterase. *Biochem J* 1999;344:625–31.
- [13] Glynn P. Neural development and neurodegeneration: two faces of neuropathy target esterase. *Prog Neurobiol* 2000;61:61–74.
- [14] Veronesi B, Ehrich M, Blusztajn JK, Oortgiesen M, Durham H. Cell culture models of interspecies selectivity to organophosphorus insecticides. *NeuroToxicology* 1997;18:283–98.
- [15] Barber D, Correll L, Ehrich M. Comparative effectiveness of organophosphorus protoxicant activating systems in

- neuroblastoma cells and brain homogenates. *J Toxicol Environ Health* 1999;57:63–74.
- [16] Barber D, Correll L, Ehrich M. Comparison of two in vitro activation systems for protoxicant organophosphorus esterase inhibitors. *Toxicol Sci* 1999;47:16–22.
- [17] Nostrandt AC, Ehrich M. Development of a model cell culture system in which to study early effects of neuropathy-inducing organophosphate esters. *Toxicol Lett* 1992;60:107–14.
- [18] Nostrandt AC, Ehrich M. Modification of mipafox-induced inhibition of neuropathy target esterase in neuroblastoma cells of human origin. *Toxicol Appl Pharmacol* 1993;121:36–42.
- [19] Flaskos J, McLean WG, Fowler MJ, Hargreaves AJ. Tricresyl phosphate inhibits the formation of axon-like processes and disrupts neurofilaments in cultured mouse N2a and rat PC12 cells. *Neurosci Lett* 1998;242:101–4.
- [20] Fowler MJ, Flaskos J, McLean W, Hargreaves AJ. Effects of neuropathic and non-neuropathic isomers of tricresyl phosphate and their microsomal activation on the production of axon-like processes by differentiating mouse N2a neuroblastoma cells. *J Neurochem* 2001;76:671–8.
- [21] Henschler D, Schmuck G, Van Aerssen M, Schiffmann D. The inhibitory effect of neuropathic organophosphate esters on neurite outgrowth in cell cultures: A basis for screening for delayed neurotoxicity. *Toxicol In Vitro* 1992;6:327–35.
- [22] Li W, Casida JE. Organophosphorus neuropathy target esterase inhibitors selectively block outgrowth of neurite-like and cell processes in cultured cells. *Toxicol Lett* 1998;98:139–46.
- [23] Schmuck G, Ahr HJ. Improved in vitro method for screening organophosphate-induced delayed polyneuropathy. *Toxicol In Vitro* 1997;11:263–70.
- [24] Casida JE, Eto M, Baron BL. Biological activity of triorthocresyl phosphate metabolite. *Nature* 1961;191:1396.
- [25] Eto M, Casida JE, Eto T. Hydroxylation and cyclization reactions involved in the metabolism of tri-*o*-cresyl phosphate. *Biochem Pharmacol* 1962;11:337–52.
- [26] Nomeir AA, Abou-Donia MB. Studies on the metabolism of the neurotoxic tri-*o*-cresyl phosphate. Synthesis and identification by infrared, proton nuclear magnetic resonance and mass spectrometry of five of its metabolites. *Toxicology* 1986;38:1–13.
- [27] Sharma RP, Watanabe PG. Time related disposition of triorthotolyl phosphate (TOTP) and metabolites in chickens. *Pharmacol Res Commun* 1974;6:475–84.
- [28] Lee VMY, Otvos Jr L, Carden MJ, Hollosi M, Dietzchold B, Lazzarini RA. Identification of the major multiphosphorylation site in mammalian neurofilaments. *Proc Natl Acad Sci U S A* 1988;85:1998–2002.
- [29] Nomeir AA, Abou-Donia MB. Studies on the metabolism of the neurotoxic tri-*o*-cresyl phosphate. Synthesis and identification by infrared, proton nuclear magnetic resonance and mass spectrometry of five of its metabolites. *Toxicology* 1986;38:1–13.
- [30] Flaskos J, Fowler MJ, Teurtrie C, Hargreaves AJ. Effects of carbaryl and trichlorfon on axon outgrowth in cultured neurons. *Toxicol Lett* 1999;110(1):79–84.
- [31] Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival – Modification to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 1986;89:271–7.
- [32] Keilbaugh SA, Prusoff WH, Simpson MV. The PC12 cell as a model for studies on the mechanism of induction of peripheral neuropathy by anti-HIV-1 dideoxynucleoside analogs. *Biochem Pharmacol* 1991;42:R5–8.
- [33] Lowry OH, Rosebrough NJ, Farr AL, Randall R. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75.
- [34] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 1970;227:680–5.
- [35] Towbin S, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci U S A* 1979;76:4350–4.
- [36] Johnson MK. Improved assay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. *Arch Toxicol* 1977;37:113–35.
- [37] Jortner BS, Ehrich M. Neuropathological effects of phenyl saligenin phosphate in chickens. *NeuroToxicology* 1987;8:303–14.
- [38] Perron JC, Bixby JL. Distinct neurite outgrowth signalling pathways converge on ERK activation. *Mol Cell Neurosci* 1999;13:362–78.
- [39] Veeranna, Amin ND, Ahn NG, Jaffe H, Winters CJ, Grant P, et al. Mitogen-activated protein kinases (ERK 1/2) phosphorylate Lys-Ser-Pro (KSP) repeats in neurofilament proteins NFH and NFM. *J Neurosci* 1998;18:4008–21.
- [40] Zhao XL, Zhu ZP, Zhang TL, Zhang CL, Yu LH, Xie KQ. Tri-*ortho*-cresyl phosphate (TOCP) decreases the levels of cytoskeletal proteins in hen sciatic nerve. *Toxicol Lett* 2004;152:139–47.
- [41] James T, Matzelle D, Bartus R, Hogan EL, Banik NL. New inhibitors of calpain prevent degradation of cytoskeletal and myelin proteins in spinal cord in vitro. *J Neurosci Res* 1998;51:218–22.
- [42] Nixon RA, Marotta CA. Degradation of neurofilament proteins by purified human brain cathepsin D. *J Neurochem* 1984;43:507–16.
- [43] Song X, Violin JD, Seidler FJ, Slotkin TA. Modeling the developmental neurotoxicity of chlorpyrifos: Macromolecule synthesis in PC12 cells. *Toxicol Appl Pharmacol* 1998;151:182–91.
- [44] Gupta RP, Lin WW, Abou-Donia MB. Enhanced mRNA expression of neurofilament subunits in the brain and spinal cord of diisopropyl phosphorofluoridate-treated hens. *Biochem Pharmacol* 1999;57:1245–51.
- [45] Brunet A, Pagès G, Pouyssegur J. Growth factor-stimulated MAP kinase induces rapid retrophosphorylation and inhibition of MAP kinase kinase (MEK1). *FEBS Lett* 1994;346:299–303.
- [46] Ehrich M, Correll L, Veronesi B. Neuropathy target esterase inhibition by organophosphorus esters in human neuroblastoma cells. *NeuroToxicology* 1994;15:309–13.
- [47] Forshaw PJ, Atkins J, Ray DE, Glynn P. The catalytic domain of human neuropathy target esterase mediates an organophosphate-sensitive ionic conductance across liposome membranes. *J Neurochem* 2001;79:400–6.
- [48] Van Teinhoven M, Atkins J, Li Y, Glynn P. Human neuropathy target esterase catalyses hydrolysis of membrane lipids. *J Biol Chem* 2002;207:20942–8.