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OKADAIC ACID INDUCES THE RAPID AND REVERSIBLE DISRUPTION OF THE NEUROFILAMENT NETWORK IN RAT DORSAL ROOT GANGLION NEURONS

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Treatment of 15-17 day old dissociated cultures of rat dorsal root ganglia with 1 μM okadaic acid caused a reduction in the mobilities of neurofilament subunits on SDS-polyacrylamide gels, signifying an increase in their phosphorylation levels. When cultures were exposed to okadaic acid for 0.5 hrs and harvested in buffer containing Triton X-100, NF-H was nearly completely redistributed to the detergent- soluble fraction while NF-M and NF-L required a longer exposure to the drug before undergoing a similar shift. This redistribution of subunits corresponded with striking changes in the immunofluorescence staining pattern for neurofilaments. Upon removal of okadaic acid from the culture medium following a 0.5 hr treatment, NF-L and NF-M returned to the Triton X-100 insoluble fraction within 2 hrs while NF-H required 10 hrs for recovery.

Neurofilaments (NFs) belong to the class of cytoskeletal proteins known as intermediate filaments and are expressed specifically in neurons. Mammalian NFs consist of three phosphoprotein subunits, NF-H, NF-M and NF-L, whose apparent molecular weights on SDS-polyacrylamide gels (SDS-PAGE) are 200 kDa, 145 kDa and 68 kDa, respectively, for the highly phosphorylated forms (1,2). The role of phosphorylation in NF function remains unclear, although it has been implicated in a variety of processes (3-8). Since phosphate moieties undergo turnover as NFs are transported down the axon (9) it appears that some aspect(s) of NF metabolism may be modulated by cyclical phosphorylation and dephosphorylation events.

Okadaic acid (OA) is a complex fatty acid derivative found in dinoflagellates which specifically inhibits protein phosphatase 1 (PP-1) and protein phosphatase 2A (PP-2A) with

Abbreviations: **DRG**, dorsal root ganglia; **EGTA**, ethyleneglycol-bis-(ß-aminoethyl ether)N,N'-tetraacetic acid; **kDa**, kilodalton; **NF**, neurofilament; **H,M** and **L** refer to the high, mid-sized and low molecular weight subunits; **OA**, okadaic acid; **PP-1** and **PP-2A** refer to protein phosphatases 1 and 2A; **SDS-PAGE**, sodium dodecylsulfate- polyacrylamide gel electrophoresis; **TBS**, tris-buffered saline; **Triton**, Triton X-100.

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IC_{50} values of 10 nM and 0.1 nM, respectively (10). The effects of this tumour promotor on different cell types is varied (see 11 for review). In fibroblasts, OA has recently been shown to increase phosphorylation of another intermediate filament protein, vimentin (12). We therefore set out to determine what effect OA would have on the three NF subunits in the neurons of cultured rat dorsal root ganglia (DRG). We show that OA caused a disruption of the NF network characterized by the hyperphosphorylation of NF subunits and disassembly of NFs. These effects were reversible within 10 hrs after removal of OA.

Materials and Methods

Materials: OA was purchased from LC Services Corp. (Woburn, MA). Insulin, progesterone, selenium, putrescine and all monoclonal antibodies (antiNF-H, N52; antiNF-M, NN18; antiNF-L, NR4) were purchased from Sigma Chemical Co. (St. Louis, MO). Transferrin was from ICN, Canada.

Cell Culture: Rat DRG were dissected from E15 embryos, dissociated with trypsin (13) and maintained in defined medium (14). Where indicated, Triton buffer contained 1% Triton X-100, 50 mM TrisHCl pH 7.5, 100 mM NaCl, 2 mM EGTA, 2 mM levamisole, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 25 μM leupeptin and 40 U/mL aprotinin. Cultures were used 15 to 17 days after plating.

Gel Electrophoresis and Western Blotting: Proteins were solubilized in SDS sample buffer and fractionated on 5% SDS-polyacrylamide slab gels (15). Proteins were electrophoretically transferred to Immobilon-P membranes (Millipore Corp.) in buffer containing 25 mM Tris, 192 mM glycine and 20% methanol. The membrane was blocked with 0.5% gelatin in TBS-Tween (20 mM TrisHCl pH 7.6, 137 mM NaCl and 0.1% Tween-20), incubated with primary antibody and detected using the ECL Western Blotting Detection Kit (Amersham) following the manufacturers instructions. Two-dimensional gel electrophoresis was performed by the method of O'Farrell et al. (16).

Immunofluorescence microscopy: Cells were rinsed, fixed with ethanol/acetic acid (90:10), blocked with goat serum for 1 hr, incubated with primary antibody for 1 hr, washed and incubated with antimouse-Texas Red conjugated antibody for 1 hr and mounted on coverslips.

Results

To begin elucidating the role of phosphorylation in NF organization and distribution we chose to treat rat DRG with OA, an inhibitor of PP-1 and PP-2A. Various concentrations of OA were tested for their effects on the behaviour of the three NF subunits on SDS-PAGE as increased phosphorylation of NF-M and NF-H is known to reduce their mobilities (2). The least phosphorylated form of NF-H did not shift noticeably until 0.1μM OA and continued to increase in apparent molecular weight at OA concentrations up to 1 μM (data not shown). Slower migrating forms of NF-L and NF-M were seen at a concentration of 0.5μM OA and persisted at 1 μM OA. We chose to use 1 μM OA for all subsequent experiments, as this concentration is recommended for intact cells (11,17,18).

Fig.1 shows the distribution of the three NF subunits in Triton-soluble (S) and insoluble (P) fractions. After treatment with OA, cells were harvested in buffer containing Triton and

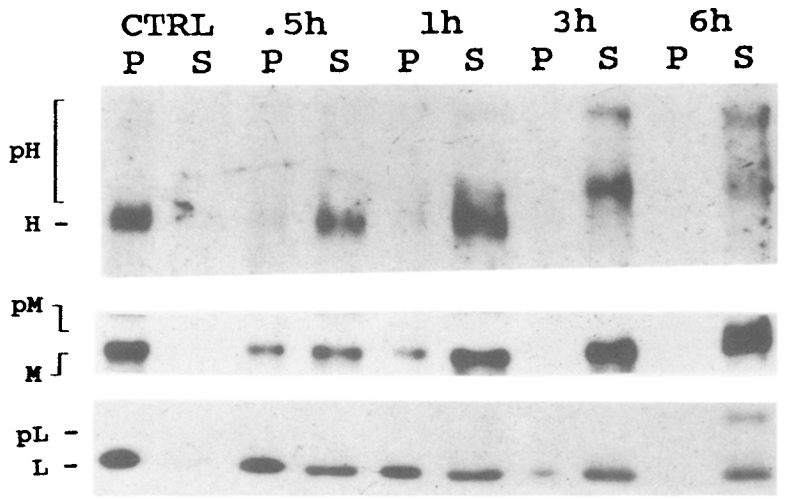

Figure 1: **Western blot analysis of Triton X-100 extracts from okadaic acid-treated dorsal root ganglion cultures**
DRG cultures were treated with OA for the stated periods of time and extracted with buffer containing 1% Triton X-100. Pellets (P) and supernatants (S) were obtained by centrifugation, fractionated by SDS-PAGE, transferred to Immobilon-P membrane and detected using antibodies specific to the three NF subunits. H, M and L refer to the native NF subunits containing low phosphate levels while pH, pM and pL refer to the more highly phosphorylated forms of the subunits.

centrifuged at 15,000xg for 20 minutes. In control cells all three subunits were found in the Triton-insoluble pellet fraction, signifying their association with the NF network. Within 0.5 hrs of OA treatment almost all of the NF-H was redistributed to the Triton-soluble supernatant along with smaller proportions of NF-M and NF-L. By 3 hrs all of the NF-M was redistributed to the supernatant fraction while it took 6 hrs for NF-L to disappear completely from the pellet. An increase in the apparent molecular weights of NF-M (to pM) and NF-L (to pL) was seen after 6 hrs of exposure to OA whereas the mobility of NF-H was seen to decrease as early as 1 hr. Furthermore, 2-dimensional gel electrophoresis showed an acidic shift in the pI values for slower migrating NF-M (pM) and NF-L (pL) consistent with their increased phosphorylation states (data not shown). Since all three NF subunits were detected in the Triton-soluble fraction within 0.5 hrs we used immunofluorescence microscopy to determine whether this shift was accompanied by changes in the cytoskeletal organization of NFs. Although no changes in electrophoretic mobility of NF subunits were seen after 0.5 hrs of OA treatment, there were marked changes in the immunofluorescence staining patterns for all three polypeptides. A representative micrograph of cultures treated with antiNF-L is shown in Fig.2. The smooth axonal staining of control cultures differed markedly from the punctate staining seen in OA-treated samples.

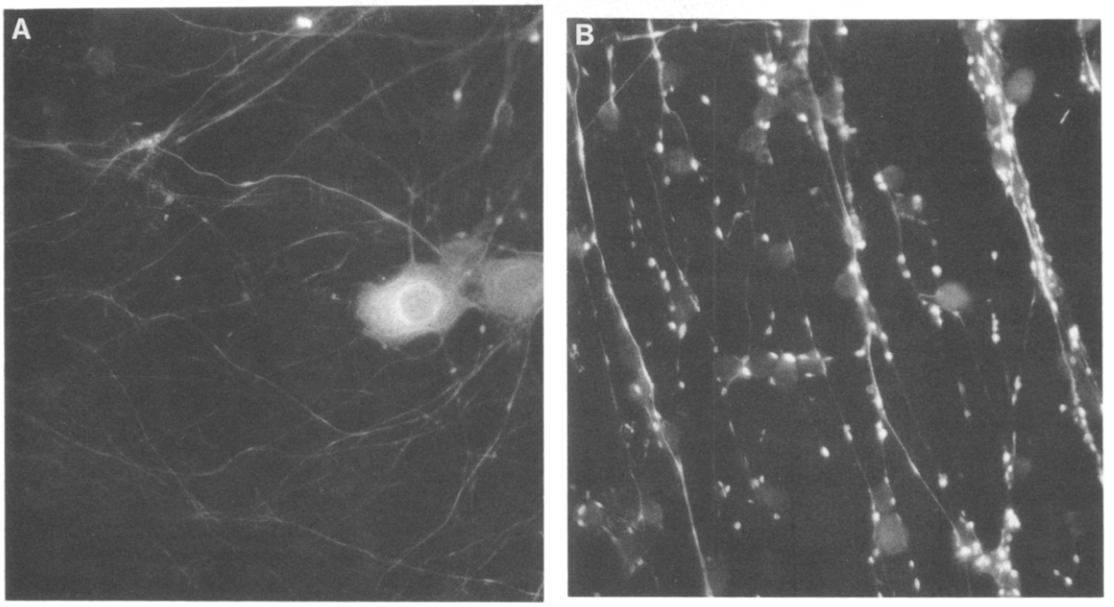

Figure 2: Immunofluorescence microscopy of control and okadaic acid-treated dorsal root ganglion cultures
DRG cultures were untreated (A) or treated with OA (B) for 0.5 hrs., fixed and stained with an antiNF-L monoclonal antibody followed by an antimouse IgG conjugated to Texas Red.

In light of the rapid disruption of NFs by OA, we wished to determine whether the process was reversible after removal of OA from the culture medium. DRG cultures were treated with OA for 0.5 hrs, washed three times with OA-free medium and allowed to recover for various times in OA-free medium containing 10% horse serum and 5% fetal bovine serum. To determine the distribution of NF subunits during recovery, cells were harvested with Triton-containing buffer at various times after removal of OA and Triton-soluble (S) and insoluble (P) fractions were separated as described for Fig.1. As seen in Fig.3, NF-L and NF-M returned to the Triton-insoluble fraction within 2 hrs while NF-H was seen in the insoluble fraction within 10 hrs after removal of OA from the medium.

These results indicate that OA causes an increase in phosphate content of NF subunits resulting in the rapid and reversible disruption of the NF network.

Discussion

The present results indicate that phosphorylation plays a major role in the dynamics of NF organization. The sequential appearance of NF subunits in the Triton-soluble fraction (Fig.1) follows the order expected from one of the proposed schemes of NF organization

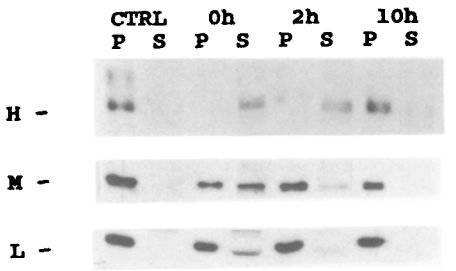

Figure 3: Western blot analysis of dorsal root ganglion cultures recovering from okadaic acid treatment
DRG cultures were treated with 1 μM OA for 0.5 hrs, washed and grown in OA-free medium containing serum for the times shown. Cells were separated into Triton X-100 soluble (S) and insoluble (P) fractions for immunoblot analysis using antibodies specific for the three NF subunits. The NF subunits are designated as described in the legend to Fig. 1.

in which NF-L is suggested to form the filament core while NF-M and NF-H have a more peripheral association (19,20). If the NFs were merely being fragmented in the presence of OA one would expect them to appear in the Triton-soluble fraction simultaneously rather than in the order seen in Fig.1. This result also suggests that the subunits are incorporated as homo- rather than heterooligomeric units during NF assembly. Fig.3 shows that the effects of short term OA treatment of DRG cultures can be reversed, with signs of recovery being apparent as early as 2 hrs post-treatment for NF-L and NF-M and 10 hrs for NF-H. The order of NF subunit reappearance in the Triton-insoluble fraction again lends support to the notion of an ordered assembly of homooligomeric units. In light of the extensive nature of the NF network in DRG neurons it is likely that a large proportion of the original subunits are reincorporated into the filamentous network.

The punctate immunofluorescence staining pattern seen in OA- treated cells (Fig.2) is intriguing considering that much of NF-L and NF-M still remained in the Triton-insoluble fraction at 0.5 hrs (Fig.1). Similar patterns were seen with antiNF-M and antiNF-H (data not shown). Since most of NF-H was Triton-soluble at this time point (cf. Fig.1), these spots may represent pools of Triton-soluble subunits precipitated during fixation. Alternatively, they may represent abnormally assembled NFs similar to those seen by Lee and Page (21) in PC12 cells.

Tryptic phosphopeptide mapping of NF subunits labelled with ^{32}P in the presence of OA may show which phosphorylated region is responsible for NF disassembly. Previous studies on other intermediate filaments, as well as on NF-L, have shown that in vitro phosphorylation of the amino terminal head domain disrupts the filaments and prevents their reassembly (8,22,23). Furthermore, protein kinase A has recently been shown to

phosphorylate the head domain of NF-M (24), although its effect on NF-M assembly has not yet been studied. Whether OA-induced disassembly of the NFs in vivo is due to head domain phosphorylation remains to be determined.

A decreased mobility on SDS-PAGE was seen for all three NF subunits (Fig.1). The appearance of a second form of NF-L (pL) was unexpected as only the 68 kDa species has been reported to date. This shift in mobility was due to phosphorylation of the protein as the pI for the slower migrating form was more acidic than that of the native form (data not shown). The lack of intermediate forms between the two NF-L species (L and pL in Fig.1) may indicate that the shift in mobility is due to one specific phosphorylation event rather than the end result of a series of phosphorylations. While phosphorylation variants of NF-M are well known (25), the physiological relevance of the slower migrating form of NF-L is unknown.

It is not clear whether the increased phosphorylation states of NF subunits in OA-treated neurons was due to the activation of protein kinase(s) and/or inhibition of NF dephosphorylation. Phosphorylated Lys-Ser-Pro sites in histone H1 analagous to the sites found in the tail domains of NF-M and NF-H have been shown to be dephosphorylated by PP-2A (26). It should be noted that PP-2A has also been reported to negatively regulate cdc2 kinase (27) and in vitro studies have shown this kinase to be capable of phosphorylating the repeated Lys-Ser-Pro sites in dephosphorylated NF-H (28,29). Perhaps a related kinase under similar regulation is involved in phosphorylating these sites in vivo.

Acknowledgments

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