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CHANGES IN MICROTUBULAR TAU PROTEIN AFTER ESTROGEN IN A CULTURED HUMAN NEUROBLASTOMA CELL LINE

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Abstract—1. Cultured human SH-SY5Y neuroblastoma cells were used to determine whether 17-β-estradiol affects the metabolism of microtubular τ protein.

2. After 24-hr treatment 17-β-estradiol (10^{-7} M) increased 50 kDa τ protein in the cytoplasmic (supernatant) fraction and decreased it in the membrane (pellet) fraction.

3. The increase in cytoplasmic τ was accompanied by increases in total protein in both cytoplasmic and membrane fractions, 50 and 70%, respectively.

4. The estrogen (10^{-7} M) also caused a 31% reduction in the total number of cells.

INTRODUCTION

The growth-promoting effects of sex steroids have been shown in a number of *in vitro* studies with explants or dissociated cell-cultures from different brain regions (Loudes *et al.*, 1983; Toran-Allerand *et al.*, 1988; Uchibori and Kawashima, 1985). However, the mechanism by which these hormones exert their neuritogenic effects and the neural elements involved in the response are still largely unknown. It has been shown that microtubule assembly plays an important role in neurite elongation (Mitchison and Kirschner, 1988). Microtubule-associated proteins MAP-1a and/or MAP-2 are involved in promoting microtubule assembly and τ protein is an important factor in determining stability (Drubin *et al.*, 1988; Matus, 1988; Ferreira *et al.*, 1990).

It is possible that sex steroids exert their effect by changing and/or enhancing the expression of microtubule-associated proteins. Using hypothalamic cell cultures Ferreira and Caceras presented evidence which showed that estrogen-enhanced neurite growth is mediated by a selective induction of the microtubule-stabilizing τ protein (Ferreira and Caceras, 1991).

τ Protein which stabilizes microtubules and promotes microtubule assembly (Drubin and Kirschner, 1986) is a major component of paired helical filaments (PHFs) which are the main components of neurofibrillary tangles which have been described in a variety of chronic neurological diseases such as lead encephalopathy and Down's Syndrome (Wisniewski *et al.*, 1979); Delacourte and Defossez, 1986). Disruption of the microtubules of cortical areas and τ protein accumulation in Alzheimer's Disease are believed to result in the absence of τ from the usual axonal sites and its appearance in PHFs, dendrites, cell bodies and presynaptic regions (Kowall and Kosik, 1987).

Sternberg *et al.* (1990) in their work on τ protein metabolism have shown the usefulness of the human neuroblastoma cell line, SH-SY5Y, in the study of normal and abnormal cytoskeletal changes with development, aging and after the use of drugs.

The aim of the present study was to determine whether estrogen affects the metabolism of τ protein in the SH-SY5Y human neuroblastoma cell line, thus affecting microtubule assembly and the transport of neurotransmitters.

MATERIALS AND METHODS

Cell culture, treatment and harvesting

Human neuroblastoma SH-SY5Y cells, established by Biedler *et al.* (1973), were obtained from the Cell Culture Facility at the University of California, San Francisco. Cells were grown in 100 × 20 mm Falcon culture dishes in a humidified incubator at 37°C with 5% CO_2 in basal medium (1:1 mixture of Ham's F-12:Dulbecco's Modified Eagle Medium, Sigma) supplemented with 10% fetal bovine serum and 1% 10 M penicillin–streptomycin (Sigma). The medium, 10 ml/dish, was changed every other day. Upon reaching confluency in 5–6 days undifferentiated cells were split 1:3. The medium was changed 1 day after plating and the cells were treated for 48 hr with 17-β-estradiol (10^{-5} 10^{-7} and 10^{-9} M). Dilutions were made immediately before use from a 10^{-2} M stock solution of 17-β-estradiol containing 2% ethanol. Control plates were treated with equimolar amounts of the diluent.

At the conclusion of the experiment the cells were washed briefly with phosphate-buffered saline (PBS) (warmed to 37°C, pH 7.4), and harvested with the aid of a rubber

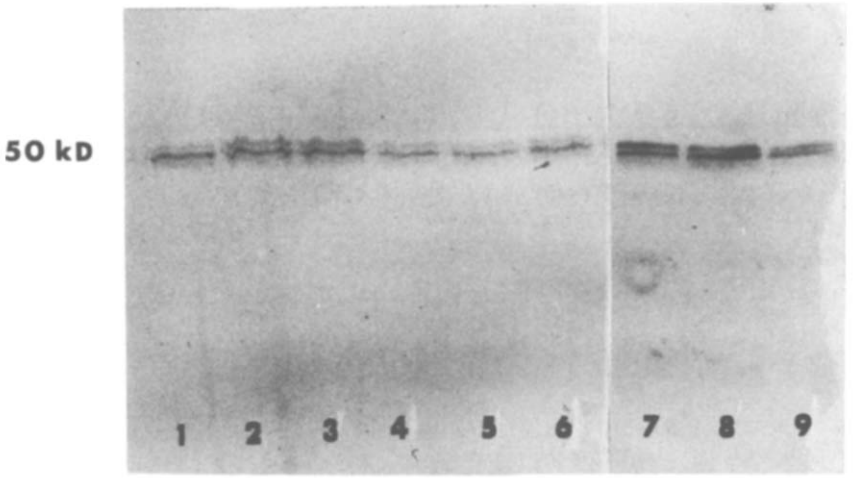

Fig. 1. Western analysis of cytoplasmic and membrane fractions of undifferentiated SH-SY5Y human neuroblastoma cells after 48 hr treatment with estradiol-17-β. Samples were prepared in Laemmli sample buffer, 40 μg of protein were loaded onto each lane and separated by 10% SDS–PAGE. Western blots were performed using a mouse monoclonal antibody to τ protein. Membrane fractions: lane 1 = 10^{-5} M estradiol-17-β; lane 2 and 3 = control; lanes 4 and 5 = 10^{-7} M estradiol-17-β; and lane 6 = control. Cytoplasmic fractions: lanes 7 and 8 = 10^{-7} M estradiol-17-β; and lane 9 = control.

policeman in 1 ml of TE PBS (10 mM Tris, 1 mM EDTA in PBS). The cells were next centrifuged for 5 min at 3000 *g* and resuspended in 1 ml TE PBS. A 10 ml aliquot was counted with the aid of a hemacytometer, and the suspension was recentrifuged. The supernatant was removed and each pellet was resuspended in 50 μl cell lysis buffer (0.01 M Tris, pH 8.3, 1 mM EDTA, 0.1 mM phenyl methyl sulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 5 mM benzamidine) and frozen at −70°C. To separate the cytoplasmic (supernatant) fraction from the membrane (pellet) fraction the samples were spun for 1 min after thawing. The pellet was resuspended in 50 μl cell lysis buffer and sonicated at 30,000 Hz for 5 sec.

SDS–PAGE and Western blot analysis

Total protein in both supernatant and pellet fractions was determined according to the method of Bradford (1976). One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to a modification of Laemmli (1970). Samples were prepared in 10% glycerol, 18.4% SDS, 0.5 M Tris, pH 6.8, with a final concentration of 0.01 M dithiothreitol added as a reducing agent. After the samples were heated at 100°C for 3 min, they were centrifuged to remove any precipitated material prior to loading gels. The supernatants were then electrophoresed in SDS polyacrylamide gels containing 12% acrylamide. Running buffer consisted of 0.025 M Tris–HCl, 0.192 M glycine, 0.1% SDS. Minigels were run at a constant voltage (90 V) until samples entered the separating gel (12% acrylamide, 0.8% bis-acrylamide, BioRad), then voltage was increased to 110 V.

Proteins separated by SDS–PAGE were blotted onto nitrocellulose membrane (Bio-Rad, 0.45 μM) for 1 hr at 12 V in a horizontal blotting apparatus (Bio-Rad) with the use of blot buffer (25 mM Tris, 192 mM glycine and 20% methanol). The nitrocellulose was incubated overnight at 4°C in blocking buffer (5% milk powder, 0.05% Tween 20 in PBS), and then re-incubated in fresh blocking buffer for 1 hr at room temperature with constant shaking. The blot was washed five times with Tris–saline (0.01 M Tris–base, 0.14 M NaCl, pH 7.6) and incubated (2 hr, 23°C) with a 1:25 dilution of mouse monoclonal antibody to τ (Boehringer–Mannheim). The blot was washed five times with Tris–saline, pH 7.6 after the incubation, and was then placed on the shaker for 5 min in fresh Tris–saline. The blot was then incubated for 30 min with an alkaline-phosphatase-conjugated second antibody (Sigma) diluted 1:1000, and then washed for 1 hr. It was then developed with the use of the substrates, nitroblue tetrazolium (50 mg/ml in 70% methyl formamide and 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in 100% dimethyl formamide) (Sigma) in Tris–saline, pH 8.8.

RESULTS

Treatment of SH-SY5Y cells for 48 hr with 17-β-estradiol (10^{-7} M) resulted in an increase in microtubular τ protein (50 kDa) in the cytoplasmic (supernatant) fraction and a decrease in the membrane (pellet) fraction (Fig. 1).

The increase in τ was accompanied by an increase in total cytoplasmic and membrane protein (50 and 70%, respectively), and a 31% reduction in the number of cells (Tables 1 and 2). These are the first

Table 1. Decrease in neuroblastoma cells after 17-β-estradiol. Each value represents the mean ± SE of three individual cultures.

	Number of cells in millions			
17-β-Estradiol	Experimental	Control	Difference (%)	*P*
10^{-5} M	1.2 ± 0.12	1.31 ± 0.04	8.3	NS
10^{-7} M	0.93 ± 0.07	1.34 ± 0.05	31	0.01
10^{-9} M	1.21 ± 0.07	1.27 ± 0.05	5	NS

P = probability based on Student–Fisher *t*-test.

Table 2. Total protein in SH-SY5Y neuroblastroma cells after 17-β-estradiol, as determined by the method of Bradford (1976), is expressed as mean ± SE of three individual cultures

	Total protein (μg/million cells)		
	17-β-Estradiol		
	10^{-5} M	10^{-7} M	10^{-9} M
Cytoplasmic fraction			
Experimental	55 ± 11	76 ± 3	51 ± 9
Control	55 ± 3	50 ± 0.4	67 ± 12
Difference (%)	0	50	24
P	NS	0.02	NS
Membrane fraction			
Experimental	89 ± 16	114 ± 9	74 ± 3
Control	75 ± 4	74 ± 4	80 ± 3
Difference (%)	19	57	8
P	NS	0.05	NS

P = probability based on Student–Fisher *t*-test.

reported findings of a change in τ protein in a neuroblastoma cell line after estrogen.

DISCUSSION

The evidence presented here confirms the important role played by gonadal steroid hormones in the growth and differentiation of neurons and their neurites. These findings are consistent with those of Ferreira and Caceres (1991) who reported on estrogen-enhanced neurite growth in cell cultures of hypothalamic neurons and presented evidence for a selective induction of τ and stable microtubules after estrogen; estradiol-17-β resulted in a 3-fold increase in the level of τ protein over the level seen in control cells.

In this investigation it is interesting that although estradiol-17-β (10^{-7} M) caused a significant reduction in the number of cells this decrease was accompanied by a significant increase in the total protein as well as in τ protein in the cytoplasmic fraction. It is noteworthy that 17-β-estradiol has been found to be critical for the growth and development of hypothalamic cells in culture (Loudes *et al.*, 1983).

Ratka *et al.* (1991) have reported on the modification of cAMP accumulation in opioid-sensitive SH-SY5Y neuroblastoma cells by estradiol and progesterone, but these are the first reported findings on a change in microtubule-stabilizing τ protein in a neuroblastoma cell line after estradiol.

According to Toran-Allerand *et al.* (1983) gonadal steroids through early effects on neurite growth, may set differentiation processes in motion, which continue to unfold over a longer period of development. In addition the findings of Toran-Allerand *et al.* (1988) in cultures of various regions of the fetal murine brain suggest that the effect of estrogen on neurite growth may involve synergistic interactions between estradiol and insulin-related peptides, and may be important in regulating estrogen-responsive neurite growth in the central nervous system (CNS).

Since hormones exert known effects at critical periods of development in accelerating as well as retarding brain maturation the possible interaction of hormones and neurohumor substances during neural growth is important. The results of Gibson and Vernadakis (1975) on the response of chick cerebellum in culture to L-DOPA and estradiol indicate that the interaction of hormones and neurotransmitters may be critical in normal CNS development and function.

It is generally recognized that genomic action of the steroid hormone can lead to changes in the synthesis of specific proteins, which may affect pre- or postsynaptic events after axonal or dendritic transport. In nongenomic effects the steroid may interact indirectly with the pre- or postsynaptic membrane or neurotransmitter receptors (McEwen, 1982).

Further investigation with this adrenergic neuroblastoma cell line is necessary to explore the action of this estrogen on microtubule-associated proteins as well as on enzymes and neurotransmitters in differentiated and undifferentiated cells.

SUMMARY

Human SH-SY5Y neuroblastoma cells were used to determine whether 17-β-estradiol affects cell number, total protein and microtubular τ protein which promotes microtubule assembly. SH-SY5Y cells were treated for 48 hr with estradiol-17-β (10^{-5}–10^{-9} M), then harvested and counted with the aid of a hemacytometer. Separation of proteins by SDS–PAGE was followed by Western blots using a mouse monoclonal antibody for τ. Estradiol-17-β (10^{-7} M) caused a 31% reduction in the number of cells as well as a decrease in microtubular τ protein (50 kDa) in the membrane (pellet) fraction. The estrogen (10^{-7} M) also caused an increase in total cytoplasmic (supernatant) and membrane (pellet) protein, 50% and 70%, respectively, as well as an increase in τ protein (50 kDa) in the cytoplasmic fraction.

REFERENCES

Biedler J. L., Helson L. and Spengler B. A. (1973) Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res.* **33**, 2643–2652.

Bradford M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein in utilizing the principle of protein dye and binding. *Analyt. Biochem.* **72**, 248–254.

Delacourte A. and Defossez A. (1986) Alzheimer's Disease: τ proteins, the promoting factors of microtubule assembly, are major components of paired helical filaments. *J. Neurol. Sci.* **76**, 173–186.

Drubin D. G. and Kirschner M. (1986) τ protein function in living cells. *J. Cell Biol.* **103,** 2739–2746.

Drubin D., Kobayashi S., Kellago D. and Kirschner M. (1988) Regulation of microtubule protein levels during cellular morphogenesis in nerve growth factor-treated PC 12 cells. *J Cell Biol.* **106,** 1583–1591.

Ferreira A. and Caceres A. (1991) Estrogen-enhanced neurite growth: evidence for a selective induction of τ and stable microtubules. *J. Neurosci.* **11,** 392–400.

Ferreira A., Busciglio J., Landa C. and Caceres A. (1990) Ganglioside-enhanced neurite growth: evidence for a selective induction of high molecular weight MAP-2. *J. Neurosci.* **10,** 293–302.

Gibson D. A. and Vernadakis A. (1975) Response of chick cerebellum in culture to L-dopa and estradiol. *Life Sci.* **15,** 751–759.

Kowall N. W. and Kosik K. S. (1987) Axonal disruption and aberrant localization of τ protein characterizing the neuropil pathology of Alzheimer's Disease. *Ann. Neurol.* **22,** 639–643.

Laemmli U. K. (1970) Cleavage of structural protein during assembly of head of bacteriophage T4. *Nature* **277,** 680–685.

Loudes C., Faivre-Baumann A., Puymirat J. and Tixier-Vidal A. (1983) Effect of hormones on the development of mouse fetal hypothalamic neurons *in vitro*. *Monogr. Neural. Sci.* **9,** 43–49.

Matus A. (1988) Microtubule-associated proteins: their potential role in determining neuronal pathology. *Ann. Rev. Neurosci.* **111,** 29–44.

McEwen B. (1982) Steroid hormone action in the brain: cellular and behavioral effects. In *Molecular Genetic Neuroscience* (Edited by Schmitt F. O., Bird S. J. and Bloom F. E.), pp. 265–275. Raven Press, New York.

Mitchison T. and Kirschner M. W. (1988) Cytoskeletal dynamics and nerve growth. *Neuron* **1,** 761–772.

Ratka A., Hochhaus G., Wissler R. N. and Simpkins J. W. (1991) cAMP accumulation in opioid-sensitive SH-SY5Y neuroblastoma cells is modified by estradiol and progesterone. *Molec. Cell Endocr.* **78,** 155–162.

Sternberg H., Mesco G., Cole G. and Timiras P. (1990) τ protein: its presence and metabolism in human neuroblastoma cells. *Adv. Exp. Med. Biol.* **265,** 283–289.

Toran-Allerand D., Leland E. and Pfeninger K. H. (1988) Estrogen and insulin synergism in neurite growth enhancement *in vitro*: mediation of steroid effects by interaction with growth factors? *Dev. Brain Res.* **41,** 87–100.

Toran-Allerand D., Hashimoto K., Greenough W. T. and Saltarelli M. (1983) Sex steroids and the development of the newborn mouse hypothalamus and preoptic area *in vitro*: III. Effects of estrogen on dendritic differentiation. *Dev. Brain Res* **7,** 97–101.

Uchibori M. and Kawashima S. (1985) Effect of sex steroids on the growth of neuronal processes in neonatal rat hypothalamus, preoptic area and cerebral cortex in primary cultures. *Int. J. Dev. Neurosci.* **3,** 381–358.

Wisniewski K., Jervis G. A., Moretz R. C. and Wisniewski H. M. (1979) Alzheimer neurofibrillary tangles in diseases other than senile and presenile dementia. *Ann. Neurol.* **5,** 288–294.