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CHANGES IN TAU PROTEIN LEVELS
OF HUMAN NEUROBLASTOMA CELLS
TREATED WITH DIETHYLSTILBESTROL
AND LEAD ACETATE

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By

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ABSTRACT

CHANGES IN TAU PROTEIN LEVELS OF HUMAN NEUROBLASTOMA CELLS TREATED WITH DIETHYLSTILBESTROL AND LEAD ACETATE

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Diethylstilbestrol (DES) and lead acetate induce aneuploidy in cultured cells involving microtubules. Tau protein promotes microtubule assembly. The aim of this research was to examine the effects of DES and lead acetate on tau protein by using a human neuroblastoma cell line, SH-SY5Y. After 48-hour treatment with DES and lead acetate, cells were harvested and counted with a Coulter counter and hemacytometer, proteins were separated using SDS-PAGE, and Western blots were performed using a mouse monoclonal antibody for tau. DES (5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8} M) decreased tau protein in the cytoplasmic (supernatant) fraction and the membrane (pellet) fraction. Lead acetate (10^{-6} M) decreased tau protein in the membrane (pellet) fraction but increased tau protein in the cytoplasmic (supernatant) fraction. These results indicate that DES and lead acetate affect microtubule assembly by altering tau protein levels and may thus contribute to aneuploidy and subsequently to carcinogenesis.

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INTRODUCTION

The mechanisms of action of neurotoxicants such as diethylstilbestrol and lead acetate remain controversial and constitute one of the more interesting areas of biological research. Diethylstilbestrol (DES), a synthesized stilbene, was first produced in London by Dodds and associates in 1938 (Dodds et al., 1938). Its biological properties are similar to naturally occurring estrogens such as estrone and 17-beta estradiol. DES was inexpensive and widely prescribed in the United States for the treatment of threatened and habitual abortions (Dodds et al., 1938 and Smith and Smith, 1949). Although it was given primarily to women who had a history of problematic pregnancies, DES was also administered to normal pregnant women (Noller and Fish, 1974). No harmful effects of DES were known until 1970 when vaginal clear-cell adenocarcinoma was reported in six young women 14-21 years old. Further studies of these young women revealed a strong association with in utero exposure to DES (Herbst and Scully, 1970). In 1971, the United States Food and Drug Administration banned the use of DES during pregnancy (U.S.F.D.A., 1971).

On the other hand, the toxicity of lead has been known for thousands of years but its mechanism of action has not

been clearly elucidated. Among the heavy metals, lead (Pb) content in water and soil is closely linked with leaded gasoline, lead-based paint, sewage sludges used as fertilizer or land disposal of sewage as well as industrial wastes (Zakrzewski, 1991). Several mechanisms of lead toxicity have been proposed such as its interference with calcium-mediated cellular processes (Simons, 1986; Markovac and Goldstein, 1988 a; Bressler and Goldstein, 1991), the release of neurotransmitters (Bressler and Goldstein, 1991 and Minnema et al., 1988), or the activation of protein kinases (Markovac and Goldstein, 1988 b; Markovac and Goldstein, 1988 a; Laterra et al., 1992). Lead may activate protein kinase C (PKC) by mimicking calcium and this may result in the production of reactive oxygen species (Nishizuka, 1986).

Both DES and lead compounds cause aneuploidy (chromosome unbalance) which is assumed to involve microtubule assembly. Tau protein was one of the first microtubule-associated proteins to be identified and purified from pig brain (Weingarten et al., 1975). Tau protein stabilizes microtubules and promotes microtubule assembly. The neuroblastoma cell line, SH-SY5Y expresses microtubule-associated tau protein. Since tau protein enhances microtubule assembly, it could be hypothesized that diethylstilbestrol and lead acetate decrease tau protein levels in SH-SY5Y neuroblastoma cells. The purpose of this investigation is to explore the effects of diethylstilbestrol and lead acetate on these tau protein-expressing

neuroblastoma cells. The three objectives of this research are to determine whether these compounds affect:

1. cell proliferation, using the Coulter counter and hemacytometer,

2. total protein of the SH-SY5Y cells, using the Bradford method, and

3. microtubule-associated tau protein levels using electrophoresis (SDS-PAGE) and Western blots.

LITERATURE REVIEW

Several laboratories have dealt with the action of diethylstilbestrol (DES) on microtubule assembly. DES has been reported to cause inhibition of microtubule polymerization under cell-free conditions as well as disruption of the mitotic spindle and the cytoplasmic microtubule complex in mammalian cells (Sawada and Ishidate, 1978; Parry et al., 1982; Sato et al., 1984; Sharp and Parry, 1985; Hartley-Asp et al., 1985; Tucker and Barrett, 1986; Wheeler et al., 1986; Sakakibara et al., 1991). DES induces aneuploidy (chromosome unbalance) in cultured cells which assumes the involvement of microtubules (Hartley-Asp et al., 1985). Some research has also examined the effects of lead compounds on microtubule assembly. In young male rats treated with lead acetate, lesions were revealed in the lumbosacral nerves, characterized by reduced neurofilaments and neurotubules (Yagminas et al., 1992). Triethyl lead chloride inhibited microtubule assembly of microtubules from porcine brain and depolymerized preformed microtubules in vitro and in living cells (Zimmermann et al., 1988). Previous observations have also shown that tau protein stabilizes microtubules and promotes microtubule assembly (Drubin and Kirschner, 1986). Since DES has been found to

inhibit microtubule assembly *in vitro* (Hartley-Asp *et al.*, 1985) and lead acetate has been found to reduce neurotubules (Yagminas *et al.*, 1992), it is hypothesized that DES and lead acetate decrease tau protein levels in SH-SY5Y human neuroblastoma cells.

Several established tumor-derived cell-lines express neuronal or glial properties. Cell culture provides three strong advantages for analyzing cellular responses to toxins: isolated cell types, controlled dose of toxic exposure and direct observation. Several direct effects of organic and inorganic lead on neural cells have been reported. One neuronal cell line that has been used in lead studies (Tiffany-Castiglioni *et al.*, 1986) is the human neuroblastoma clonal cell line SH-SY5Y which has a stable diploid karyotype and an adrenergic neuronal phenotype. These neuroblastoma cells express multiple isoforms of the microtubule-associated protein tau which is required for axonal neurite elaboration (Shea *et al.*, 1992). Microtubules assembled from tubulin subunits contribute to both neurite structure and axonal transport.

Neuroblastoma is one of the most common childhood extracranial solid tumors, arising from embryonal neural crest and accounting for 15% of all childhood cancer deaths (Cotran *et al.*, 1994). When analyzing karyotypes from 35 human neuroblastomas, 70% of these cases showed a structural aberration of the short arm of chromosome 1 (1p) (Petkovic *et al.*, 1993; Tonini *et al.*, 1993). In addition to the

abnormalities of chromosome 1p, abnormalities involving only two other chromosome segments occurred with significant frequency (in 20% or more of cases) in this cancer. These abnormalities involved trisomies for the long arms of chromosomes 1 and 17. The gene changes produced by the abnormalities of chromosome 1p in neuroblastoma may play a primary role in the development of this cancer and are presumed to contribute to tumor progression (Gilbert et al., 1984).

The parental cell line SK-N-SH was established from a bone marrow aspiration after excision of a large thoracic primary tumor from a 4-year-old girl with continuing elevated levels of urinary catecholamines and vanillymandelic acid. From the SK-N-SH cell line, the neuroblast-like SK-N-SH5Y cell line was cloned. The SK-N-SH cell lines express some neuronal features under standard conditions. SK-N-SH lines are predominantly adrenergic and are comparable to early normal embryonic autonomic neurons in their fine structure: free ribosomes, moderately-developed Golgi complexes and usually scant granular endoplasmic reticulum. These cells also express neurotransmitter-synthesizing enzymes (Barnes et al., 1981).

The SH-SY5Y cell line has served for many years as a model for neuronal differentiation, responding to nerve growth factor by neurite outgrowth (Matsushima and Bogenmann, 1990; Perez-Polo et al., 1979; Sonnefield and Ishii, 1982) and induced electrical excitability (Kuramoto et al., 1981).

The cells also differentiate in response to phorbol ester, tetradecanoylphorbol acetate (TPA) by exhibiting growth inhibition, neurite extension (Pahlman et al., 1981), elevated norepinephrine content (Pahlman et al., 1983), voltage-regulated calcium channels (Akerman et al., 1984) and the depolarization-evoked release of norepinephrine (Scott et al., 1986). In contrast to TPA responses, retinoic acid induces a less mature, more cholinergic phenotype in SH-SY5Y cells (Haussler et al., 1983; Pahlman et al., 1983; Sidell and Horn, 1985; Slack, 1992). SH-SY5Y cells also express several subtypes of muscarinic receptors including M_1, M_2, M_3, M_4, and M_5 (Baumgold et al., 1992; Fraeyman et al., 1991; Heikkila et al., 1991; Koman et al., 1993). It is believed that muscarinic receptors of SH-SY5Y are predominantly of the M_3 and M_1 subtype while M_2, M_4, and M_5 are expressed in small quantities (Baumgartner et al., 1993). In SH-SY5Y cells the M_3 muscarinic receptor couples to both calcium mobilization and stimulation of cyclic AMP accumulation (Heikkila et al., 1991). SH-SY5Y cells also express a single class of neuronal/nicotinic acetylcholine receptors which are calcium permeable and, when activated, can cause substantial depolarization (Gould et al., 1992; Lukas et al., 1992). Mu and delta opioid receptors have also been found in SH-SY5Y cells. These two types of opioid receptors couple to different G protein subtypes (Kazmi et al., 1986; Laugwitz et al., 1993; Toll, 1992). Platelet-derived growth factor (PDGF) alpha and beta receptors are also expressed in

cultured SH-SY5Y cell lines. When the SH-SY5Y cells are differentiated by TPA, PDGF alpha-receptor expression decreases, whereas beta-receptor expression persists. Therefore, the down-regulation of the alpha-receptor in differentiated SH-SY5Y cells suggests that the expression of this receptor subtype is linked to early differentiation stages of peripheral nervous system neuroblasts (Pahlman et al., 1992). These SH-SY5Y neuroblastoma cells express multiple isoforms of the microtubule-associated protein tau, which is required for axonal elaboration (Shea et al., 1992). Microtubules assembled from tubulin subunits contribute to both neurite structure and axonal transport (Wang et al., 1992). Other microtubular components include the microtubule-associated proteins (MAP's): MAP I and MAP II. "Tau" represents a class of several closely related proteins which are referred to collectively as tau protein. Tau proteins are the low molecular weight microtubule-associated proteins of the brain ranging from 50 to 70 kD (55,000 to 62,000 molecular weight). Tau proteins have similar amino acid content consisting of 300 to 400 amino acids (4 to 5 polypeptides) which are immunologically related (Sternberg et al., 1990).

In the adult brain, MAP-II has been found primarily in the dendrites, while tau protein appears to be restricted primarily to the axons. Tau protein is found chiefly in the white matter of the brain and appears to be synthesized in the cell body and transported to the axon. Tau protein has

been described as a rod-like structure associated with microtubules with arm-like projections (Sternberg et al., 1990). Tau protein increases the polymerization rate of individual microtubules and slows their rate of depolymerization. This dynamic nature of microtubule array permits rapid changes in cell shape such as the changes occurring during axonal extension by neurites (Drechsel et al., 1992). Tau protein has been reported to be phosphorylated on multiple sites by cyclic AMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase II, and protein kinase C. The phosphorylation of tau influences both its biological and chemical properties. This phosphorylation appears to inhibit microtubule assembly by altering its interaction with tubulin and affecting the stability of the microtubule cytoskeleton (Scott et al., 1993). Dephosphorylation by alkaline phosphatase increases the ability of tau protein to stimulate tubulin polymerization (Arioka et al., 1993).

Tau protein is also an antigenic component of paired-helical filaments (PHF's), pathological structures that develop within neurons in Alzheimer's disease. PHF's accumulate in dystrophic neurites and form neurofibrillary tangles in the cell bodies and proximal dendrites of neurons located in the neocortex and hippocampus. PHF's have also been implicated in the pathology of Parkinson's disease (Scott et al., 1993).

Microtubule-associated protein tau is required for axonal neurite elaboration by neuroblastoma cells (Shea et al., 1992). Although some progress has been made, little is known about the mechanisms involved in regulating the presence of tau protein forms, the enzymes involved in tau protein generation, and the cellular roles and functions of tau protein (Sternberg et al., 1990).

Diethylstilbestrol may affect tau protein synthesis since it is well-known that DES causes inhibition of microtubule assembly while enhancing the disassembly process (Sharp and Parry, 1985). DES (0.025 mM) caused 30% inhibition of the assembly of porcine brain tubulin in vitro (Albertini et al., 1988). At 10-200 uM DES was shown to be an inhibitor of microtubule assembly using microtubule proteins isolated from porcine brains (Sato et al., 1984). DES also induced a decrease in microtubule fibers, with an EC_{50} (effective concentration required for induction of microtubule disruption in 50% of the cells) of 48 uM for MCF-7 human breast cancer cells and 50 uM for MDA-MB-231 human breast cancer cells (Aizu-Yokota et al., 1994). The decrease in cellular microtubule fibers induced by DES was reversible. The cells lacking a microtubule network were treated with DES for one hour. Recovery of the normal microtubule network was observed in both cell lines within three hours after replacement of the DES-treated medium with fresh medium. Recent studies on the mechanism of the DES-mediated inhibition of microtubule polymerization have shown that DES

has two binding sites on the tubulin heterodimer which is the major component of microtubule proteins (Metzler and Pfeiffer, 1995). One of the binding sites of DES is similar to that of colchicine since both compounds compete for this site (Metzler and Pfeiffer, 1995 and Sharp and Parry, 1985). Binding to this site could cause the inhibition of microtubule assembly. The other DES binding site seems to have no importance in microtubule polymerization as it also binds 17-beta estradiol, which displays no inhibitory effect on microtubule assembly (Metzler and Pfeiffer, 1995). DES was shown to bind competitively with radiolabelled colchicine but without maximal inhibition of binding. Colchicine is known to bind specifically to the proteinaceous subunits of microtubule protein, the tubulin dimer. It is possible that DES could be binding to other proteins in the sample of microtubule proteins prepared from Syrian hamster brains including tau and high molecular weight proteins that co-purify along with the tubulin subunits (Sharp and Parry, 1985).

Inhibition of microtubules in intact cells may lead to the induction of micronuclei and aneuploidy (chromosome gene unbalance) and thereby, contribute to estrogen-mediated carcinogenesis (Metzler and Pfeiffer, 1995; Sato et al., 1984; Tucker and Barrett, 1986). DES caused significant increase in aneuploidy with a narrow range of high concentrations (50-100 uM) (Wheeler et al., 1986). DES has also been shown to induce neoplastic transformation in the

absence of measurable mutations at specific loci in Syrian hamster embryo cells (Tucker and Barrett, 1986).

DES not only causes aneuploidy by inhibiting microtubule assembly but also by decreasing the number of spindle microtubules. Concentrations of DES that cause aneuploidy also produce abnormal or arrested mitotic spindles. Therefore, DES may disrupt spindle microtubules and produce aneuploidy that results in disordered gene expression and eventual neoplastic transformation (Tucker and Barrett, 1986). It was found that the mitotic index increased while spindle fiber formation was inhibited with increasing DES concentration (10-20 ug/ml). After exposure to DES, the metaphase spindle was generally shorter than controls. DES treatment resulted in more of the chromosome cluster type (Wheeler et al., 1986). Among estrogens, DES is the most potent mitotic arrestant. At 25 uM of DES, there was a complete metaphase arrest when applied to cells in vitro (Chinese hamster strain Don). 17-beta estradiol induced micronuclei at a greater frequency than did DES. DES inhibited spindle assembly and disassembled the cytoplasmic microtubule complex whereas 17-beta estradiol at similar concentrations, arrested mitosis in a manner that allowed spindle assembly. DES appears to inhibit the cell cycle to a greater degree than other estrogens such as 17-beta estradiol (Wheeler et al., 1986). In human lymphocyte cultures, DES prolongs the cell cycle by extending the G2 phase (Hill and Wolff, 1983).

The noncovalent interaction with tubulin and subsequent inhibition of microtubule assembly is only one among several mechanisms whereby estrogens can cause chromosomal damage (Schuler et al., 1995). DES inhibits mitosis in mammalian cells and causes chromosome lagging or malorientation during recovery. Electron microscopy indicates that DES causes disruption of the mitotic spindle, centriole elongation, and unusual chromosome associations due to interkinetochore microtubules. No apparent damage to kinetochores was noted in lagging or maloriented chromosomes. Interkinetochore microtubules induced by DES may account for some types of malorientation of chromosomes in anaphase. On the other hand, abnormal chromosome segregation in DES-treated cells appears not to be due to faulty, damaged, or missing kinetochores, or lack of spindle microtubule association (Brinkley et al., 1985).

Metzler proposed that in vivo effects of estrogens require metabolic activation. In fact, the effect of a compound on microtubule assembly observed under cell-free conditions may be different in intact cells where metabolism of estrogen can either diminish or enhance the propensity for tubulin binding (Metzler, 1981 and Metzler and Pfeiffer, 1995). Indenestrol A (IA) is a metabolite of DES and indenestrol B (IB) is an analog of IA. The (+)-, (-)-, and (+/-)-indenestrols A and B were shown to be inhibitors of microtubule assembly in vitro using microtubule proteins from porcine brain (Oda et al., 1993). Other DES analogues,

dienestrol, meso-hexestrol, and *dl*-hexestrol not only have an inhibitory effect on microtubule assembly but also accumulate twisted ribbon structures (Sato et al., 1987 and Chaudoreille et al., 1987). E-diethylstilbestrol (1.25×10^{-5} M) inhibits microtubule formation by 15% but no twisted ribbon structures form (Chaudoreille et al., 1987). The hydroxylated metabolites of DES, such as the 4-hydroxyderivative of DES dimethyl ether, have greater cytotoxic activities than DES, although epoxidation of DES leads to a product which can be broken down more readily than the parent compound (Oda et al., 1995). An increased inhibition of microtubule assembly might be expected for all compounds capable of quinone formation. The peroxidation of stilbene-type estrogens such as diethylstilbestrol and indenestrol A proceeds via semiquinoid and quinoid intermediates. Catechol metabolites of steroidal estrogens such as 2-hydroxy-estradiol and the benzene metabolite hydroquinone also yield semiquinones and quinones upon oxidation. These reactive quinone metabolites covalently bind to thiol groups of the alpha- and beta-subunit of tubulin essential for microtubule polymerization. Formation of the covalent tubulin adducts inhibits microtubule assembly and may also impair mitotic spindle formation contributing to chromosomal nondisjunction and aneuploidy induction (Epe et al., 1990).

A recent analysis of DES and its metabolites indicates that neither DES nor its metabolites are mutagenic. However, the reactive metabolic intermediates are predicted to have a

wide spectrum of carcinogenic effects while DES itself has a narrow spectrum. Therefore, the metabolites are very important in studying DES carcinogenesis as well as DES-inhibited microtubule assembly (Cunningham et al., 1996).

The effects of estrogens on the nervous system are confined to cells which contain intracellular estrogenic receptors. Once estrogen binds to its receptor, the estrogen-receptor complex is then translocated to the nucleus. In the nucleus the hormone-receptor complex affects the production of messenger RNA's involved in the synthesis of proteins. In summary, DES could be affecting tau protein synthesis by genomic and nongenomic mechanisms:

1. The metabolites of DES such as DES semiquinone and DES quinone could be binding to DNA forming DNA adducts (Gladek and Liehr, 1989) and thereby, interfere with transcription, RNA production, and tau protein synthesis.

2. DES could be acting in a nongenomic manner by disrupting the microtubules and interfering with the levels of tau protein.

Diethylstilbestrol was first used in the prevention of miscarriages but later was found to be carcinogenic in the female offspring of treated mothers. Even though the use of DES was banned in 1971, several compelling reasons support ongoing research of this synthetic estrogen. First, more information is needed to counsel persons that have been exposed to DES about potential cancer risks and to develop strategies for risk reduction, early detection, and prevention (Giusti et al., 1995). Second, DES exposure serves as an excellent model for assessing the potential

toxicity of a broad range of compounds that affect estrogen production or metabolism or mimic estrogen action (Colburn et al., 1993; Davis et al., 1993). These compounds, which are environmental toxicants, include some chlorinated organic compounds, polycyclic aromatic hydrocarbons, herbicides, and pharmaceutical agents. Such compounds have been proposed to contribute directly or indirectly to increasing rates of breast cancer (Davis et al., 1993) and disorders of the male reproductive tract (Sharpe and Skakkebaek, 1993). Dichlorodiphenyltrichlorethane (DDT), dioxin, and methoxychlor (an estrogenic pesticide currently used as a substitute for DDT) have been shown to have reproductive effects similar to those of DES after in utero exposure in rodents (Mably et al., 1992; Walters et al., 1993; Kaldas and Hughes, 1989). The development of functional assays designed to identify compounds with activity similar to that of DES could be used for the toxicologic evaluation of the environmental estrogenic compounds (McLachlan, 1993).

Estrogenic contaminants are obviously not the only identified neurotoxicants present in the environment. Heavy metals such as lead also produce severe central nervous system (CNS) symptoms such as ataxia, convulsions, and coma or lesser CNS deficits including learning disorder, hyperactivity, and headache (Needleman et al., 1990). High-dose exposure to lead may penetrate the blood-brain barrier even in adults. The hippocampus is known to accumulate lead to a higher degree than other divisions of the brain (Peters

et al., 1994). Children are much more susceptible than adults to lead toxicity; even low doses cause neuronal dysfunction. Lead in vivo has been found to interfere with normal neuronal development. Exposure of fetal and neonatal rodents to inorganic lead results in changes in the fine structure of neurons and their synaptic connections (Audesirk et al., 1991).

Many studies examine the effects of lead on various neuroblastoma cell lines. Much of the focus of this research has explored ion channel activation and inactivation. A novel type of ion channel has been found to be activated by lead in mouse N1E-115 neuroblastoma cells. This metal ion-activated channel is activated by cadmium and aluminum as well as lead (Oortgiesen et al., 1990 a). In contrast, inorganic lead (1 nM to 3 mM) concentrations selectively block neuronal nicotinic acetylcholine response in the same cell line. Therefore, the modification of the neuronal nicotinic receptor function may contribute, at least in part, to lead neurotoxicity (Oortgiesen et al., 1990 b). Later experiments showed that lead affects neuronal nicotinic acetylcholine receptors in N1E-115 neuroblastoma cells in a dual manner. At nanomolar concentrations, lead blocks these receptors while at submillimolar concentrations, the blocking effect is reversed (Oortgiesen et al., 1995). The serotonin 5-HT3 receptor-ion channel complex is less sensitive to lead compared to the nicotinic acetylcholine receptor-ion channel complex. Internal lead also causes activation of calcium-

activated potassium channels in N1E-115 cells. Therefore, lead could affect synaptic transmission by blocking presynaptic voltage-dependent calcium channels. However, the nicotinic acetylcholine receptor may also be involved in the toxic effects of lead on the nervous system (Oortgiesen et al., 1993). Triethyl lead acetate inhibited acetylcholinesterase activity slightly at 5 x 10^{-5} M and 10^{-4} M concentrations. Consequently, it is proposed that acetylcholine transmission (synthesis and release) is susceptible to lead neurotoxicity (Hoshi et al., 1991).

In other studies involving the same N1E-115 mouse neuroblastoma cell line, two types of voltage-sensitive calcium channels were isolated and treated with free lead ion concentrations. Type I voltage-sensitive calcium channels are the low voltage activated and rapidly inactivating T-type channels while Type II voltage-sensitive calcium channels are the high voltage activated and slowly inactivating L-type. Both types are reversibly inhibited in a dose-dependent manner at free lead ion concentrations ranging from 20 nM to 14 uM (Audesirk and Audesirk, 1991). The human neuroblastoma cell line SH-SY5Y has two types of voltage-activated calcium channels which are equivalent to the N and L types and both types were equally blocked by lead acetate in a concentration-dependent (1-30 uM) and reversible manner. Blockade of the calcium channels by lead results in deficits in synaptic transmission. Therefore, calcium channels play

an important role in neurological diseases and lead intoxication (Reuveny and Narahashi, 1991).

In another study involving the SH-SY5Y cell line, lead acetate (0.1-1000 mM) temporarily inhibited cell proliferation if the cells were treated one day (but not three days) after plating (Tiffany-Castiglioni et al., 1986). Lead acetate was also found to amplify L-glutamate-induced oxidative stress. In other words, lead acetate increased L-glutamate-induced production of reactive oxygen species, decreased cellular glutathione, and induced cytotoxicity in the human neuroblastoma SH-SY5Y cells. Lead may also cause its neurotoxicity through the amplification of glutamate-induced oxidative stress by protein kinase C activation (Naarala et al., 1995).

Intracellular calcium may also have a role in mediating the toxicity of lead. Lead elevates the free calcium ion concentration in the neuroblastoma x glioma hybrid cell line NG108-15 (Schanne et al., 1989). The influence of lead and calcium on the metabolism of a nuclear matrix protein called p32/6.3 has been studied in mouse neuroblastoma 2a (NB2a) cells. The expression of this protein was increased by lead. This protein is normally abundant only in neural tissues. In chronically lead-intoxicated animals, p32/6.3 was increased in intranuclear inclusion bodies of kidney tubule-lining cells. P32/6.3 accumulates slowly in intact animals over a period of weeks to months. However, enriched levels of p32/6.3 exist in mouse NB2a cells. The relative abundance of

the p32/6.3 protein increased in these cells after one- and three-day exposures to lead (Klann and Shelton, 1989). The intranuclear inclusion bodies appear as fibrous aggregates with dense amorphous centers. The nuclear bodies are found in the nuclear interior separated from both the nucleolus and the nuclear envelope by zones of apparently normal chromatin. The inclusion bodies are lead and protein-rich while being devoid of nucleic acid (Shelton et al., 1993).

It is also known that lead stimulates neurite initiation in N1E-115 mouse neuroblastoma cells. Lead increased neurite numbers and the neurite length in these cells which are peripherally-derived (Audesirk et al., 1991). Therefore, lead impacts multiple regulatory processes that affect neuron survival and differentiation.

Although many lead neurotoxicity studies use neuroblastoma cell lines, even more lead toxicity tests involve the use of rats. Triethyl lead and its metabolites, diethyl lead and lead, accumulate in a dose-dependent manner in blood, liver, kidney, brain of male weanling rats. Triethyl lead accumulated preferentially in the liver while inorganic lead accumulated in the kidney. Levels of serum calcium decreased while levels of phosphorus elevated in a dose-dependent fashion. Serum cholesterol and alkaline phosphatase were elevated in the high dose groups (0.2, 0.5, and 1.0 mg triethyl lead/kg body weight for 91 days, five days per week). Lactate dehydrogenase was significantly lower than the control in the lead acetate (200 mg/kg body

weight/day)-treated group but microsomal aniline hydroxylase was elevated compared to the control (Yagminas et al., 1990).

Regions of the brain differ in their sensitivity to lead acetate. Wistar rat pups receiving 400 ug lead acetate/g body weight/day revealed significant increases in noradrenaline in the hippocampus, cerebellum, hypothalamus, brainstem, and accumbens-striatum. The elevated concentrations persisted in all regions except for the hypothalamus even after rehabilitation. However, the dopamine levels increased in the hypothalamus and decreased in the brainstem and the hypothalamus recovered after rehabilitation. Also the serotonin levels were elevated significantly in the hippocampus, brainstem, and motor cortex, while after 100 days of rehabilitation, the increase only persisted in the motor cortex. After 60 days of 100 ug lead acetate/g body weight/day exposure, a significant decrease of glutamic acid decarboxylase in the cerebellum and glutamate in the motor cortex was observed (Shailesh-Kumar and Desiraju, 1990). Exposure of cultured cells from rat telencephalon to 10^{-6} M to 10^{-4} M lead acetate for nine days also showed a decrease of glutamic acid decarboxylase and glutamine synthetase markers. These same effects were observed after prolonged treatment (50 days) with 10^{-7} M lead acetate (Zurich et al., 1994). Therefore, regional factors influence the vulnerability of the axon terminals to lead neurotoxicity even though all projections originate from

similar neurons in the brainstem (Shailesh-Kumar and Desiraju, 1990).

Kala and Jadhav (1995) also examined brain regions for dopamine, serotonin and their metabolites. Dopamine contents of the nucleus accumbens and hypothalamus were significantly reduced by the subchronic lead exposure. However, dopamine levels in the frontal cortex and hippocampus were not affected by low levels (25 and 50 ppm) of lead and were increased by exposure to 500 ppm lead. Dopamine metabolites, homovanillic acid and 3,4-dihydroxyphenyl-acetic acid showed changes similar to dopamine. Serotonin content, in contrast, showed consistent decreases in the nucleus accumbens, frontal cortex, and brainstem. The serotonin metabolite, 5-hydroxyindole acetic acid was decreased only in the frontal cortex. Therefore, the nucleus accumbens appears to be the most susceptible area of the brain for lead-induced neurotoxicity.

The steady-state levels of monoamines were essentially unaltered after postnatal lead exposure in rats, while functional aspects of striatal dopamine transmission were affected after exposure to 8 mg/kg lead acetate. Therefore, lead-induced changes in motor skills and exploratory behavior may be related to altered dopamine transmission (Luthman et al., 1994). Exposure to lead at an early age could also result in a learning disability persisting at a much later age even after discontinuation of exposure (Shailesh-Kumar and Desiraju, 1992). However, chronic lead exposure also

reinforced motivational behavior in adult male rats (Burkey and Nation, 1994).

Exposure of rats to lead increased tyrosine hydroxylase activity and decreased sodium-potassium ATPase activity (Chin et al., 1992). These data imply that the catecholaminergic nervous system in the pons-medulla, telencephalon and midbrain could be selectively affected by lead. Subclinical lead administration exerts its effect by slowing cell proliferation in the very early growth phase of the brain (Agodi et al., 1990). A metabolic compensative response to the subtoxic effect of lead acetate may arise in the cerebellum and hippocampus during critical phases of nervous system development between fifteen and thirty days of age.

Young rats show much greater sensitivity to toxic metals compared with adults. Eight day-old rats were injected with one or five doses of lead acetate (0, 3.5, or 7.0 mg/kg). Five doses of lead acetate (3.5 or 7 mg/kg) caused a 25% and 40% inhibition respectively of hepatic delta-aminolevulinic acid dehydratase (ALA-D) and an increase of 1.4 fold and 2.6 fold of blood enzyme respectively (Rocha et al., 1995). Chronic prenatal lead exposure delays the age-dependent decrease in mRNA expression, ADP-ribosylation and photoaffinity labeling of alpha i subunit of G protein while chronic adult lead exposure does not cause these changes (Singh, 1993).

Lead has also been shown to alter the immunogenicity of myelin basic protein and glial fibrillary acidic protein

(GFAP). For example, a significant increase in interleukin-6 production was seen in mice immunized with lead-altered myelin basic protein (Waterman et al, 1994). Prolonged lead exposure was also found to alter GFAP and vimentin in rat brain astrocytes (Selvin-Testa et al., 1995). The p32/6.3 nuclear matrix protein as mentioned previously is another protein implicated in chronic lead toxicity (Klann and Shelton, 1989).

Investigators are greatly interested in the molecular interaction of lead compounds with target molecules. Triethyl lead chloride was found to interact with two thiol groups of the tubulin dimer (Zimmermann et al., 1988). The molecular basis of interaction of triethyl lead with tubulin was studied by monitoring the reactivity of the cysteine moieties of the protein. It was demonstrated that in the presence of triethyl lead the amount of reactive thiol groups was decreased by 1.9 $\pm$ 0.2 mole of thiol per mole of tubulin dimer.

Although there are few studies on changes in microtubules after lead acetate, it was found that application of 50 uM triethyl lead to tubulin preparations inhibited microtubule assembly (Zimmermann et al., 1988). A similar effect was produced with colchicine at a comparable concentration. Addition of triethyl lead to preassembled microtubules caused disassembly in fifteen minutes. The microtubule network of human fibroblast cells in culture was destroyed after two-hour incubation with 10^{-6} M triethyl

lead. The depolymerization of microtubules in kangaroo rat cells also occurred over a two-hour incubation with 10^{-6} M triethyl lead. Triethyl lead (10^{-6} M) not only affects microtubules in the cytoplasm of interphase cells but also the microtubules involved in the architecture of the mitotic apparatus (Zimmermann et al., 1988).

In addition, 10^{-6} M triethyl lead inhibits cellular growth by 80% without affecting cell viability in human fibroblasts (Wi-38 cells) and kangaroo rat cells (PtK-1) after two-hour treatment (Zimmermann et al., 1985). Unlike colchicine, triethyl lead does not induce a strong increase of the mitotic index (Dustin, 1959; Zieve, et al., 1980). Growth inhibition was caused by the prevention of cytokinesis which results in the formation of binucleate cells with some micronuclei (Zimmermann et al., 1988).

Since microtubules are abundant in nerve cells, the interaction of lead compounds with microtubules and tubulin in vivo is likely to be at least partly responsible for the neurotoxicity of triethyl lead. The induction of aneuploidy by triethyl lead (Ahlberg et al., 1972) as well as the prevention of cytokinesis (Zimmermann et al., 1985) are also significant events which are directly caused by the interaction of triethyl lead with tubulin and microtubules.

Consequently, both diethylstilbestrol, a carcinogenic and synthetic estrogen, and triethyl lead, a well-known neurotoxicant and environmental contaminant, cause inhibition of microtubule assembly.

Inhibition of microtubules in intact cells may lead to the induction of micronuclei and aneuploidy (chromosome gene unbalance) and thereby, contribute to estrogen-mediated carcinogenesis and lead neurotoxicity. Tau protein stabilizes microtubules and promotes microtubule assembly. Since estrogens and lead compounds have been found to inhibit microtubule assembly and induce aneuploidy, diethylstilbestrol and lead acetate should decrease microtubule tau protein levels in treated human neuroblastoma SH-SY5Y cells.

HYPOTHESIS

This research is based on the hypothesis that diethylstilbestrol and lead acetate inhibit cell proliferation by reducing tau protein concentrations in human neuroblastoma SH-SY5Y cells.

MATERIALS AND METHODS

Culture of human neuroblastoma SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells, established by Biedler, J., Helson, L., and Spengler, B.(1973), were obtained from the Cell Culture Facility at the University of California, San Francisco. Cells were grown in 100 x 20 mm Falcon culture dishes or in 75 cm^2 Corning tissue culture flasks in a humidified incubator at 37.5°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, 1% 10 M penicillin-streptomycin, and 1% L-glutamine (Sigma). The medium, 10 ml/dish or 25 ml/flask was changed every 48 hours. Upon reaching confluency in 5 to 6 days cells were split 1:3 before treatment.

Dose-response curve for diethylstilbestrol

40,000 SH-SY5Y cells were plated in each well of a Falcon 24-multiwell plate (area 1.88 cm^2 per well) with 2 ml of medium. After 24 hours, old medium was aspirated and each concentration of test compound in medium was added to each of 3 wells. After 48 hours, the medium was aspirated and 250 ul Trypsin-EDTA (1x)/PBS 1:1 was added to 3 wells at one time. After 3 minutes of trypsinization, 250 ul of 2% serum in PBS

was applied to the detached cells. Detachment was checked under an inverted microscope. The cells were then pipetted up and down 10 times (500 ul in well) for equal cell suspension. 100 ul of the cells/trypsin/serum was added to 10 ml isotonic saline in a plastic vial for use with the Coulter counter. The vial was inverted 2-3 times while avoiding air bubbles. Three counts were made of each sample and an average count was calculated. There was enough isotonic saline for 4 counts in case the electrode became clogged. The following formula was required to determine the actual counts obtained by the Coulter counter: Count x dilution factor = cells/ml. Then to obtain the total number of cells in each well, the cells/ml were multiplied by 0.5 ml (500 ul of trypsin-EDTA (1x)/PBS and 2% serum/PBS). Counts were performed after all of the cell samples were added to isotone.

The dilution of DES for the dose-response curve

The concentrations used (10^{-4} M-10^{-12} M DES) were prepared in the following manner:

		Concentration in Medium (1:1000 dilution)
Stock #1:	10^{-1} M .0268 g DES in 1 ml ethyl alcohol (Molecular weight of DES = 268.34 g)	10^{-4} M
Stock #2:	10^{-2} M 100 ul of Stock #1 900 ul of ethyl alcohol	10^{-5} M

Stock #3: 10^{-3} M — 10^{-6} M
100 ul of Stock #2
900 ul of ethyl alcohol

Stock #4: 10^{-4} M — 10^{-7} M
100 ul of Stock #3
900 ul of ethyl alcohol

Stock #5: 10^{-5} M — 10^{-8} M
100 ul of Stock #4
900 ul of ethyl alcohol

Stock #6: 10^{-6} M — 10^{-9} M
100 ul of Stock #5
900 ul of ethyl alcohol

Stock #7: 10^{-7} M — 10^{-10} M
100 ul of Stock #6
900 ul of ethyl alcohol

Stock #8: 10^{-8} M — 10^{-11} M
100 ul of Stock #7
900 ul of ethyl alcohol

Stock #9: 10^{-9} M — 10^{-12} M
100 ul of Stock #8
900 ul of ethyl alcohol

In order to make the dilutions in the medium, a 1:1000 dilution (1 ul of stock in 1000 ul of medium) (0.1% ethyl alcohol in medium) was made. For example, for the 3 wells of 10^{-12} M, 8 ul of 10^{-9} M stock were added to 8000 ul medium. 6 ml of medium were actually needed to fill 3 wells but 8 ml were made to have an extra supply.

Determination of cell proliferation, total protein, and tau protein in SH-SY5Y cells after DES treatment

Culture of neuroblastoma SH-SY5Y cells **(see page 28)**

Cell treatment

The medium was changed one day after plating and the cells were treated for 48 hours with diethylstilbestrol (5 x 10^{-7} M, 10^{-7} M, 5 x 10^{-8} M, and 10^{-8} M). Dilutions were made immediately before use from a 10^{-1} M stock solution of diethylstilbestrol. Controls were exposed to 0.1% ethanol in untreated medium while all the experimental dishes were exposed to 0.1% ethanol after diluting the dissolved DES in medium.

Cell harvesting

Cells were harvested in 1 ml TEPBS (10 mM Tris-base, pH 7.5 and 1 mM EDTA in phosphate-buffered saline). Cells were then centrifuged at 3000 g for 5 minutes and resuspended in 1 ml TEPBS. A 20 ul aliquot was counted with a Coulter counter (Model = Z_f, Coulter Electronics, Inc., Hialeah, FL). The suspension was then recentrifuged at 3000 g for 5 minutes. The supernatant was removed and each pellet was resuspended in 35 ul of cell lysis buffer and frozen at -70°C. Cell lysis buffer contains 0.01 M Tris-base, pH 8.3, 0.001 M EDTA, and 0.1 mM phenyl methyl sulfonyl fluoride in addition to the following enzyme inhibitors: 10 ug/ml leupeptin, 10 ug/ml pepstatin A, 10 ug/ml aprotinin, and 5 uM benzamidine. After thawing, the samples were spun for 2 minutes at 8800 g. The membrane (pellet) fraction was resuspended in 35 ul of cell lysis buffer and sonicated at 30,000 Hz for 5 seconds.

Determination of Total Protein

This method is based on the binding of Coomassie Blue to the protein molecule (Bradford, 1976). Protein Assay Dye Reagent Concentrate obtained from Bio-Rad is used and contains Coomassie blue which is a red dye that turns blue after binding with the protein. The optimum wavelength in which this complex is measured with the spectrophotometer is 595 nanometers. The protein dye reagent concentrate was diluted: 20% protein dye reagent and 80% double distilled water. Standards were made up using bovine serum albumin (BSA) (1 mg/ml) and frozen at -70°C. Two blanks (protein dye reagent only) and 8 standards were prepared: 95 ul, 85 ul, 65 ul, 50 ul, 35 ul, 20 ul, 10 ul, and 5 ul. 3 ml of protein dye reagent were pipetted into each test tube. Five ul of each unknown were pipetted into a test tube which was covered with parafilm and inverted to mix. A spectrophotometer (Spectronic 20, Milton Roy Company, Rochester, N.Y.) was used to measure the standards and samples. The amount of protein in each sample was calculated from the standard curve.

SDS-PAGE

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to a modification of Laemmli (1970). Cytoplasmic and supernatant fractions were prepared in 10% glycerol, 18.4% SDS, 0.5 M Tris-HCl, pH 6.8 (buffer), 20% saturated

bromophenol blue in 80% glycerol (tracking dye) with a final concentration of 0.01 M dithiothreitol added as a reducing agent. The fractions were heated at 100°C for three minutes, and then centrifuged to remove any precipitated material, prior to loading gels. First, the SDS-PAGE marker was added to the first lane of the gel, then 40 ug of each protein sample was loaded into separate lanes. Running buffer was 0.025 M Tris-Base, 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% bisacrylamide, Bio-Rad), then voltage was increased to 110 V.

Western blot analysis

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Bio-Rad, 0.45 uM) using a horizontal blotting apparatus (Bio-Rad) and blot buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for one hour at 24 V. The nitrocellulose was incubated overnight at 4°C in blocking buffer (5% milk powder, 0.05% Tween 20 in PBS) and then reincubated with fresh blocking buffer for 1 hour at room temperature with constant shaking. The blot was then washed five times with Tris/saline (0.01 M Tris-base, 0.14 M NaCl, pH 7.6) and incubated (2 hours, 23°C) with a mouse monoclonal antibody to tau (tau-1, 1:1000 from Dr. Binder, University of Alabama through Dr. G. Mesco and tau antibody from Boehringer Mannheim, 1:200). After incubation the blot was washed 5 times with Tris/saline, pH 7.6, and placed on

the shaker for 5 minutes in fresh Tris/saline. The blot was incubated 60 minutes with an alkaline phosphatase-conjugated second antibody (Fisher) diluted 1:1000, washed for 1 hour and subsequently developed with the use of substrates, nitroblue tetrazolium (50 mg/ml in 70% dimethyl formamide) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) (50 mg/ml in 100% dimethyl formamide) in Tris/saline, pH 8.9.

Determination of cell proliferation, total protein, and tau protein in SH-SY5Y cells after lead acetate

Cell culture (See page 28)

Treatments

The medium was changed one day after plating and the cells were treated for 48 hours with lead acetate (10^{-4} M, 10^{-6} M, and 10^{-8} M). Dilutions were made immediately before use from a 10^{-2} M stock solution of lead acetate containing double distilled water. The control cells were treated with the same amount of double distilled water as the experimental cells at each concentration.

Cell harvesting, counting, determination of total protein and tau protein

The same procedures involved in the DES experiments were also used with the lead acetate-treated cells except for the cell counting methods. A hemacytometer was used to count the lead-acetate-treated cells. A 10 ul sample of the 1 ml cells

in TEPBS was counted with the hemacytometer. The formula used to determine cell counts with the hemacytometer was as follows: Cells per ml = the average count per square x dilution factor x 10^4 (10 squares counted).

RESULTS

Treatment of neuroblastoma SH-SY5Y cells (Figures 1-4) with diethylstilbestrol (10^{-4} M) showed a 52% decrease ($P < 0.01$) in cell proliferation while a lower concentration of diethylstilbestrol (10^{-10} M) showed a 21% increase ($P < 0.04$) in cell proliferation (Table 1). No significant changes in cell numbers were obtained with 10^{-5} M--10^{-9} M, 10^{-11} M, and 10^{-12} M DES (Tables 1 and 2). Treatment with DES also did not change the amount of total protein (Table 3). However, treatment with diethylstilbestrol (5×10^{-7} M, 10^{-7} M, and 5×10^{-8} M) decreased 50 kD tau protein (phosphorylated and non-phosphorylated) in the cytoplasmic (supernatant) fraction (Figure 5). Figure 7 shows decreases in non-phosphorylated tau only after DES treatment (5×10^{-7} M, 5×10^{-8} M and 10^{-8} M) in the cytoplasmic (supernatant) fraction. DES (5×10^{-7} M, 10^{-7} M, and 5×10^{-8} M) decreased 50 kD tau protein in the membrane (pellet) fraction (Figure 6).

On the other hand, treatment of neuroblastoma SH-SY5Y cells with lead acetate (10^{-6} M) showed a 34% decrease ($P < 0.05$) in total protein of the cytoplasmic (supernatant) fraction and a 33% decrease ($P < 0.05$) in total protein of

the membrane (pellet) fraction of these cells (Table 5). As Figure 9 indicates, lead acetate (10^{-6} M) also decreased 50 kD tau protein in the membrane (pellet) fraction of SH-SY5Y cells while lead acetate increased 50 kD tau protein in the cytoplasmic (supernatant) fraction as indicated in Figure 8. A 59% increase ($P < 0.01$) in cell proliferation with lead acetate (10^{-6} M) treatment was also observed (Table 4).

Table 1. Diethylstilbestrol (DES) dose-response

DES	Experimental	Control	% Difference	P value
	Number of Cells/Culture			
10^{-4} M	28448 ± 2535	59327 ± 5800	- 52	< 0.01
10^{-5} M	55314 ± 3588	59242 ± 6751	+ 7	n.s.
10^{-6} M	53911 ± 11020	52009 ± 3649	+ 4	n.s.
10^{-7} M	57894 ± 5275	58372 ± 3334	- 1	n.s.
10^{-8} M	52194 ± 1162	47593 ± 6093	+ 10	n.s.
10^{-9} M	50477 ± 5333	43127 ± 3353	+ 17	n.s.
10^{-10} M	52884 ± 3958	43710 ± 3067	+ 21	< 0.04
10^{-11} M	50679 ± 1043	44137 ± 3432	+ 15	n.s.
10^{-12} M	45770 ± 2957	43418 ± 3288	+ 5	n.s.

Each value represents the mean ± SE of 3 individual cultures.

Statistical analysis was performed using simple main effects of 2 x 9 Factorial ANOVA within subjects.

Table 2. Proliferation of SH-SY5Y cells treated with diethylstilbestrol (DES)

DES	Experimental	Control	% Difference	P
	Number of Cells in Millions			
5×10^{-7} M	1.48 ± .37	1.51 ± .24	2	n.s.
10^{-7} M	1.81 ± .52	2.10 ± .42	16	n.s.
5×10^{-8} M	1.70 ± .31	2.16 ± .23	27	n.s.

P = Probability based on Student-Fisher "t"
Each value represents the mean ± SE of 6 individual cultures.

Table 3. Total protein in SH-SY5Y cells treated with DES

Diethylstilbestrol	10^{-5} M	10^{-7} M	10^{-9} M
	Total Protein (ug/million cells)		
Membrane (pellet) fraction			
Experimental	128 ± 54	68 ± 18	93 ± 47
Control	183 ± 70	54 ± 18	115 ± 59
% Difference	43	26	24
P	n.s.	n.s	n.s.
Cytoplasmic (supernatant) fraction			
Experimental	44 ± 14	50 ± 19	41 ± 21
Control	77 ± 42	53 ± 22	61 ± 46
% Difference	75	6	49
P	n.s.	n.s.	n.s.

P = Probability based on Student-Fisher "t"
(As determined by method of Bradford (1976) and expressed as mean ± SE of 3 individual cultures.)

Table 4. Proliferation of SH-SY5Y cells treated with lead acetate

Lead acetate	Experimental	Control	% Difference	P
	Number of Cells in Millions			
10^{-4} M	1.35 ± .17	1.42 ± .16	5	n.s.
10^{-6} M	3.02 ± .27	.82 ± .16	↑59	< 0.01
10^{-8} M	1.06 ± .21	.89 ± .05	19	n.s.

P = Probability based on Student-Fisher "t" test.
Each value for 10^{-6} M represents the mean ± SE of 5 individual cultures while each value for 10^{-4} M and 10^{-8} M represents the mean ± SE of 4 individual cultures.

Table 5. Total protein in SH-SY5Y cells treated with lead acetate

Lead Acetate	10^{-4} M	10^{-6} M	10^{-8} M
	Total Protein (ug/million cells)		
Membrane (pellet) fraction			
Experimental	119 ± 7	62 ± 5	102 ± 18
Control	84 ± 13	93 ± 9	101 ± 7
% Difference	42	↓33	1
P	n.s.	< 0.05	n.s.
Cytoplasmic (supernatant) fraction			
Experimental	202 ± 27	135 ± 2	205 ± 52
Control	148 ± 50	204 ± 9	196 ± 62
% Difference	36	↓34	5
P	n.s.	< 0.05	n.s.

P = Probability based on Student-Fisher "t".
(As determined by method of Bradford (1976) and expressed as mean ± SE of 4 individual cultures.)

Figure 1. SH-SY5Y neuroblastoma cells newly plated

This figure shows human SH-SY5Y neuroblastoma cells-undifferentiated--newly plated cells showing rounded shape and clusters. *Total magnification 2000 x.*

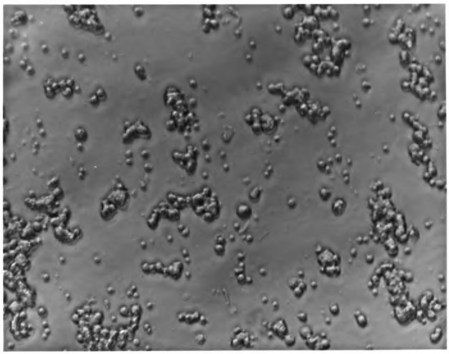

Figure 1

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Figure 2. SH-SY5Y cells after one week in culture

This figure shows human SH-SY5Y neuroblastoma cells-undifferentiated--grown for one week in culture medium. Cells display immature neuroblast-like morphology, with rounded cell bodies and occasional short processes. *Total magnification 4000 x.*

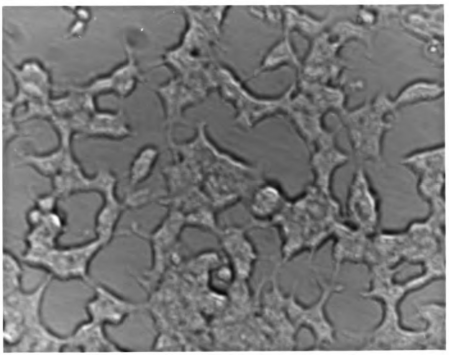

Figure 2

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Figure 3. SH-SY5Y cells after two weeks in culture

This figure shows human SH-SY5Y neuroblastoma cells--undifferentiated--grown for two weeks in culture medium. Cells display immature neuroblast-like morphology, with rounded cell bodies and occasional short processes. *Total magnification 8000 x.*

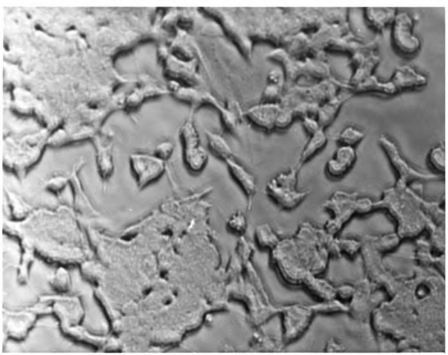

Figure 3

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Figure 4. SH-SY5Y cells showing confluency

This figure shows human SH-SY5Y neuroblastoma cells--undifferentiated--grown for two weeks in culture medium and displaying confluency. *Total magnification 8000 x.*

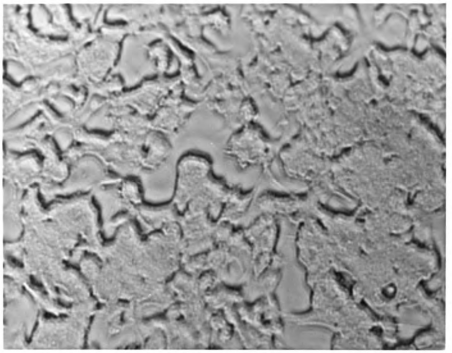

Figure 4

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Figure 5. Western blot of tau protein in the cytoplasmic fraction of SH-SY5Y cells treated with DES using tau antibody from Boehringer-Mannheim

This figure shows the Western analysis of cytoplasmic (supernatant) fraction of SH-SY5Y adrenergic human neuroblastoma cells after 48 hr. treatments with diethylstilbestrol (5×10^{-7} M, 10^{-7} M, 5×10^{-8} M). Samples were prepared in Laemmli sample buffer; 40 ug of protein were loaded onto each gel and separated by SDS-PAGE, Western blots were performed using an antibody to tau from Boehringer-Mannheim (1:200) which recognizes both phosphorylated and non-phosphorylated forms of tau.

Lane 1 = 5×10^{-7} M DES **Lane 2 = Control**
Lane 3 = 10^{-7} M DES **Lane 4 = Control**
Lane 5 = 5×10^{-8} M DES **Lane 6 = Control**

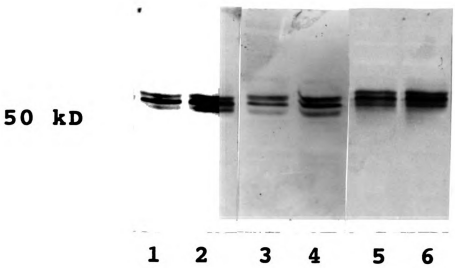

Figure 5

Figure 6. Western blot of tau protein in the membrane fraction of SH-SY5Y cells treated with DES

This figure shows the Western analysis of membrane (pellet) fraction of SH-SY5Y adrenergic human neuroblastoma cells after 48 hr. treatments with diethylstilbestrol (5×10^{-7} M, 10^{-7} M, 5×10^{-8} M). Samples were prepared in Laemmli sample buffer; 40 ug of protein were loaded onto each gel and separated by SDS-PAGE, Western blots were performed using an antibody to tau from Boehringer-Mannheim (1:200).

Lane 1 = 5×10^{-7} M DES **Lane 2 = Control**
Lane 3 = 10^{-7} M DES **Lane 4 = Control**
Lane 5 = 5×10^{-8} M DES **Lane 6 = Control**

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Figure 7. Western blot of tau protein in the cytoplasmic fraction of SH-SY5Y cells treated with DES using tau-1 antibody

This figure shows the Western analysis of cytoplasmic (supernatant) fraction of SH-SY5Y adrenergic human neuroblastoma cells after 48 hr. treatments with diethylstilbestrol (DES)(5×10^{-7} M to 10^{-8} M). Samples were prepared in Laemmli sample buffer; 40 ug of protein were loaded onto each gel and separated by SDS-PAGE, Western blots were performed using an antibody to tau (tau-1, 1:1000 from Dr. Binder, University of Alabama through Dr. G. Mesco which recognizes non-phosphorylated tau).

Lane 1 = 5 x 10^{-7} M DES	**Lane 2 = Control**
Lane 3 = 5 x 10^{-8} M DES	**Lane 4 = Control**
Lane 5 = 10^{-8} M DES	**Lane 6 = Control**

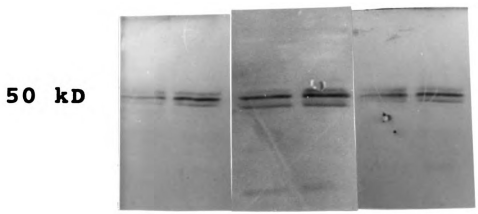

Figure 7

Figure 8. Western blot of tau protein in the cytoplasmic fraction of SH-SY5Y cells treated with lead acetate

This figure shows the Western analysis of cytoplasmic (supernatant) fraction of SH-SY5Y adrenergic human neuroblastoma cells after 48 hr. treatments with lead acetate (10^{-6} M). Samples were prepared in Laemmli sample buffer; 40 ug of protein were loaded onto each gel and separated by SDS-PAGE, Western blots were performed using an antibody to tau from Boehringer-Mannheim (1:200).

Lane 1 = 10^{-6} M Lead Acetate Lane 2 = Control
Lane 3 = 10^{-6} M Lead Acetate Lane 4 = Control

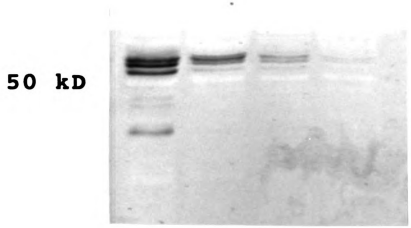

Figure 8

Figure 9. Western blot of tau protein in the membrane fraction of SH-SY5Y cells treated with lead acetate

This figure shows the Western analysis of membrane (pellet) fraction of SH-SY5Y adrenergic human neuroblastoma cells after 48 hr. treatments with lead acetate (10^{-6} M). Samples were prepared in Laemmli sample buffer; 40 ug of protein were loaded onto each gel and separated by SDS-PAGE, Western blots were performed using an antibody to tau from Boehringer-Mannheim (1:200).

Lane 1 = 10^{-6} M Lead Acetate Lane 2 = Control
Lane 3 = 10^{-6} M Lead Acetate Lane 4 = Control

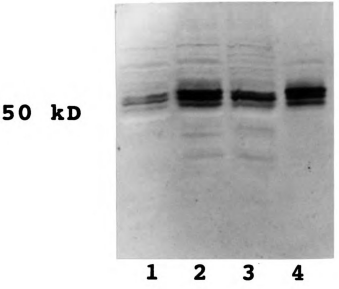

Figure 9

DISCUSSION

Diethylstilbestrol (DES) is a human cancer-causing agent that causes cervical and vaginal carcinoma in female offspring. Although diethylstilbestrol was banned in 1971, questions on the possible health effects on the third-generation remain unanswered (Giusti et al., 1995). A key step in solving these problems would be to elucidate the mechanism of action of DES. However, the mechanism of action of DES remains controversial with evidence for both genotoxic and nongenotoxic mechanisms. (Cunningham et al., 1996).

Several significant reasons exist for studying the mechanism of action of diethylstilbestrol. Most importantly, this research is needed for counseling persons exposed to DES and possible effects on the third-generation. Elucidation of this mechanism would also further the understanding of environmental and pharmacologic compounds similar to DES (McLachlan, 1993). Diethylstilbestrol studies would also contribute to a better understanding of the role of estrogens in normal reproductive development, hormonal imprinting, and carcinogenesis (Walker, 1989).

Estrogens have been found to exert their action by modifying and/or enhancing the expression of microtubule-associated tau protein. No increase in tubulin, microtubule-

associated protein 1a (MAP-1a) or MAP-2 protein expression has been demonstrated. 17-beta estradiol-treated neurons showed a three-fold increase in the levels of tau protein over the controls which was followed by an increase in stable microtubules and in neurite length of medial basal hypothalamic neurons maintained in culture (Ferreira and Caceres, 1991). It is also noteworthy that 17-beta estradiol (10^{-7} M) increased tau protein in the cytoplasmic (supernatant) fraction of cultured human SH-SY5Y neuroblastoma cells (Lew, 1993). Previous observations have shown that tau protein is capable of stabilizing microtubules in vivo (Drubin and Kirschner, 1986). Consequently, MAP-1a and/or MAP-2 are important factors involved in promoting microtubule assembly and tau protein is essential in determining stability (Ferreira and Caceres, 1991). In addition, overexpression of tau leads to the formation of microtubule bundles (arrays of parallel filaments) and neurite extension (Ksiezak-Reding et al., 1992).

However, results from this study are the first reported findings dealing with diethylstilbestrol, a non-steroidal synthetic estrogen, and its effects on tau protein. This present study demonstrates that diethylstilbestrol, a synthetic carcinogenic estrogen, can decrease the expression of tau protein, possibly, acting like an antiestrogen. This decrease could lead to inhibition of microtubule assembly and subsequent aneuploidy and carcinogenesis.

However, the results obtained in the dose-response curve for DES also indicate a tendency for the lower concentrations of DES to increase the number of cells. Although this increase was only found to be statistically significant at 10^{-10} M DES, it is possible that a longer treatment period would have revealed a significant increase in the numbers of cells with all lower concentrations of this drug. In addition, the decrease in neuroblastoma cell proliferation as observed with 10^{-4} M DES indicates harmful effects on this cell line.

It is interesting to note that estrogens may play a role in the maturation of neurons. 17-beta estradiol influences neuronal survival *in vitro* (Faivrebaum *et al.*, 1981: Gahr and Konishi, 1988). 17-beta estradiol promotes neurite extension and arborization in organotypic cultures (Toran-Allerand, 1976; Ferreira and Caceres, 1991; Uchibori and Kawashimi, 1985). Estradiol also stimulates synapse formation *in vivo* and thereby, affects neuronal plasticity (Frankfurt *et al.*, 1990; Arai and Matsumoto, 1978).

The decrease in tau protein obtained in this investigation is consistent with the finding that DES inhibits the microtubule assembly of porcine brain microtubules *in vitro* (Hartley-Asp *et al.*, 1985). For this inhibitory effect on tubulin assembly, DES acts directly with tubulin 6S on sites analogous to the colchicine-site (Chaudoreille *et al.*, 1991). DES has two binding sites on the tubulin heterodimer (Pfeiffer *et al.*, 1994). Binding to

one of these sites causes inhibition of microtubule assembly while the other site demonstrates no inhibition of microtubule assembly with binding. The formation of twisted ribbon structures upon the binding of estrogenic drugs to microtubular protein and tubulin has been shown to be strongly magnesium-dependent (Chaudoreille et al., 1991).

The effects of estrogens on the nervous system may be confined to cells which contain intracellular estrogenic receptors. Estrogens bind to estrogen receptors and the estrogen receptor-complex binds to estrogen responsive elements in chromatin, where estrogens act as transcription factors regulating gene expression (Kangas, 1992). Antiestrogens prevent estrogens from expressing their full effects on target tissues, acting as antagonists. Antiestrogens and estrogens may interact with intracellular calcium ion signalling mechanisms (Lee and Wurster, 1994). Some antiestrogens such as clomiphene have notable similarities in structure to DES (Katzenellenbogen et al., 1979).

One previous study involved the transfection of a human estrogen receptor into the human SK-N-BE neuroblastoma cell. This generated cell-line called SK-ER3 was then treated with 17-beta estradiol. The 17-beta estradiol inhibited cell proliferation by activating the estrogen receptor. After this growth arrest, the SK-ER3 cells differentiated and expressed tau protein and synaptophysin, two proteins synthesized in differentiating neurons (Ma et al., 1993).

Further studies demonstrate a strict relationship between estrogen receptor and monoamine oxidase A activity. Estrogens inhibit monoamine oxidative activity (Ma et al., 1995). On the other hand, insulin growth factor-I receptors are upregulated in the kidney by short term exposure of Syrian hamsters to a carcinogenic dose of DES (Chen and Roy, 1996). This upregulation of insulin growth factor-I receptors is supported by data demonstrating that insulin and related growth factors (IGF-I and IGF-II) could activate the estrogen receptor to control the growth and differentiation of the human neuroblastoma cell line (SK-ER3) (Ma et al., 1994).

The spectrum of the physiological actions of estrogens may exceed that of receptor-mediated hormone effects. Estrogens may serve as free radical scavengers, although this remains to be proven (Kuhl, 1993). Estrogens exert multiple and complex effects on the central nervous system. 17-beta estradiol increases brain glucose transport and utilization (Namba and Sokoloff, 1984; Kostanyan and Nazaryan, 1992; Bishop and Simpkins, 1994), regulates key synthetic and degrading enzymes in norepinephrine (Johnson et al., 1985), dopamine (Chiodo and Caggiula, 1980; Crowley, 1982), and acetylcholine (Ball et al., Heritage et al., 1980) neurons and interacts with neurotrophic neurons (Gibbs and Pfaff, 1992; Singh et al., 1993; Toran-Allerand, 1992). 17-beta estradiol modulates the expression of genes (Jennes, 1990; Weisz and Rosales, 1990) and appears to exert direct effects

on the plasma membrane to alter electrical activity of neurons (Wong and Moss, 1992; Kelley et al., 1977) and the cross-membrane flow of ions (Rambo and Szego, 1983; Morley et al., 1992). Diethylstilbestrol (DES) has been found to be metabolized to reactive intermediates such as DES semiquinone and DES quinone by nuclear cytochromes P-450. These metabolites have been shown to covalently bind to DNA (Liehr et al., 1989; Williams, et al., 1993) and to microsomal and microtubular proteins (Pfeiffer and Metzler, 1992; Haff et al., 1987). Low levels of DES-DNA adducts are found in hamsters treated with high doses of DES (200 mg/kg). In vitro, DNA adducts are formed only in the presence of hydroperoxide cofactors required for the oxidation of DES. Therefore, stilbene-DNA adduction may occur only under conditions of oxidative stress (Bhat et al., 1994). The unexpected finding that structurally diverse estrogens induce identical covalently modified nucleotides demonstrates a unique mechanism of DNA adduction. Therefore, it is concluded that estrogens induce the binding of the same unknown endogenous compound to target tissue DNA. This estrogenic property may play a key role in estrogen-induced carcinogenesis (Liehr, et al., 1986). However, 17-beta estradiol has been proven to exert higher microtubule-disruptive activity than DES in cultured Chinese hamster V79 cells indicating that some natural estrogens cause microtubule disruption in a nongenomic manner (Aizu-Yokota et al., 1995). Consequently, more research is necessary to

elucidate the mechanism of DES-induced carcinogenesis. Since DES has been found to decrease the levels of tau protein in the cytoplasmic (supernatant) fraction and the membrane (pellet) fraction of neuroblastoma cells, DES may also inhibit microtubular assembly and neuronal differentiation.

Results from this investigation are also the first reported studies dealing with the effects of lead acetate on tau protein. These results show that lead acetate (10^{-6} M) decreased tau protein in the membrane (pellet) fraction of SH-SY5Y neuroblastoma cells. These data on decreased tau protein levels help to elucidate the mechanism of microtubule inhibition after lead which is partly responsible for the neurotoxicity of this compound. Although there are not many investigations dealing with lead and microtubules, one study did find that triethyl lead chloride inhibited microtubule assembly and depolymerized preformed microtubules *in vitro* and in living cells which is consistent with the decrease in tau protein found in this present study (Zimmermann *et al.*, 1988). However, lead acetate (10^{-6} M) also increased tau protein in the cytoplasmic (supernatant) fraction and increased cell proliferation by 59%. The increase in cell number after 48 hours is consistent with the increase in numbers of neurites and neurite length as reported by Audesirk *et al.*, 1991. In contrast, Tiffany-Castiglioni (1986) reported that lead acetate temporarily inhibited SH-SY5Y neuroblastoma cell proliferation if the cells were treated for one day after plating while no inhibition was

observed if the cells were treated for three days. Therefore, the reported increase in cell proliferation after two days of treatment is possible. Certainly, this demonstrates that the length of exposure to lead acetate can influence its effect on cell proliferation of the SH-SY5Y cells.

Although lead acetate (10^{-6} M) decreased total protein in these studies, it has been found to increase certain proteins such as p32/6.3 protein after one- and three-day exposures (Klann and Shelton, 1989). Likewise, tau protein was also increased in the cytoplasmic (supernatant) fraction of the SH-SY5Y cells.

More research needs to focus on lead neurotoxicity since lead exposure is a well-known environmental hazard. In recent years heavy metals such as lead have become ubiquitous in the environment due to industrialization and urbanization (Miranda and Ilangovan, 1996). The developing brain is particularly susceptible to lead toxicity but the mechanism of action of lead is still not well understood. Children exposed to what once were considered low concentrations of lead are now having learning disabilities and behavioral problems (McMichael et al., 1988). Lead has been implicated among the causes of several neurological diseases (Campbell et al., 1970), presenile dementia with Alzheimer type changes (Niklowitz and Mandybur, 1975), diffuse demyelination of the cerebral white matter (Verhaart, 1942) and brain tumors in children (Schreir et al., 1977).

There are a variety of mechanisms by which lead may exert its neurotoxicity. Kern et al. (1993) demonstrated that inorganic lead may inhibit neurite development in cultured rat hippocampal neurons through hyperphosphorylation and hypothesized that intracellular Pb^{2+} stimulates calmodulin activity which in turn stimulates calmodulin-dependent protein kinase, which hyperphosphorylates important cytoskeletal proteins. Consequently, hyperphosphorylation of cytoskeletal elements disrupts neurite initiation. Prolonged lead exposure was found to modify the astrocyte cytoskeletal proteins, glial fibrillar acidic protein (GFAP) and vimentin in the rat brain (Selvin-Testa et al., 1995).

Likewise, this research shows alteration of tau protein levels in SH-SY5Y human neuroblastoma cells after treatment with lead. Accumulation of tau develops in paired-helical filaments that develop within neurons in Alzheimer's disease (Scott et al., 1993) and lead toxicity has also been associated with Alzheimer type changes (Niklowitz and Mandybur, 1975). These earlier studies support the present finding that lead acetate affects tau protein.

No single neuronal system has been identified as the primary target of lead toxicity (Shailesh-Kumar and Desiraju, 1990). Lead can induce inhibition of monoamine oxidase (Unni and Caspers, 1985), phenylethanolamine-N-methyl-transferase (Caspers, 1982), brain adenyl cyclase (Nathanson and Bloom, 1975), central nervous system myelination (Toews et al., 1980, 1983), and acetylcholine release (Silbergeld et al.,

1974). Another study with lead acetate with SH-SY5Y cells indicates that lead may cause its neurotoxicity by amplifying glutamate-induced oxidative stress, possibly through protein kinase C activation (Naarala et al., 1995) which is confirmed by the finding that glutamate increases the levels of Ca^{2+} in SH-SY5Y cells (Naarala et al., 1993). Lead has been found to block the N and L types voltage-activated calcium channels present in this cell line (Reuveny and Narahashi, 1991). Glutamate activates at least three receptors which are present in the SH-SY5Y cells: the N-methyl-D-asparate (NMDA), the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and the kainate receptor subtypes (Monaghan et al., 1989). It is well-known that glutamate induces oxidative stress and that the brain is especially susceptible to oxidative attack because of its high lipid content, high rate of oxidative metabolism, and low levels of free-radical scavenging enzymes (Bondy and Lee, 1993; Lafon-Cazal et al., 1993; Halliwell, 1992; Halliwell and Gutteridge, 1985; LeBel and Bondy, 1991; Bondy, 1992).

Recent studies also show that lead severely impairs neuronal growth even at submicromolar concentrations (Cline et al., 1996). The effect of lead on neuronal morphology may be due to impaired assembly or stability of the cytoskeleton which is consistent with the aforementioned finding of lead interference with calcium-dependent events. Consequently, the neurotoxicity of lead acetate may be partially due to its effects on microtubular tau protein.

Further investigation with this adrenergic neuroblastoma cell line is necessary to explore the action of diethylstilbestrol and lead acetate on all microtubule-associated proteins as well as on enzymes and neurotransmitters in differentiated and undifferentiated cells. The immunohistochemical staining of lead acetate and DES-treated microtubules would clearly demonstrate the effects of lead acetate and DES on microtubule assembly in these human neuroblastoma cells. Low concentrations of diethylstilbestrol and lead acetate may have chronic effects so longer treatments may be necessary to explore the mechanism of action of these neurotoxicants. Exploration of estrogen and glutamate receptors would clarify possible nongenomic action while the effects of these agents on tau mRNA would explore the possible genomic mechanisms of action.

SUMMARY

The SH-SY5Y human neuroblastoma cell line was used in this investigation to examine the effects of diethylstilbestrol (DES) and lead acetate on cell number, total protein levels, and tau protein levels. After 48-hour treatment with DES and lead acetate, SH-SY5Y cells were harvested and counted with a Coulter counter and hemacytometer, respectively, proteins were separated using SDS-PAGE and Western blots were performed using a mouse monoclonal antibody to tau.

Treatment of SH-SY5Y cells with DES (10^{-4} M) showed a 52% ($P < 0.01$) decrease in cell number while 10^{-10} M diethylstilbestrol treatment demonstrated a 21% ($P < 0.04$) increase in cell number of the human SH-SY5Y neuroblastoma cells. On the other hand, no significant change in total protein of these cells was observed after DES treatment. However, after 48 hours, DES (5×10^{-7} M, 10^{-7} M, and 5×10^{-8} M) decreased 50 kD tau protein (phosphorylated and non-phosphorylated) in the cytoplasmic (supernatant) fraction. Nonphosphorylated tau also decreased after 48-hour treatment with DES (5×10^{-7} M, 5×10^{-8} M, and 10^{-8} M) in the cytoplasmic (supernatant) fraction. DES treatment

(5×10^{-7} M, 10^{-7} M, and 5×10^{-8} M) decreased 50 kD tau protein in the membrane (pellet) fraction.

In contrast, lead acetate (10^{-6} M) caused a 59% increase ($P < 0.01$) in cell proliferation of the SH-SY5Y human neuroblastoma cells after 48-hour treatment. Lead acetate treatment (10^{-6} M) caused a 34% decrease ($P < 0.05$) in total protein of the cytoplasmic (supernatant) fraction of the SH-SY5Y human neuroblastoma cells and a 33% decrease ($P < 0.05$) in total protein of the membrane (pellet) fraction. A decrease of 50 kD tau protein in the membrane (pellet) fraction and an increase of 50 kD tau protein in the cytoplasmic (supernatant) fraction of SH-SY5Y cells was also observed after 48-hour treatment with lead acetate (10^{-6} M).

These results represent the first studies involving the interactions of DES and lead acetate on microtubule-associated tau protein. Both DES and lead acetate decrease the expression of tau protein which could lead to the inhibition of microtubule assembly followed by aneuploidy and carcinogenesis. DES could be exerting its effect on tau protein synthesis by genotoxic and nongenotoxic mechanisms. DES metabolites such as the DES quinones could bind to DNA and thereby, interfere with DNA transcription, RNA production and tau protein synthesis. Lead may also exert its neurotoxicity by a variety of mechanisms. Lead may inhibit neurite development by the hyperphosphorylation of cytoskeletal proteins. Lead may also exert its neurotoxicity by amplifying glutamate-induced oxidative stress, possibly

through protein kinase C activation and thereby, interfering with calcium-dependent events. DES and lead acetate could be acting in a nongenomic manner by disrupting microtubules and interfering with tau protein levels.

This research clearly demonstrates that DES and lead acetate influence tau protein levels. It would be interesting to examine the effects of DES and lead acetate on the microtubule assembly of these SH-SY5Y cells in differentiated as well as undifferentiated cells to further elucidate the mechanism of action of these neurotoxicants.

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