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This study is dedicated with love to my parents, Leena and Tapio Naarala.

Tämä tutkimus on omistettu rakkaudella vanhemmilleni, Leena ja Tapio Naaralalle.

ABSTRACT

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Diss.

Excitotoxicity and oxidative stress are two phenomena that have repeatedly been described as being involved in a wide range of disorders of the nervous system including epilepsy, transient cerebral ischemia, Alzheimer's disease, Parkinson's disease and Huntington's disease. Glutamate, aspartate, and acetylcholine are the major excitatory neurotransmitters in the brain. The coexistence and co-release of neurotransmitters and their association in neuronal events, and coupling mechanisms have recently evoked considerable interest in the way these are related to excitatory neuronal damage and death. In the present study, oxidative stress evoked by glutaminergic and muscarinic receptors, and its modulation by an environmental toxin, lead, was studied in a human neuroblastoma cell line.

The results of the present study show that human SH-SY5Y neuroblastoma cells express glutamate receptor subtypes. Activation of these receptors elicits functional intracellular calcium responses. Their stimulation by glutamate increases the production of reactive oxygen species although this is seen only after the impairment of the cellular glutathione defense system. Glutamate together with lead causes sustained production of reactive oxygen intermediates and subsequent oxidative stress. Protein kinase C may have an important role in the onset of oxidative stress because the production of reactive oxygen species by glutamate is completely inhibited by a protein kinase C inhibitor. Moreover, protein kinase C inhibition together with lead treatment causes oxidative stress. This may be due to modulation of the phosphorylation status of protein kinase C target proteins. The stimulation of cholinergic muscarinic receptors seems to increase the production of reactive oxygen intermediates in this cell line; cholinergic-induced oxidative stress seems to be protein kinase C-controlled and to be due to increased production of the superoxide anion.

The principal route for phorbol ester-induced differentiation of SH-SY5Y cells may be down-regulation of protein kinase C. Also the differentiation of these cells promoted by lead seems to be under the partial control of protein kinase C.

The present findings shed new light on the role of the glutaminergic and cholinergic neurotransmitter systems in oxidative stress. Also, the SH-SY5Y cell line proved to be an effective tool in investigating the effects of stimulation of different receptor systems and their modulation by lead. This study proposes a new mechanism for lead neurotoxicity through amplification of glutamate-mediated cellular activation.
Key words: Neurotoxicity; oxidative stress; excitatory amino acid receptors; cholinergic muscarinic receptors; lead; protein kinase C; differentiation.

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Contents

List of original publications

This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals:

I Naarala, J., Nykvist, P., Tuomala, M. & Savolainen, K. 1993: Excitatory amino acid-induced slow biphasic responses of free intracellular calcium in human neuroblastoma cells. - FEBS Lett. 330: 222-226.

II Naarala, J., Loikkanen, J., Ruotsalainen, M. & Savolainen, K. 1995: Lead amplifies glutamate-induced oxidative stress. - Free Rad. Biol. Med. 19: 689-693.

III Naarala, J., Loikkanen, J. & Savolainen, K. 1996: The combination of lead with a protein kinase C inhibitor causes oxidative stress in human neuroblastoma cells. - Neurosci. Res. Commun. 19: 135-143.

IV Naarala, J., Loikkanen, J., Haapasalo, A. & Savolainen, K. 1997: Effects of lead and protein kinase C inhibition on differentiation of human neuroblastoma cells (manuscript submitted to J. Neurochem.).

V Naarala, J., Tervo, P., Loikkanen, J. & Savolainen, K. 1997: Cholinergic-induced production of reactive oxygen species in human neuroblastoma cells. - Life Sci., in press.

Abbreviations

ACh	acetylcholine
1S,*3R*-ACPD	(1S,*3R*)-1-aminocyclopentane-1,3-dicarboxylic acid
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APV	D-2-amino-5-phosphonovalerate
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	intracellular calcium concentration
CCh	carbachol
CNS	central nervous system
DAG	diacylglycerol
DEM	diethylmaleate
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
EAA	excitatory amino acids
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid
FCS	fetal calf serum
GABA	γ-aminobutyric acid
GF 109203X	a bisindolylmaleimide, a protein kinase C inhibitor
GMP	guanine monophosphate
GSH	glutathione
HBSS	Hanks' balanced salt solution
H-7	1-(5-isoquinolinesulphonyl)-2-methylpiperazine, a protein kinase C inhibitor
IC_{50}	50% inhibitory concentration
IP_3	inositol 1,4,5-trisphosphate
K252a	a bisindolylmaleimide, a protein kinase C inhibitor
L-AP4	L-2-amino-4-phosphonobutyrate
L-CCGI	(2S,1'S,2'S)-2-(carboxycyclopropyl)glycine
LDH	lactate dehydrogenase
mAChR	muscarinic cholinergic receptor
MBCL	monochlorobimane
mGluR	metabotropic glutamate receptor
NADPH	nicotinamide adenine dinucleotide phosphate
NMDA	*N*-methyl-D-aspartate
$NO^{\bullet}$	nitric oxide radical
O_2	molecular oxygen
$O_2^{\bullet}$	superoxide radical
$OH^{\bullet}$	hydroxyl radical
$ONOO^-$	peroxynitrite
PDBu	phorbol 12,13-dibutyrate

PIP_2	phosphatidylinositol(4,5)bisphosphate
PKC	protein kinase C
PMA	phorbol myristate acetate
QNB	quinuclidinylbenzilate
Ro	a protein kinase C inhibitor
Ro 31-7549/ Ro 31-8220	bisindolylmaleimides, protein kinase C inhibitors
ROS	reactive oxygen species
-SH	sulfhydryl group

1 INTRODUCTION

Glutaminergic and cholinergic receptor systems are the most ubiquitous receptors involved in the excitation of neuronal cells and tissue. These receptors are also linked to overt cellular excitation causing cellular damage and death, which is termed excitotoxicity. There is increasing evidence that both glutaminergic and cholinergic receptor systems participate in the production of reactive oxygen species and subsequent oxidative stress in neuronal cells. Also, the involvement of these receptors seems to be important in mediating the actions of environmental toxins on the nervous system. In many neurological disorders both excitotoxicity and oxidative stress are thought to be involved. Several neurotoxic compounds elicit excitotoxicity and simultaneously increase the production of reactive oxygen species. It seems likely that free radical-mediated neuronal damage may be a common pathway for injury in which excitotoxic and other forms of neuronal injuries of the central nervous system converge.

The use of lead may have begun about 2000 B.C. when lead was obtained as a byproduct of silver melting. Heavy metals were generally known already by the ancient Romans. In ancient Rome lead was used commonly in dishes combined with tin. This caused chronical lead poisoning which has been even postulated to have contributed to the fall of the Roman Empire. "If we were to judge of the interest excited by any medical subject by the number of writings to which it has given birth, we could not but regard the poisoning by lead as the most important to be known of all those that have been treated of, up to the present time" (Orfila 1817, see Goyer 1995). In spite of a plethora of research on the mechanisms of toxicity of lead in the nervous system, the underlying cellular and molecular mechanisms of lead neurotoxicity have remained largely obscure. Lead has been shown to interfere with calcium-mediated cellular processes and also with the ligand-binding properties of different classes of receptors. There is also evidence that lead may affect neuronal differentiation.

This thesis focuses on the role of the glutaminergic and cholinergic receptor

systems in the production of reactive oxygen species and subsequent oxidative stress in a neuronal cell line. It seems likely that protein kinase C is involved in both glutamate- and carbachol-induced production of reactive oxygen species. The modulative effects of an environmental toxin, lead, was studied on glutaminergic receptor-induced oxidative stress. In addition, the effects of lead on the differentiation of the cells were explored. The results of this thesis indicate that reactive oxygen species are produced by glutaminergic and cholinergic receptor stimulation in human SH-SY5Y neuroblastoma cells. Lead has a dramatic amplifying effect on glutamate-induced oxidative stress. This amplification may be a new mechanism for lead neurotoxicity. These data also suggest that lead may induce differentiation of neuronal cells. The present study suggests that the SH-SY5Y human neuroblastoma cell line is an effective tool for investigating the effects of stimulation of different receptor systems and their modulation by lead.

2 REVIEW OF THE LITERATURE

2.1 Excitotoxicity

The term "excitotoxicity" was first introduced by Olney in 1978. He suggested that neuronal death occurred as a consequence of excessive exposure of neurons to excitatory amino acids (EAA) glutamate and aspartate. The hypothesis of excitotoxicity (Olney 1978, Rothman & Olney 1987, Choi 1988) proposes a cascade of events leading to neuronal death, and consists of three postulations: 1) depolarization of neurons leads to release of excitatory amino acids (EAA); 2) both excitation and toxicity are mediated through a common EAA receptor; and 3) propagation of cellular events directs neuronal cells down a path to dysfunction and death. Both basic research and clinical trials have led to the general proposal that excess neuronal activity caused by abnormal extracellular levels of EAA have a role in cerebral ischemia, Alzheimer's disease, epilepsy, Huntington's disease and Parkinson's disease (Bondy & LeBel 1993). Many extrinsic and cell-intrinsic factors are thought to determine neuronal vulnerability to excitotoxic injury. An important extrinsic factor may be the availability of endogenous excitatory inputs. Prominent intrinsic components may include the types and numbers of the excitatory receptors in the cell membrane, mechanisms available to buffer or extrude intracellular calcium ions, and radical scavenging machinery (Choi 1992).

During depolarization of neuronal membranes by EAA, ion- and water fluxes are produced to and from extracellular and intracellular compartments. When excessive, these fluxes result in an abnormal accumulation of ions and water inside the neurons causing acute toxic swelling and rupture of neurons (Van Harreveld 1959, Watkins 1978). In addition, it has been shown that these ion- and water fluxes not only cause excessive accumulation of ions but also initiate intracellular

metabolic cascades leading to delayed cellular damage (MacDermott et al. 1986, Choi 1988). Mechanisms linked to these delayed toxic effects of excitation are associated with excessive accumulation of calcium ions, subsequent impairment in mitochondrial function, and induction of enzyme activation leading to cytoskeletal alterations, EAA release, impaired EAA uptake, and the production of reactive oxygen species (Nieminen et al. 1988, Meldrum & Garthwaite 1990, Whetsell 1996).

Both glutaminergic and cholinergic inputs have been implicated in a number of cerebral disorders, and both neurotransmitters induce seizures and neuronal damage (Wade et al. 1987, Savolainen & Hirvonen 1992, Coyle & Puttfarcken 1993, Hirvonen et al. 1993, Schoepp & Conn 1993). Cholinergic convulsions, and associated brain damage, can be prevented by pretreating animals with a cholinergic antagonist, atropine. However, atropine was ineffective if it was given after the onset of convulsions (Jope et al. 1986). By using specific *N*-methyl-D-aspartate (NMDA) receptor antagonists, both cholinergic and kainic acid-induced convulsions and associated neuronal damage could be prevented, indicating that neurotransmitters other than acetylcholine are responsible for the final deletorious effects of excessive cholinergic neuronal stimulation (Piredda & Gale 1985, Savolainen & Hirvonen 1992). Moreover, increased glutamate release into extracellular fluid in the CNS has been found after the induction of convulsions in rats with kainic acid or soman (Wade et al. 1987). These findings have been substantiated by the observations of Docherty et al. (1987) which show that cholinergic stimulation of neurons may be associated with a co-release of the excitatory amino acids aspartate and glutamate.

The two types of excitatory receptors discussed in this context are the subtypes of glutaminergic receptors and cholinergic muscarinic receptors.

2.1.1 Glutaminergic receptors

The excitatory amino acid, glutamate, causes the entry of calcium into neuronal cells through ionotropic receptor-gated calcium channels (Monaghan et al. 1989, Bettler & Mulle 1995, Mori & Mishina 1995), and a release of calcium from intracellular nonmitochondrial calcium stores through actions on metabotropic receptors (Pin & Duvoisin 1995).

The endogenous excitatory amino acid, glutamate, activates at least three distinct ionotropic receptors in neuronal cells: the N-methyl-D-aspartate (NMDA), the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and the kainate receptor subtypes (Monaghan et al. 1989, Bettler & Mulle 1995, Mori & Mishina 1995). These three receptor classes are further divided into two groups: the NMDA receptor group consisting of NMDA receptors, and the non-NMDA receptor group consisting of AMPA and kainate receptors. The channels gated by all three

receptor subtypes are permeable to both sodium and potassium ions. The NMDA receptor subtype is the most clearly defined among ionotropic glutamate receptors since highly specific ligands such as NMDA and D-2-amino-5-phosphonovalerate (APV) have been developed. The NMDA receptor channel is unique in that it is gated by both ligands and voltage changes. Moreover, the NMDA receptor channel is highly permeable to calcium ions unlike most of the non-NMDA channels (MacDermott et al. 1986).

In 1985 it became apparent that glutamate had a more complex role in neuronal excitation since it was reported to stimulate phospholipase C through a receptor which did not belong to the ionotropic glutamate receptor families (Sladeczek et al. 1985). These metabotropic glutamate receptors (mGluR) are coupled to G protein(s), and they are either positively coupled to phospholipase C, or negatively coupled to adenylyl cyclase (Sladeczek et al. 1985, Sugiyama et al. 1987, Nicoletti et al. 1988, Schoepp et al. 1990, Masu et al. 1991, Pin & Duvoisin 1995). To date there are at least eight members in this receptor family, and in addition, several splice variants. According to their amino acid sequence identity, metabotropic glutamate receptors can be classified into three groups (I, II, and III). The most potent agonists for groups I, II, and III are quisqualate, (2S,1´S,2´S)-2-(carboxycyclopropyl)glycine (L-CCG-I), and L-2-amino-4-phosphonobutyrate (L-AP4), respectively. A widely used selective agonist for the metabotropic glutamate receptor is (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) which is active on group I and II mGluRs whereas group III receptors are insensitive to it. For group I mGluRs, the most important signal transduction coupling is the stimulation of phospholipase C, and for groups II and III the major signal transduction route is the inhibition of adenylyl cyclase. Stimulation of phospholipase C increases the formation of two inositol lipid-derived second messengers, inositol-1,4,5-trisphosphate and diacylglycerol (Berridge & Irvine 1989, Berridge 1993, Divecha & Irvine 1995). Inositol-1,4,5-trisphosphate elevates free intracellular calcium and this in turn regulates a number of cellular signal transduction processes (Clapham 1995), including the activation of protein kinase C and calcium/calmodulin dependent protein kinase. Intracellular calcium also plays a critical role in toxic cell killing and programmed cell death (Orrenius et al. 1989, Orrenius et al. 1992). Another second messenger formed, diacylglycerol, activates protein kinase C in the cell membrane (Nishizuka 1986). Inhibition of adenylyl cyclase by mGluRs (Tanabe et al. 1992) is antagonized by pertussis toxin, indicating that the G protein of the G_i family mediates this response. G_i proteins may also have inhibitory action on potassium and calcium channels, and they may also activate cyclic GMP phosphodiesterase (Neer 1995). Adenylyl cyclase inhibition will reduce the amount of cyclic AMP in cells resulting in diminished activity of cyclic AMP-dependent protein kinase and this decreased phosphorylation of a number of cytoplasmic and nuclear proteins (Francis & Corbin 1994, Lalli & Sassone-Corsi 1994).

2.1.2 Cholinergic muscarinic receptors

Cholinergic muscarinic receptors (mAChR) are the predominant receptors in many brain regions such as the limbic forebrain, caudate, and hippocampus (Kuhar & Yamamura 1976). In addition to glutaminergic receptors, they are also the principal excitatory receptors in the brain (Coyle & Puttfarcken 1993, Savolainen et al. 1994). In human behavior, muscarinic receptors play an important role in complex processes such as memory, arousal, and learning (Felder 1995), and they may also be linked to neuronal growth and maturation (Pinkas-Kramarski et al. 1992, Felder et al. 1993). Moreover, cholinergic deficits have been implicated in the etiology of a number of neurological disorders such as Alzheimer's disease, Down's syndrome, and Parkinson's disease (Yates et al. 1980, Dubois et al. 1983, McKinney & Coyle 1991). Cholinergic systems are also crucial in many forms of chemically-induced convulsions, and may play a role in the triggering and maintenance of seizure activity in human epilepsy (Olney et al. 1986, Turski et al. 1989). However, the mechanisms whereby cholinergic muscarinic excitation of neurons may cause deleterious effects in the brain have remained obscure.

Five mAChR subtypes (m1-m5) have been identified by molecular cloning (Bonner 1989, Felder 1995). Three of these receptor subtypes have been also pharmacologically characterized by utilizing specific antagonists (Lambert & Nahorski 1990). Stimulation of m1, m3, or m5 muscarinic receptor subtypes activates multiple G-protein-coupled effector cascades causing simultaneous activation of phospholipases A2, C, and D, as well as tyrosine kinase, and opening of voltage-insensitive calcium channels (Abdel-Latif 1986, Caulfield 1993, Felder 1995). The m2 and m4 receptor subtypes may activate phospholipase A2 in addition to inhibiting the activity of adenylate cyclase (Hosey 1992, Caulfield 1993, Felder 1995). Activation of phospholipase C by m1, m3, or m5 muscarinic receptor subtypes promotes the hydrolysis of phosphatidylinositol(4,5)bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge 1984, 1993).

After binding to its receptor on non-mitochondrial vesicles (Putney 1987), IP_3 induces calcium mobilization from these intracellular calcium stores and elevates the levels of free intracellular calcium, whereas DAG activates protein kinase C (PKC) (Nishizuka 1986, 1995). Thus, the signal transduction route elicited by m1, m3, or m5 resembles the processes elicited by group I metabotropic glutamate receptors. Phospholipase A_2 activation by m1, m3, and m5 receptors generates free arachidonic acid and the corresponding lysophospholipid from membrane phospholipids (Felder 1995). Arachidonic acid metabolism may have an important role in the production of reactive oxygen species (ROS) (Lafon-Cazal et al. 1993).

Thus, several muscarinic receptor subtypes may be potentially linked to the ROS production through multiple effector cascades, i.e. by elevating intracellular

calcium levels, activating PKC, or by increasing the formation of arachidonic acid through phospholipase A_2 activation. Inositolphosphate metabolism and subsequent PKC activation have been shown to be important factors in the initiation of the production of reactive oxygen species in immunological cells (Ruotsalainen & Savolainen 1995, Heiskanen & Savolainen 1996).

2.2 Oxidative stress

Oxidative stress in the central nervous system elevates free intracellular calcium levels, releases excitatory amino acids, increases lipid peroxidation, and depletes neuronal glutathione stores (Halliwell 1992, Savolainen et al. 1994, 1996). The brain is especially susceptible to ROS attack because of its high lipid content, high rate of oxidative metabolism, and low levels of free-radical scavenging enzymes (Halliwell & Gutteridge 1985, LeBel & Bondy 1991, Bondy 1992, Halliwell 1992). Both neuronal and glial cells possess antioxidant capabilities, though these are more prominent in the glial cells (Raps et al. 1989). Moreover, hypoxia-induced oxidative stress is associated with the depletion of glutathione from neuronal cells (Siesjö et al. 1989). However, besides many harmful effects, reactive oxygen species may also have an important role in cell signalling since they also function as second messengers (Schreck et al. 1991, Pahl & Baeuerle 1994).

2.2.1 Free radicals and reactive oxygen species

Neuronal ATP is primarily formed in mitochondria through oxidative phosphorylation. In this process, molecular oxygen (O_2) is reduced to water (H_2O) through the addition of four electrons and four hydrogen atoms (Halliwell & Cutteridge 1989). These reactions involve the generation of the superoxide radical ($O_2^{\bullet}$), the oxidizing agent hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($OH^{\bullet}$). Free radicals are atoms or molecules containing an unpaired electron in one or more orbitals (Halliwell & Cutteridge 1989, Halliwell & Cross 1994). Free radicals are usually very reactive because pairing of electrons spinning in opposite directions impairs their stability. They are capable of reacting with and damaging, a variety of biological molecules, including proteins, lipids, and DNA, and they can also mediate programmed (apoptotic) cell death (Wolff et al. 1986, Ratan et al. 1994).

There are many potential sources of oxygen free radicals within cells. About 2% of the oxygen consumed by mitochondria is incompletely reduced, and escaped electrons from electron transport machinery induce the formation of $O_2^{\bullet}$ and $OH^{\bullet}$ radicals. The hydroxyl radical is a very reactive oxidizing agent and is likely to be

the prime mediator of oxidative damage in cells. The superoxide anion is considerably less toxic and it induces rather limited direct damage by itself. However, $O_2^{\bullet}$ is a serious hazard to neuronal cells because it can react with the nitric oxide radical ($NO^{\bullet}$) to form the extremely reactive peroxynitrite ($ONOO^-$) and $OH^{\bullet}$ radicals (Beckman et al. 1990). $NO^{\bullet}$ is generated by a family of enzymes called nitric oxide synthases (NOS). Constitutive forms of NOS, which are expressed in neuronal cells, are activated by a time-limited cytosolic calcium increase which leads to the release of $NO^{\bullet}$ which lasts for several minutes. Inducible NOS isoforms are expressed after inflammatory stimuli, producing large amounts of $NO^{\bullet}$, for up to several days (Nathan & Xie 1994, Brüne et al. 1995). Both superoxide and nitric oxide can be produced in response to elevated intracellular levels of calcium. After activation of phospholipase A_2 by calcium, arachidonic acid is released and further metabolized to form $O_2^{\bullet}$. Also, calcium may activate a protease which converts xanthine dehydrogenase to xanthine oxidase which then catalyzes the oxidation of xanthine to uric acid and $O_2^{\bullet}$.

2.2.2 The relationship between excitotoxicity and oxidative stress

Many neurological disorders may involve both neuronal excitation and subsequent oxidative stress, and several neurotoxic substances may trigger both of these events simultaneously (Maridonneau-Parini et al. 1986, Bondy & LeBel 1993). Antioxidants are able to protect against cytotoxicity induced by high intracellular levels of calcium in the central nervous system. In addition, decreased intracellular calcium levels may reduce hydrogen peroxide-elicited DNA damage, and blocking of calcium channels can prevent cyanide-induced peroxidation of membrane lipids and subsequent cell death (Maduh et al. 1988, Cantoni et al. 1989). It seems likely that free radical-mediated neuronal damage may be a common pathway for injury in which excitotoxic and other forms of neuronal injuries of the central nervous system converge. Reactive oxygen species also induce the release of glutamate from hippocampal slices and inhibit glutamate uptake by glial cells (Pellegrini-Giampietro et al. 1990, Volterra et al. 1994).

Glutamate toxicity has been recently linked to the production of reactive oxygen species and the onset of oxidative stress in neuronal preparations (Murphy et al. 1989, Bondy & Lee 1993, Lafon-Cazal et al. 1993, Reynolds & Hastings 1995). The most likely signal transduction effectors contributing to the production of reactive oxygen species after excitatory stimulation of neuronal cells are phospholipases C and A_2, activation of nitric oxide synthase, activation of oxidative enzyme systems including cyclo-oxygenase or lipoxygenase, P_{450} enzymes and xanthine oxidase (Dugan & Choi 1994). Intracellular calcium levels are elevated and protein kinase C is activated after the activation of phospholipase C.

Impairment of mitochondrial enzyme machinery and subsequent leakage of

reactive oxygen species may contribute to, and amplify EAA toxicity (Dugan & Choi 1994). Recent studies suggest a critical role for mitochondria in the production of ROS in association with glutamate excitotoxicity (Dugan et al. 1995, Reynolds & Hastings 1995). These studies show that NMDA receptor-mediated, calcium-dependent uncoupling of mitochondrial electron transport may contribute to the ROS production elicited by glutamate exposure. In more detail, mitochondrial calcium accumulation and the subsequent permeability transition may be critical early events leading to the onset of cell death during excitotoxicity (Nieminen et al. 1996, Schinder et al. 1996, White & Reynolds 1996).

Protein kinase C is an important enzyme in the initiation of the production of reactive oxygen species through the phosphorylation of NADPH oxidase e.g. in immunological cells (Ruotsalainen & Savolainen 1995, Heiskanen & Savolainen 1996). The role of protein kinase C in EAA-induced production of reactive oxygen species in neuronal cells has remained open but Cid & Ortega (1993) observed that in cultured chick cerebellar glial cells both glutamate and its structural analogue kainate evoked an increase in the number of binding sites for [^{3}H]phorbol 12,13-dibutyrate. This was mediated via the activation of AMPA/kainate receptors. These findings are in agreement with a possible role of PKC in glutamate-evoked production of ROS in neuronal cells.

Activation of calcium-stimulated phospholipase A_2 by calcium influx initiated by NMDA receptor activation can lead to increased arachidonic acid metabolism followed by increased production of reactive oxygen intermediates (Pellerin & Wolfe 1991, Lafon-Cazal et al. 1993, Dugan & Choi 1994). Oxidative activity generated by phospholipase A_2 may also impair gamma-aminobutyrate (GABA) receptor channels which are the major neuronal inhibitory receptor complexes in the central nervous system (Schwartz et al. 1988). Impairment of inhibitory receptors may lead to the overt excitation of neuronal cells and subsequent increase in oxidative stress. Calcium also activates the oxidative burst in polymorphonuclear leukocytes, these cells are known to accumulate in post-ischemic cerebral tissues, leading to the production of ROS (Zimmerman et al. 1989, Ruotsalainen et al. 1995). In addition, calcium activates the proteolytic conversion of xanthine dehydrogenase to xanthine oxidase (McCord 1987).

There is evidence that nitric oxide may also mediate the neurotoxicity of glutamate by activating guanyl cyclase and by increasing the production of cyclic GMP. Agonists of NMDA subtype of glutaminergic receptors stimulate nitric oxide synthase, and subsequently nitric oxide can interact with superoxide anion to form the very reactive peroxynitrite radical (Dawson et al. 1991, Kiedrowski et al 1992, Bonfoco et al. 1995, Moncada & Higgs 1995).

2.3 Lead

Lead is the most ubiquitous toxic heavy metal and can be found in practically all compartments of the inert environment and in all biological systems. Lead is toxic to most living organisms at high doses, and there is no known biological requirement for lead. Specific concerns vary with the age and condition of the host, with the principal risk being toxicity to the nervous system. The principal route of exposure in humans is via food, and sources that cause surplus exposure to lead include lead-based paints, and lead in environmental dusts, drinking water, in air from combustion, and hand-to-mouth activities of young children living in polluted environments. Moreover, significant exposure to lead occurs in the manufacturing of lead-glazed pottery. Also lead dust brought home by industrial workers on their clothes may cause additional exposure of workers and their families (Goyer & Rhyne 1973, Goyer 1993).

Organic lead compounds (e.g. triethyllead and trimethyllead) may have even greater impact on the central nervous system than inorganic lead substances (Chang 1990). Organometal compounds are difficult to eliminate from the central nervous system, and injuries may lead to permanent neurological deficits. Organolead compounds interfere with GABA and glutamate metabolism, as well as mitochondrial function, and intracellular calcium homeostasis (Verity 1990).

Adults may absorb 5 to 15% of ingested lead but less than 5% of ingested lead is retained in the body. In children, the absorbtion may be much more effective, even 40% of the ingested inorganic lead salts may be absorbed. Also, the retention of lead in tissues is greater in infants than in adults. It has been shown that almost 30% net retention of ingested lead may occur in children (NRC 1993).

2.3.1 Effects of lead on cellular signalling, and differentiation of neuronal cells

The biochemical and molecular mechanisms of lead toxicity are poorly understood. Recent data suggest that some of the effects of lead may be due to its interference with calcium-mediated cellular processes (Simons 1986, Markovac & Goldstein 1988a, Bressler & Goldstein 1991), such as the release of neurotransmitters dopamine, norepinephrine, and acetylcholine. These effects of lead are most likely mediated through interference with calcium metabolism and/or synaptic functioning (Minnema et al. 1988, Rius et al. 1988, Bressler & Goldstein 1991), or the effects on the activities of protein kinases, calmodulin, tyrosine hydroxylase, glucocorticoid receptor binding, and choline acetyltransferase, or on brain energy metabolism (Markovac & Goldstein 1988a,b, Laterra et al. 1992, Ronnback & Hansson 1992, Tong et al. 1996, Tonner et al. 1997).

Lead may activate protein kinase C (PKC) by mimicking calcium (Markovac & Goldstein 1988a,b, Long et al. 1994), and this may result in the production of

reactive oxygen species (Nishizuka 1986). Accordingly, both organic and inorganic lead increase lipid peroxidation and the production of reactive oxygen species in several cell types (Rehman 1984, Ali & Bondy 1989, LeBel et al. 1990). High doses of lead may produce either severe central nervous system (CNS) symptoms such as ataxia, convulsions, and coma, or lesser CNS deficits including learning disorders, hyperactivity, and headache (Needleman et al. 1990, Tong et al. 1996). High-dose exposure to lead may disrupt the blood-brain barrier, even in the adult brain. However, infants are much more susceptible than adults to lead toxicity, and even low doses of lead may cause neuronal dysfunction in children (Goldstein et al. 1974, McMichael et al. 1988, Tong et al. 1996).

Lead has also been linked to differentiation and growth of neuronal cells (Audesirk et al. 1991, Kern et al. 1993). Audesirk et al. (1991) have reported that lead may have an impact on multiple regulatory processes that influence neuronal survival and differentiation, and that its effects show varying dose-dependencies. They have also suggested that lead may have different effects depending on the cell type studied. Lead activates calcium-sensitive enzyme PKC in cell-free preparations (Markovac & Goldstein 1988a, Long et al. 1994) and in brain microvessels (Markovac & Goldstein 1988b). However, lead may also have inhibitory effects in the micromolar concentration range on PKC subtypes (I, II, and III) purified from the rat brain (Murakami et al. 1993). Also Rajanna and coworkers have reported inhibitory actions on partially purified rat brain PKC by micromolar concentrations of lead acetate (Rajanna et al. 1995). In addition, the results of Tomsig & Suszkiw (1995) indicate that lead may be a partial agonist of PKC, capable of both activating or inhibiting the enzyme depending on the lead concentration. One source of difficulty in characterizing lead-PKC interactions has been the existence of several phospholipid/calcium-dependent (α, β, and γ) and phospholipid-dependent/calcium-independent (δ, ϵ, and ζ) PKC isozymes, which may exhibit different sensitivity to activation or inhibition by polyvalent metal cations (Maurer et al. 1992).

Protein kinase C (PKC) is the key regulatory enzyme in many important cellular functions (Nishizuka 1986, Nishizuka 1995), including the cell cycle (Nishizuka 1986, Soma et al. 1994), growth (Weinstein 1988, Battaini et al. 1990, Mischak et al. 1993), and differentiation (Wada et al. 1989, Clemens et al. 1992, Leli et al. 1993). PKC has been proposed to be one of the principal enzymes involved in differentiation of neuroblastoma cells (Heikkilä et al. 1989, Miñana et al. 1990, Leli et al. 1992). Both long-term PKC activation by phorbol esters and PKC inhibition by specific PKC inhibitors have been reported to induce differentiation in neuronal cells (Heikkilä et al. 1989, Felipo et al. 1990, Leli et al. 1992) indicating a possible role of PKC down-regulation or direct inhibition in cellular differentiation. Since lead may have profound effects on the activity of PKC, lead may also have an impact on cellular processes such as growth, proliferation and differentiation.

2.3.2 Effects of lead on excitotoxicity

Lead, and other metal ions have several important properties which greatly influence their effects on membranes of the cells. Metal ions may bind electrostatically to membrane proteins and lipids, and they may also bind chemically to membrane channel proteins and receptors. Furthermore, the existence of specific metal receptors has also been suggested (Kiss & Osipenko 1994). Specifically, lead has multiple effects on the function of various distinct types of voltage-, receptor-, and calcium-activated cell membrane ion channels (Kiss & Osipenko 1994, Vijverberg et al. 1994). By altering the properties of channels which govern the transport of ions across the plasma membrane, and also by interfering with the membranes of vesicles which function as intracellular calcium stores, lead may have profound effects on cellular excitability and subsequent oxidative stress (Vijverberg et al. 1994).

Vijverberg and coworkers (1994) have investigated the direct effects of lead on ion channels in cultured N1E-115 neuroblastoma cells. In these cells, lead elicits both inhibitory and activating effects on ion channels. Voltage-activated ion channels were blocked by lead at micromolar or submillimolar concentrations. Similar block of voltage-activated ion channels by lead has, in fact, been reported by many groups (Audesirk & Audesirk 1991, Reuveny & Narahashi 1991, Audesirk & Audesirk 1993, Büsselberg et al. 1994a,b). The receptor-activated ion channels recently studied include the neuronal nicotinic acetylcholine receptor which was inhibited by nanomolar concentrations of lead, and activated by micromolar lead concentrations. However, serotonin receptors were only inhibited by lead (Vijverberg et al. 1994). Since calcium is involved in the regulation of learning and memory (Bressler & Goldstein 1991), a number of biochemical and physiological studies have addressed the effects of lead on the glutaminergic ionotropic NMDA receptor (Alkondon et al. 1990, Ujihara & Albuquerque 1992, Guilarte & Miceli 1992, Uteshev et al. 1993, Ishihara et al. 1995). Lead reduces NMDA-activated channel currents without reducing glutamate- or kainate-activated currents (Alkondon et al. 1990) and without changing the voltage characteristics of the channel current (Ujihara & Albuquerque 1992, Uteshev et al. 1993).

It has been shown that lead may elicit numerous changes also in cholinergic functions in the central nervous system. These include alterations in cerebral acetylcholine levels, release of acetylcholine, acetylcholinesterase activity, and binding of agonists towards their muscarinic receptors (Bondy & Agrawal 1980, Costa & Fox 1983, Cory-Slechta & Pokora 1995). The most consistent effects of lead are decreased acetylcholine release and diminished cerebral cholinergic activity (Shih & Hanin 1978, Hrdina et al. 1980, Bielarczyk et al. 1994). In contrast, Cory-Slechta & Pokora (1995) have reported that exposure to lead may significantly increase the sensitivity of muscarinic cholinergic receptors to oxotremorine but not to arecoline, and attenuate the ability of atropine to antagonize the cholinergic

effects of arecoline.

Ion fluxes through metal ion-activated ion channels may also mediate some of the effects of lead (Kiss & Osipenko 1994, Vijverberg et al. 1994). External lead, at high concentrations, directly activates a slow inward current in N1E-115 cells (Oortgiesen et al. 1990, Vijverberg et al. 1994). This current is not blocked by channel blockers or receptor antagonists indicating that it is not mediated via any previously described membrane ion channel (Vijverberg et al. 1994).

2.3.3 Effects of lead on oxidative stress

Although lead does not readily undergo the valence changes characteristic of transition metals, the catalysis of peroxidative reactions by lead may be a major contributing factor to the toxic effects of this metal (Donaldson & Knowles 1993, Stohs & Bagchi 1995).There are reports suggesting that lead increases dose- and time-dependently hepatic production of peroxides (Lawton & Donaldson 1991), and that in rats lead causes lipid peroxidation which is prevented by vitamin E (Ramstoeck et al. 1980). Lead-induced lipid peroxidation has also been detected in several brain regions, mostly in the frontal cortex (Gerber et al. 1978, Rehman 1984, Ali & Bondy 1989). However, hippocampal and cerebral membranes have not shown any increases in lipid peroxidation (Ali & Bondy 1989). Bondy & Guo (1996) have reported that lead markedly promoted iron-initiated production of ROS in rat cerebral synaptosomal suspension, and was similar to aluminum in this respect (Bondy & Kirstein 1996). They suggested that the possible mechanism could be the formation of an active chelate of iron on the surface of insoluble lead salts (Bondy & Guo 1996).

Since the formation of free radicals and subsequent oxidative stress are closely related to the increased levels of cytosolic calcium, there may be a close connection of metals capable of mimicking calcium with the initiation of free radical formation (Olanow & Arendash 1994). By mimicking calcium, lead may have profound effects on calcium-activated proteases, calcium-activated NOS, and on calcium-activated PLA_2 which all are linked to the free radical formation in the cells. Metals have been shown to take part in the development of many neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis. It is also established that in the pathogenesis of these diseases, free radicals play a major role (Bondy & LeBel 1993, Olanow & Arendash 1994).

3 AIM OF THE STUDY

The aim of the present thesis was to explore the mechanisms of oxidative stress caused by glutaminergic or cholinergic stimulation of human SH-SY5Y neuroblastoma cells. The modulating effects of an environmental toxin, lead, were also investigated on glutamate-induced neuronal activation, oxidative stress, and neuronal differentiation.

The specific aims of this study were:

1. To assess the expression of glutamate receptor subtypes and $[Ca^{2+}]_i$ responses in human SH-SY5Y neuroblastoma cell line (I).

2. To study the involvement of glutaminergic and cholinergic stimulation in oxidative stress in human SH-SY5Y cells (II, V).

3. To assess the role of the environmental toxin, lead, in the production of reactive oxygen species and subsequent oxidative stress (II, III).

4. To study the role of protein kinase C in the onset of oxidative stress (II, III, V).

5. To explore the involvement of lead and protein kinase C in the differentiation of human SH-SY5Y neuroblastoma cell line (IV).

4 SUMMARY OF MATERIALS AND METHODS

4.1 Cell culture

Human SH-SY5Y neuroblastoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 7 or 10% inactivated fetal calf serum (FCS) and 50 U/ml penicillin and 50 µg/ml streptomycin, and incubated at +37°C in 5% CO_2 in an air-ventilated humidified incubator. The cells used in the experiments were cultured to confluency and harvested with 0.02% EDTA.

4.2 Measurement of free intracellular calcium

The levels of free intracellular calcium ($[Ca^{2+}]_i$) were measured using the acetoxymethyl ester of the fluorescent calcium probe, fura-2 (fura-2/AM) (Grynkiewicz et al. 1985). Aliquots (1 ml) of the cells were loaded with 3 µM fura-2/AM with a constant agitation for 45 min at +37°C. After loading, the cells were washed twice with ice cold Hanks' balanced salt solution (HBSS) and resuspended in HBSS (without Ca^{2+} and Mg^{2+}, including 1 mg/ml bovine serum albumin and 10 mM D-glucose) to give a final cell concentration of 3 x 10^6 cell/ml. One ml of the cell suspension and 1 ml of prewarmed HBSS were placed in a quartz cuvette, in which the final cell concentration was 1.5 x 10^6 cells/ml. Free intracellular calcium was measured using a Hitachi F-4000 spectrofluorometer equipped with a thermostated cuvette holder and a magnetic stirrer applying the method described by Tsien et al., (1985). The fluorescence was monitored at an excitation wavelength of 340 nm (bandpass 5 nm) and an emission wavelength of 510 nm (bandpass 5 nm). Free intracellular calcium was calculated using the equation

$$[Ca^{2+}]_i = K_D(F - F_{min})/(F_{max} - F),$$

where K_D = 224 nM, F = the relative level of intracellular fluorescence, F_{min} = the relative level of fura-2 fluorescence in 7.5 mM EGTA after cell lysis with 10% sodium dodecyl sulphate, and F_{max} = the relative level of fluorescence with 7 mM calcium added after the determination of F_{min}. Before the measurements, $CaCl_2$ was added directly into the cuvette to give a final concentration of calcium of 1.2 mM in the medium. Twenty μM of $MnCl_2$ was used to quench the extracellular fura-2/AM.

4.3 Measurement of soluble lead concentration

The actual, soluble lead concentration in the incubation medium (HBSS including 10 mM D-glucose and 1 mg/ml bovine serum albumin) was measured by using an atomic absorption spectrometer (Perkin-Elmer 306) applying a flame detection technique. The lead which was precipitated in the medium was spun down to obtain a clear solution containing only the soluble lead. The lead standards were prepared both in water and in the medium to check out the possible effect of different matrices. The medium had no effect on the standard values.

4.4 Measurement of intracellular reactive oxygen species

For the measurement of the production of reactive oxygen species, neuroblastoma cells were loaded with 25 μM 2',7'-dichlorofluorescin diacetate (Bass et al. 1983) for 15 min at +37°C in 1 ml aliquots (3 x 10^6 cells/ml) of the cell suspension. After loading, the cells were washed twice with HBSS including 10 mM D-glucose and 1 mg/ml bovine serum albumin. The cells were then incubated (final concentration 1.5 x 10^6 cells/ml) with the experimental compounds. The formation of the fluorescent compound, dichlorofluorescein, was monitored at an excitation wavelength of 488 nm (bandpass 5 nm) and an emission wavelength of 525 nm (bandpass 20 nm) using a Hitachi F-4000 spectrofluorometer equipped with a thermostated cuvette holder and a magnetic stirrer.

4.5 Measurement of intracellular glutathione levels

For the measurement of changes in intracellular glutathione (GSH) levels,

neuroblastoma cells were incubated with the experimental compounds at +37°C in a shaking water bath. After the treatments, the cells were loaded with 100 µM monochlorobimane (MBCL) (Shrieve et al. 1988) for 15 min in 1 ml aliquots (3 x 10^6 cells/ml) of the cell suspension. The loaded cells were spun down and suspended in HBSS for the measurement of the intracellular levels of GSH (final concentration was 1.5 x 10^6 cells/ml). The formation of the fluorescent MBCL-GSH-complex was monitored at an excitation wavelength of 395 nm (bandpass 5 nm) and an emission wavelength of 470 nm (bandpass 5 nm) using a Hitachi F-4000 spectrofluorometer.

4.6 Receptor binding assays

Specific displacement of L-^{3}H-glutamate by glutamate or specific ionotropic (NMDA, AMPA, or kainate) or metabotropic (trans-ACPD) glutamate receptor agonists was measured using intact SH-SY5Y neuroblastoma cells. All incubations were done in Tris-buffer (50 mM Tris, pH 7.4 adjusted with acetic acid) to avoid Na^+ and Cl^- ions which may interfere with the binding of the agonists with the glutamate receptors (Young & Fagg 1990). The cells were simultaneously incubated with 10 nM L-^{3}H-glutamate, and with 10 nM - 1 mM of unlabeled agonist for 15 min at +4°C. Incubation volume was 250 µl (100-200 µg protein/sample). After that, the cells were centrifuged , and the cell pellets were washed three times with ice cold Tris-buffer to remove unbound label. The washed pellets were agitated for 24 hours in liquid scintillant, and thereafter the samples were subjected to liquid scintillation counting. Nonspecific binding ranged between 9 and 26% of the total binding.

Quinuclidinylbenzilate (QNB) binding was assayed using intact SH-SY5Y neuroblastoma cells. All incubations were carried out in HBSS buffer including 10 mM D-glucose and 1.3 mM $CaCl_2$. All phases of the experiments were conducted in this solution except dilutions of ^{3}H-QNB which were made in HBSS including 10 mM D-glucose, 1.3 mM $CaCl_2$, and 1 mg/ml bovine serum albumin. Reaction volume was 100 µl (ca. 100 µg protein/sample) and the reaction was carried out at room temperature for 20 min by using gentle orbital shaking. Total binding was measured utilizing eight ^{3}H-QNB concentrations (0.25 - 50 nM), and nonspecific binding was determined in the presence of 100 µM atropine. Nonspecific binding was always less than 30% of the total binding. The reactions were stopped with ice-cold HBSS, and the cells were harvested to Whatman GF/C glassfibre filters which were presoaked in HBSS for 30 min. Unbound label was removed from the filters by suction using a Millipore equipment. The filters were washed twice with ice-cold HBSS and agitated overnight in liquid scintillant. Thereafter the filters were subjected to liquid scintillation counting. The binding data were analyzed using Scatchard analysis.

4.7 Measurement of protein kinase C translocation

Phorboldibutyrate (PDBu) binding assays were conducted by measuring total and nonspecific binding to intact human SH-SY5Y neuroblastoma cells utilizing eight ^{3}H-PDBu concentrations (0.5 - 30 nM) for 20 min at room temperature using gentle orbital shaking. The nonspecific binding was determined in the presence of 10 μM phorbol myristate acetate, and it was always less than 30% of the total binding. The reactions were carried out in HBSS containing 10 mM D-glucose, 1 mg/ml bovine serum albumin, and 1.3 mM $CaCl_2$, and dilutions of ^{3}H-PDBu were made using the same buffer. Reaction volume was 100 μl (80-100 μg protein/sample). The reactions were started by adding the cell suspension and stopped with ice-cold HBSS, and the cells were harvested to Whatman GF/C glassfibre filters which were presoaked in HBSS for 30 min. The filters were washed twice with ice-gold HBSS using a Millipore suction equipment to remove unbound label. The filters were agitated overnight in liquid scintillant, and thereafter the filters were subjected to liquid scintillation counting. The binding data were analyzed using Scatchard analysis.

4.8 Measurement of DNA and protein synthesis

Measurement of thymidine and leucine incorporation were utilized to assess neuronal DNA and protein synthesis, respectively. Five thousand cells per well were grown in Costar 96 well plates. The cells were allowed to attach to the wells overnight. After cell attachment, cells were incubated with the selected experimental compounds for various time periods (3-72 h). Then, 0.1 μCi of ^{3}H-thymidine (20 Ci/mmol, 1.0 mCi/ml) or 1.0 μCi of ^{3}H-leucine (85 Ci/mmol, 1.0 mCi/ml) in 50 μl of DMEM was added, and the cells were incubated for 2.5 h. After the incubation, the cells were detached by shaking and then they were aspirated to a glass fibre filter (Filtermat A, Wallac, Finland) using a TomTec cell harvester (TomTec Inc., USA). In preliminary experiments, washes after incubations were used. They were, however, later omitted because of the loose attachment of SH-SY5Y cells to the plates. After harvesting of the cells, the wells and the filter were washed three times using a TomTec cell harvester with an automated program. Filtermat A was dried for 1 h at 37°C and a melt-on scintillator MeltiLex A (Wallac, Finland) was melted on the filter. After this procedure, the radioactivity incorporated in the filter containing MeltiLex A was counted using MicroBeta 1450 microplate scintillation counter (Wallac, Turku, Finland).

4.9 Assessment of morphological differentiation

Morphological differentiation was measured from photographs of several randomly chosen fields of the cultures and was expressed as the percentage of cells with processes longer than 50 µm (Påhlman et al. 1981). Neurites from at least 200 cells were calculated.

4.10 Measurement of total cell number

The cells were cultured on 6-well plates at a seeding density of 5×10^4 cells/well. The cells were allowed to attach to the wells overnight. After incubation with drugs and drug combinations, the cells were detached by 0.02% EDTA in phosphate-buffered saline, and counted using a Bürker counting chamber.

4.11 The cell viability assays

The cell viability was determined using propidium iodide fluorescence to detect dead cells. SH-SY5Y cell suspension (1×10^6 cells/ml) was washed once with HBSS buffer, and the cells were then suspended in 1 ml aliquots in HBSS containing 10 mM D-glucose and 1 mg/ml bovine serum albumin, and this cell suspension was added to a quartz cuvette with 1 ml of buffer (final concentration was 5×10^5 cells/ml). Propidium iodide (10 µM) was added to the cuvette, and after 5 min incubation, the baseline fluorescence values were measured. Thereafter, fluorescence values were measured every 30 min for 180 minutes. At the end of the experiments 15 µg/ml digitonin was added for 10 min to permeabilize the cells and obtain the maximal fluorescence values corresponding to 100% cell death. Fluorescence was monitored at an excitation wavelength of 515 nm (bandpass 10 nm), and at an emission wavelength of 620 nm (bandpass 10 nm) using a Hitachi F-4000 spectrofluorometer. Percent viability was calculated as follows: $V = 100 \times (B - X) / (B - A)$, where V is percent viability, B is the fluorescence after permeabilizing of the cells, X is the fluorescence at any given time point, and A is the baseline fluorescence.

The cell viability was also assayed by using the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant (Cytotoxicity Detection Kit (LDH), Boehringer Mannheim).

In addition, Trypan Blue exclusion assay was used to determine cell viability. The cells were incubated with experimental substances at +37 °C in a shaking water

bath at a concentration of 1 x 10^6 cells/ml. After incubation, the aliquot of the cells (100 µl) was removed and suspended with the same amount of 0.4% Trypan Blue solution. The cells were incubated with Trypan Blue for 2 min before counting the cells using a Bürker counting chamber.

4.12 Data analysis

The data were analyzed with one-way analysis of variance and Duncan's multiple-range test. $P < 0.05$ was considered statistically significant. SPSS/PC+ computer program was used for statistical analysis. Prism 2.01 computer program was used to calculate Scatchard analysis.

5 REVIEW OF THE RESULTS

5.1 Glutamate-induced changes in $[Ca^{2+}]_i$ and expression of glutamate receptors (I)

High concentrations (500 μM - 3 mM) of glutamate and of specific ionotropic glutamate receptor agonists induced a rapid initial increase in $[Ca^{2+}]_i$ which was followed by a sustained decrease of $[Ca^{2+}]_i$. Low concentrations (< 500 μM) of glutamate receptor agonists caused only a sustained decrease in $[Ca^{2+}]_i$. Glutamate, N-methyl-D-aspartate (NMDA), and kainate all caused dose-dependent increase in $[Ca^{2+}]_i$ at a concentration range between 500 μM and 3 mM. However, amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) did not cause a significant increase in $[Ca^{2+}]_i$. This is not, however, surprising because AMPA is known to affect mainly sodium fluxes via the AMPA receptor (Monaghan et al. 1989). The metabotropic glutamate receptor agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) increased $[Ca^{2+}]_i$ only at a 3 mM concentration (at 17 sec). The time to reach the maximum elevation of $[Ca^{2+}]_i$ was dose-dependently shortened by glutamate and kainate. After glutamate, the time to reach the $[Ca^{2+}]_i$ maximum decreased from 29 (500 μM) to 19 sec (3 mM), whereas after kainate it decreased from 6 (1 mM) to 3 sec (3 mM). On the contrary, AMPA prolonged the time for the maximum in $[Ca^{2+}]_i$ from 19 (300 μM) to 64 sec (3 mM). NMDA did not have any consistent effect on the calcium peaking time which varied between 12 and 17 sec.

The initial increase in $[Ca^{2+}]_i$ produced by glutamate, NMDA, kainate, AMPA, and 1*S*,3*R*-ACPD was followed by a sustained decrease in $[Ca^{2+}]_i$ which reached a low point at 3 min; there was always a dose-dependent decrease in $[Ca^{2+}]_i$ which was, however, reversed at high doses. At low agonist concentrations,

however, only a decrease in $[Ca^{2+}]_i$ occurred. The levels of $[Ca^{2+}]_i$ remained decreased, at least for the follow-up period of 10 min. The basal $[Ca^{2+}]_i$ in unstimulated SH-SY5Y neuroblastoma cells was 131 ± 4 nM (mean ± SEM). The maximal decrease was 30 - 55 nM depending on the agonist.

The receptor binding data suggest that both ionotropic and metabotropic glutamate receptor agonists exhibit specific binding to their receptors in human SH-SY5Y neuroblastoma cells.

5.2 Effects of glutaminergic stimulation on oxidative stress; amplification by lead (II)

L-glutamate (1 mM) was effective in increasing the production of reactive oxygen species (ROS) in human SH-SY5Y neuroblastoma cells; this effect was observed, however, only when cellular glutathione (GSH) levels were depleted with diethylmaleate (DEM). GSH levels were about 34% of the control after 30 min, and 22% of the control after 120 min of incubation with 0.02% DEM. DEM incubation alone had no effect on the production of ROS in SH-SY5Y neuroblastoma cells. L-glutamate alone did not affect either the levels of cellular GSH or cell viability. Lead acetate (1 mM), or a protein kinase C inhibitor, Ro 31-7549 (1 μM; Twomey et al. 1988), alone did not have any effects on the production of ROS, the levels of GSH, or the viability of human SH-SY5Y neuroblastoma cells. When the cells were exposed to a new PKC inhibitor, Ro 31-7549 (1 μM), together with L-glutamate (1 mM) in the presence of DEM (0.02%), glutamate-induced production of ROS was completely blocked.

Co-exposure of cells to lead acetate (1 mM) and L-glutamate (1 mM) always markedly increased the production of ROS as compared to the effects of these compounds alone. The production of ROS induced by lead-glutamate combination also markedly exceeded that induced by L-glutamate alone in the presence of 0.02% DEM. The maximal per cent increase from the control value in the production ROS, measured by relative fluorescence at 60 min, was 219 ± 62% (mean ± SEM) for lead and L-glutamate. The amplification of glutamate-induced production of ROS by lead was especially remarkable because neither of the compounds released reactive oxygen species on their own, except for glutamate when cellular glutathione was depleted.

When the cells in the present study were co-exposed to lead acetate and L-glutamate, the production of ROS increased almost linearly for 60 min. Also the GSH levels were depleted when the cells were exposed to this drug combination. The decrease in cell viability followed exactly the same pattern when this drug combination was used.

There was a 10 min lag-phase prior to the beginning of the increased

production of ROS in the experiment in which lead, together with L-glutamate was used. During this lag-phase, the fluorescence increased only slightly, or stayed at the control level. After 60 min, the production of reactive oxygen species, the depletion of GSH, and the loss of cell viability reached a plateau which lasted for at least two hours. L-glutamate, lead acetate, or Ro 31-7549 did not cause any cytotoxicity on their own.

5.3 Effects of lead and protein kinase C inhibition on the production of reactive oxygen species (III)

Exposure of human SH-SY5Y neuroblastoma cells to lead acetate (1 mM) or a protein kinase C inhibitor, Ro 31-7549 (1 μM), alone did not affect the production of reactive oxygen metabolites, the levels of intracellular glutathione, or the viability of these cells.

Co-exposure of the cells to lead acetate (1 mM) and a protein kinase C inhibitor, Ro 31-7549 (1 μM), always markedly increased the production of ROS. The maximal per cent increase from the control value in the ROS production, measured by relative fluorescence at 60 min, was 313 ± 71% (mean ± SEM). The actual total soluble lead concentration in our experiments was in the micromolar range (10-20 μM).

When the cells in the present study were co-exposed to lead acetate and a PKC inhibitor, ROS production increased almost linearly for 60 min. Also the GSH levels were depleted when the cells were exposed to this drug combination and the decrease in cell viability followed exactly the same pattern. Cellular levels of GSH were 76 ± 3% of control values at 60 min, and cell viability was 49 ± 1% when observed at 30 min.

There was a 10 min lag-phase prior to the beginning of the increased ROS production in the experiment in which lead, together with a protein kinase C inhibitor, was used. During this lag-phase, the fluorescence increased only slightly, or stayed at the control level. After 60 min, the production of ROS, the depletion of GSH, and the loss of cell viability reached a plateau which lasted for at least two hours.

5.4 Involvement of lead and protein kinase C in the differentiation of SH-SY5Y cells (IV)

Phorbol myristate acetate (PMA; 16 nM), a protein kinase C activator, promoted differentiation of SH-SY5Y cells after 72 hours of incubation. DNA synthesis, as

measured by thymidine incorporation, decreased to 60% of control already at 24 h, i.e., before the differentiation of the cells by PMA. Also protein synthesis, as measured by leucine incorporation, decreased marginally, to 85% of control, after 72 h of incubation with PMA. Total cell number decreased to 49% of the control values after 72 h of incubation of the cells with PMA. The viability of the cells, as measured with propidium iodide staining or LDH release, decreased to 13% of the control values at 72 h.

When 10 μM PKC inhibitor, Ro 31-8220 (Ro), was used, DNA synthesis decreased rapidly, within 3 h, to 50% of control values. Also protein synthesis decreased within 3 h to 80% of control values, and within 24 h to 50% of the control values. However, Ro did not promote any differentiation of SH-SY5Y cells as compared to the control cells. Ro decreased the total cell number to 56% of control values within 72 h of incubation. Likewise, cell viability decreased to 57% of the control values after 72 h of incubation of the cells with Ro, consistent with the total cell number data.

Co-incubation of the cells with 16 nM PMA and 10 μM Ro markedly amplified the differentiation of the cells already at 6 h, as compared to the effect induced by the compounds alone. Cell differentiation was markedly enhanced, with respect to that induced by either substance alone, from 6 h to 48 h (ca. 45% and 70% of the cells differentiated, respectively), but returned to the level induced by PMA alone after 72 h of incubation. The combination of PMA and Ro decreased DNA and protein synthesis in a manner similar to that induced by Ro alone. The total cell number was 45% of the control values after 72 h of incubation of the cells with PMA and Ro. Cell viability decreased to 83% of the control values after 72 h of incubation of the cells with PMA and Ro.

The cells did not differentiate when they were exposed to 10 nM concentration of lead, and their DNA or protein synthesis was not affected. However, the total cell number increased slightly, to 129% of the control values, after 72 h of incubation of the cells with 10 nM lead. A higher, micromolar lead concentration (100 μM) induced differentiation (ca. 55% of the cells differentiated) of the cells after 72 h of incubation. The high lead concentration also decreased thymidine (to 60% of control values), and leucine (to ca. 80% of control values) incorporation at 72 h. The total cell number decreased to 89% of the control values after 72 h of incubation with the high concentration of lead. Lead-induced alterations in the viability of the cells were similar to changes in the total cell numbers induced by lead.

Co-incubation of the cells with 10 nM lead and 10 μM Ro did not induce differentiation of the cells. Lead and Ro, however, in combination rapidly decreased both DNA and protein synthesis. The total cell number was 59% of the control values after 72 h of incubation, which corresponds well to the decrease in the cell viability subsequent to their exposure to lead and Ro.

When the cells were co-incubated with 100 μM lead acetate and 10 μM Ro

there was a moderate differentiation (ca. 40% differentiated) of the cells after 72 h. DNA and protein synthesis decreased rapidly in a manner similar to that induced by Ro alone. The total number of the cells decreased to 51% of the control values after 72 h of incubation with this combination of agents. The viability of the cells also decreased to 27% of the control values after 72 h of incubation with this combination.

5.5 Effects of cholinergic stimulation on the production of reactive oxygen species (V)

Stimulation of human SH-SY5Y neuroblastoma cells with one millimolar carbachol (CCh) increased both the levels of free intracellular calcium and the production of ROS. The levels of free intracellular calcium became elevated without any delay, whereas the increase in the production of ROS started within 30 min of incubation with 1 mM CCh, and the difference from the control cells was about three fold at 3 h. With the doses of 100 or 500 μM of CCh, the ROS production remained at a control level, or there was only a very slight increase in the ROS production, which was not, however, statistically different from the control. With 3 mM CCh, ROS production was about the same as with 1 mM CCh. Thus, there seemed to be a threshold of 1 mM before CCh-induced ROS production in human SH-SY5Y neuroblastoma cells in these experimental conditions. ROS production was completely blocked by the muscarinic receptor antagonist, atropine, and by a PKC inhibitor, Ro 31-8220. Superoxide dismutase inhibited ROS production totally, whereas catalase only partially inhibited ROS production.

Intracellular glutathione levels were slightly decreased (80% of control) after 120 min of incubation with 1 mM CCh although the values obtained did not differ statistically significantly from the control values. However, after 180 min of incubation, the GSH levels had returned to the control level (93.5% of control). Three millimolar CCh caused a slight decrease in the GSH levels (88.5% of control), whereas other CCh concentrations (100 and 500 μM) had no effect.

Scatchard analysis revealed that QNB binding had both high (20% of the total amount) and low (80% of the total amount) affinity binding sites. For a two site fit, the regression coefficient (r) was on average 0.85 (mean of 24 Scatchard-plots). For a one site fit, the r was on average only 0.47. There was a marked increase in the number of the low affinity binding sites after one hour of incubation with 1 mM CCh. Also the number of the high affinity binding sites increased after one hour of incubation with CCh, but the increase was not statistically significantly different from the basal binding. One hour of incubation with 1 mM CCh tended to decrease ligand affinity both to the low and high affinity binding sites. After 3 h of incubation, the binding parameters returned back to the basal level.

PDBu binding, which reflects translocation of PKC to the cell membrane, revealed a marked increase in the number of binding sites after 20 min of incubation with 1 mM CCh, but a decrease in binding site affinity. Scatchard analysis revealed only one type of binding site for PDBu.

Cell viability was not affected by the incubation of the cells with 1 mM CCh.

6 DISCUSSION

The results of the present study indicate that glutaminergic (I, II) and cholinergic (V) excitatory receptor systems are capable of producing reactive oxygen species and of causing subsequent oxidative stress in a human SH-SY5Y neuroblastoma cell line. Protein kinase C and possibly the overall phosphorylation status of the cells seem to have a vital role in the production of reactive oxygen species in this cell line (II, III, V). This study provides a new alternative to explain the mechanism of lead neurotoxicity, and suggests that lead amplifies glutamate-induced oxidative stress and this is involved in the subsequent neurotoxicity (II). It seems that the differentiation of this cell line may be associated with the down-regulation of protein kinase C, and that the differentiation may have a protein kinase C-dependent and -independent component (IV). Lead-induced differentiation of neuroblastoma cells seems to be under the partial control of protein kinase C (IV).

6.1 Glutaminergic stimulation elicits a functional intracellular calcium response in SH-SY5Y cells (I)

Functional or morphological expression of glutamate receptors in human SH-SY5Y neuroblastoma cells has not been reported earlier. The present study shows that both glutamate and selective ionotropic and metabotropic glutamate receptor agonists induce a slow biphasic response in free intracellular calcium levels ($[Ca^{2+}]_i$) in human SH-SY5Y neuroblastoma cells. The rapid initial increase is followed by a sustained decrease in cell calcium, suggesting both stimulatory effects on $[Ca^{2+}]_i$ and sequestration of excess calcium after ionotropic and metabotropic glutamate receptor stimulation. Specific binding of glutamate and other glutamate receptor

agonists, together with the biphasic calcium response produced by a glutamate receptor agonist, suggests that human SH-SY5Y neuroblastoma cells express both ionotropic and metabotropic glutamate receptors. There is one report in the literature on the expression of NMDA receptors in the SH-SY5Y cell line by Nair and coworkers (Nair et al. 1996) in which they show the presence of NMDA-R1 mRNA in this cell line. They have used this cell line to study the interaction of NMDA and dopamine receptors.

The differences between the effects of glutamate, NMDA, kainate and AMPA on $[Ca^{2+}]_i$ are probably partially due to conformational changes that take place in receptor-gated calcium channels. The increase of $[Ca^{2+}]_i$ by 1S,3R-ACPD occurred only at a high concentration (3 mM) at 17 sec. It is, however, in agreement with the time for the onset of an increase in $[Ca^{2+}]_i$ induced by other agonists coupled to calcium-mobilizing receptors (Furuya et al. 1989, Tuomala et al. 1992, Tuomala et al. 1993). These data suggest that ionotropic rather than metabotropic glutamate receptors dominate the temporal pattern (Nicoll 1988) of intracellular calcium increases after stimulation of human SH-SY5Y neuroblastoma cells by glutamate or specific ionotropic glutamate receptor agonists. In addition to the opening of receptor-gated calcium channels (Bertolino & Llinás 1992) and intracellular release of calcium (Berridge 1993), secondary depolarization of neuronal membrane and subsequent opening of voltage-dependent calcium channels (Nicotera & Orrenius 1992, Reuveny & Narahashi 1993) may contribute to the elevation of $[Ca^{2+}]_i$. This study describes an integrated calcium response to glutamate receptor agonists, which does not exclude, however, rapid calcium oscillations (Berridge 1993) with a wide range of local alterations in $[Ca^{2+}]_i$. In fact, excitable cells are continuously susceptible to depolarization via firing of other neuronal cells, and this direct depolarization may also contribute to the elevated $[Ca^{2+}]_i$ through voltage-dependent calcium channels (Nicotera & Orrenius 1992, Reuveny & Narahashi 1993). If calcium is not removed from the cytoplasm, it may reach toxic or desensitizing levels (Blaustein 1988, Nicotera & Orrenius 1992).

The normal physiological range of $[Ca^{2+}]_i$ in unstimulated cells is between 50 and 200 nM (Carafoli 1987); maintaining $[Ca^{2+}]_i$ in the physiological concentration range is essential for neuronal cells (Tsien et al. 1988). There are several effective mechanisms in SH-SY5Y neuroblastoma cells to stabilize $[Ca^{2+}]_i$, i.e. calcium pumps to remove calcium from the cytosol to the extracellular space, or to intracellular calcium stores, such as endoplasmic reticulum, mitochondria and nucleus (Blaustein 1988, Baimbridge et al. 1992, Carafoli 1992, Nicotera & Orrenius 1992). Moreover, calcium binding by cytosolic proteins, including calmodulin, parvalbumin, and vitamin D-dependent calcium binding protein, are effective buffering processes (Blaustein 1988) essential for the protection of excited neuronal cells against calcium-induced cell death (Nicotera & Orrenius 1992).

The sustained decrease of $[Ca^{2+}]_i$ below the resting level was an unexpected finding. Excessive glutamate receptor stimulation may be followed by receptor

desensitization (Catania et al. 1991) which may cause a change in the balance between $[Ca^{2+}]_i$ elevations, calcium buffering, and calcium removing systems. Moreover, glutamate receptor-induced elevation of $[Ca^{2+}]_i$ may be associated with neuronal hyperpolarization (Nicoll 1988) which would result in a relative desensitization of voltage-dependent calcium channels toward depolarizing firing. The biphasic $[Ca^{2+}]_i$ response upon glutamate receptor stimulation provides evidence that two separate processes are involved in these calcium-mediated changes. Even though the integrated decrease in $[Ca^{2+}]_i$ always remained within the physiological range, rapid oscillations in $[Ca^{2+}]_i$ are possible.

In conjunction with the slow biphasic calcium responses to glutamate receptor activation, the receptor binding data also provide evidence for the expression of glutamate receptors in human SH-SY5Y neuroblastoma cells. Both receptor-gated calcium channels, and the stimulation of G-protein coupled calcium-mobilizing second messenger systems, are likely to be involved in the glutamate-induced changes of $[Ca^{2+}]_i$. This increases the functional diversity and plasticity of human SH-SY5Y neuroblastoma cells after glutamate receptor stimulation. Thus, human SH-SY5Y neuroblastoma cells may be useful in exploring excitatory amino acid-induced processes in human neuronal cells.

6.2 Glutamate causes production of ROS which is amplified by lead (II)

Glutamate increased the production of reactive oxygen species in human SH-SY5Y neuroblastoma cells. In agreement with the present results, others have shown that NMDA receptor stimulation with high doses of NMDA (1 mM) or L-glutamate (3 mM) induces superoxide production in cultured cerebellar granule cells (Lafon-Cazal et al. 1993), and that glutamate receptor agonists induce the production of reactive oxygen species in the synaptoneurosomal fraction, also at high doses (Bondy & Lee 1993). In this study, glutamate-induced oxidative stress was, however, only observed when cellular GSH levels were depleted by DEM. This may be due to activation of gene expression intended to protect cells against oxidative damage (Reed et al. 1991). The human SH-SY5Y neuroblastoma cell line contains naturally high levels of bcl-2 gene products which are known to neutralize ROS (Reed et al. 1991). The present results also indicate that the GSH defence system against oxidative stress seems to be very effective in this cell line.

The production of reactive oxygen species by L-glutamate in this study was completely blocked by a protein kinase C (PKC) inhibitor, indicating a role for PKC in glutamate-induced ROS production. The present findings suggest that glutamate increases the production of reactive oxygen species through stimulation of its cell surface receptors and by elevating $[Ca^{2+}]_i$ (I). Elevations of $[Ca^{2+}]_i$ contribute to

PKC activation and subsequent cellular events (Nishizuka 1986). The involvement of both ionotropic and metabotropic glutamate receptors is possible in Ca^{2+}-mediated cell stimulation. The production of ROS may also be partially routed through stimulation of mitochondrial oxidative metabolism (Kane et al. 1993).

The importance of PKC in the glutamate-induced oxidative burst is emphasized by the finding that glutamate may also increase the number of membrane phorbol dibutyrate (PDBu) binding sites (Cid & Ortega 1993) via the translocation of PKC to the membrane. L-glutamate may increase the influx of lead and calcium to the cell interior through glutamate receptor-coupled calcium channels (I), or through voltage-dependent calcium channels. This is possible because lead, in aqueous solutions, closely mimicks calcium (Simons 1986). These conclusions are also in agreement with the finding that lead directly stimulates PKC by mimicking calcium (Markovac & Goldstein 1988a, b, Long et al. 1994). Thus, lead may potentially induce PKC-mediated production of ROS in neuronal cells.

It was interesting that there was a 10-minute lag-time before the amplification of glutamate-induced production of ROS by lead. However, this lag-time is in a good agreement with the incubation time needed to see an increase in the number of PDBu binding sites after L-glutamate (Cid & Ortega 1993). This time may have been needed to allow the translocation and subsequent activation of PKC.

The amount of ROS produced by combination of lead and glutamate reached an apparent plateau after 60 min of cell stimulation. It is possible that within 60 min of ROS production, GSH synthesis becomes activated to neutralize these reactive oxygen species. Increased GSH synthesis could explain the plateau in the production of ROS, as well as the plateaus in GSH depletion, and in the loss of cell viability.

The production of reactive oxygen species reflects an important endpoint in a cascade of complex cellular events. The present results add a new dimension to the significance of the finding that lead increases the activity of purified PKC in an acellular system (Markovac & Goldstein 1988a), an observation that is consistent with the present findings. The actual total soluble lead concentration in our experiments was in the micromolar range (10-20 μM) as measured by atomic absorption spectrometry (Simons 1986). This concentration is higher than those found to be highly toxic to humans (Bressler & Goldstein 1991). However, the intracellular free lead concentrations to which PKC and other systems were exposed may be considerably lower in the present situation.

The present results suggest that lead causes neuronal damage by amplifying glutamate-induced oxidative stress. L-glutamate is an ubiquitous neurotransmitter in the brain (Nicholls 1993), and long-term exposure to lead may disrupt the blood-brain barrier (McMichael et al. 1988). This may cause the accumulation of lead in the brain (Rehman 1984). Thus, lead may, through PKC activation, amplify the excitotoxic effects of L-glutamate on neuronal functions.

6.3 PKC inhibition enhances the ability of lead to induce oxidative stress in SH-SY5Y cells (III)

Combination of lead with a PKC inhibitor, Ro 31-7549, increased the production of ROS, decreased intracellular GSH levels, and increased cytotoxicity in human SH-SY5Y neuroblastoma cells. This finding is of special interest since neither of these agents had any effects on their own on ROS production, GSH levels, or cell viability. The present results suggest that the state of PKC activity may be an important factor in lead neurotoxicity. Inhibition of phosphorylation of PKC target proteins involved in ROS production may render them active, resulting in a synergistic interaction between lead and PKC in ROS production.

A novel PKC inhibitor, bis-indolyl maleimide, Ro 31-7549, is structurally related to staurosporine and K252a (Twomey et al. 1990). Ro 31-7549 has a similar inhibitory potency for PKC as staurosporine, and is more potent than K252a. However, it is much more selective for PKC than staurosporine or K252a with an IC_{50} value of 50 - 80 nM for PKC in a cell free system, but its IC_{50} value against protein kinase A is 4.2 - 5.1 μM and against Ca^{2+}/calmodulin-dependent kinase it is 15 μM (Twomey et al. 1990). Ro 31-7549 shows a slight selectivity for PKC α over β, γ, and ϵ, and it competes with ATP for binding to the enzyme (Wilkinson et al. 1993). Ro 31-7549 should, therefore, cause a potent and specific inhibition of PKC, and a subsequent inhibition of PKC-mediated phosphorylation (Combadière et al. 1993) of target proteins. Accordingly, Ro 31-7549 dramatically attenuated the glutamate-induced oxidative burst in SH-SY5Y cells (II).

Protein kinase C inhibition by Ro 31-7549 may affect the functional properties of calcium channels in the cell membrane by altering their phosphorylation state (Levitan 1994). By mimicking calcium, lead may gain access to the cell through cell surface calcium channels which may not be phosphorylated by PKC. After entering the cell, lead may have calcium-like effects on a variety of cellular enzymatic systems (Simons 1986). It has been shown by others (Audesirk 1993) that under comparable experimental conditions, lead may enter the cell mainly via voltage-sensitive calcium channels. Increased influx of lead is consistent with the result of the present study that inhibition of calcium channel phosphorylation may enhance the influx of lead into the cell. When the SH-SY5Y neuroblastoma cells were stimulated with lead and glutamate, there was also an increase in oxidative stress (II), possibly due to the entry of lead into the cell through glutamate receptor-coupled calcium channels. However, lead-induced direct activation of PKC (Markovac & Goldstein 1988a, b, Long et al. 1994) does not seem to be the mechanism to account for this lead-PKC inhibitor interaction because PKC activity is inhibited by Ro 31-7549 alone (Twomey et al. 1990).

Lead may interfere with sulfhydryl (-SH) groups of proteins (Viarengo & Nicotera 1991), and through this action it may directly affect cellular GSH levels.

GSH depletion by lead may lead to accumulation of sufficient ROS to overwhelm cellular antioxidant defenses, and to lead to increased oxidative stress. Lead may affect GSH levels in two ways, by increasing ROS production and by inhibiting the activity of ROS-scavenging enzymes. Others have shown that lead may also affect the cell membrane antioxidant enzymes, superoxide dismutase, catalase, and glutathione peroxidase, or cellular GSH levels (Viarengo & Nicotera 1991, Somashekaraiah et al. 1992). Lead may also cause cell membrane lipid peroxidation (Donaldson 1991, Somashekaraiah et al. 1992, Dominguez et al. 1995), an effect which could be reversed by the antioxidant, ethoxyquin (Donaldson 1991).

The production of ROS with the combination of lead and the PKC inhibitor reached a plateau after 60 min of cell stimulation and continuous ROS production. It is possible, therefore, that within 60 min after the onset of the oxidative burst, GSH synthesis becomes activated and is capable of neutralizing ROS. Increased GSH synthesis could explain the plateau in the ROS production, GSH depletion, and cytotoxicity. The plateau in the ROS production may also be due to decreased cell viability, i.e. due to a decrease of the number of ROS-producing cells, induced within 30 min by simultaneous incubation of the cells with lead and Ro 31-7549. Lead may also, like calcium, be subject to a concentration gradient accross the cell membrane (Markovac & Goldstein 1988a). The relatively high lead concentrations used here in the medium may be required to demonstrate the observed effects in intact cells. Others have used purified PKC or brain microvessels to explore the effects of lead on PKC (Markovac & Goldstein 1988a, b, Long et al. 1994).

It seems likely that lead requires the presence of additional factors before it is able to induce oxidative stress in human neuroblastoma cells. PKC inhibition may enhance the ability of lead to induce the oxidative burst, possibly by allowing lead to penetrate calcium channels in the cell membrane. Also, perturbation of protein phosphorylation by PKC inhibition may render enzymes in a form in which they may be activated by lead. The resulting activation of calcium-sensitive enzymes, important in ROS production, may be essential for lead-induced neurotoxicity; a similar interaction occurred when neuroblastoma cells were exposed to lead and glutamate (I, II).

6.4 Lead and PKC have a role in the differentiation of SH-SY5Y cells (IV)

The PMA-induced prolonged PKC activation, the associated down-regulation of the enzyme, and subsequent differentiation of the cells are corroboration of the findings of other investigators (Påhlman et al. 1981, Jalava et al. 1992). Prolonged exposure of the cells to PMA may cause down-regulation of PKC which is associated with cell differentiation (Heikkilä et al. 1989, Leli et al. 1993). These findings emphasize

the importance of the state of PKC activity, as well as the amount of PKC, in the cascade of events leading to cell differentiation.

The present study shows that PMA decreased DNA synthesis and increased cell differentiation. Both findings are consistent with a decreased rate of cell division, and neuronal differentiation, and emphasize the role of PKC in these events (Spinelli et al. 1982). Protein synthesis also decreased concomitantly with the decreased DNA synthesis and the differentiation of the cells. Moreover, PMA decreased the total number of the cells after 72 h of incubation. Decreased cell number, associated with increased differentiation of the cells, may be due to the activation of programmed cell death in this cell population, possibly due to down-regulation of PKC (Dypbukt et al. 1994).

It has been reported that the reduction of PKC activity by PKC inhibitors or neutralizing PKC-specific antibodies may also induce neuronal differentiation (Morioka et al. 1985, Felipo et al. 1990, Miñana et al. 1990, Leli et al. 1992, Chakravarthy et al. 1995). Furthermore, endogenous PKC inhibitors may also play a role in modulating cellular processes regulated by PKC, including differentiation (Chakravarthy et al. 1995). However, contrary to earlier reports, in the present study, inhibition of PKC did not have any effect on the differentiation of SH-SY5Y neuroblastoma cells, although a PKC inhibitor decreased DNA and protein synthesis in these cells. A PKC inhibitor, Ro 31-8220, which is more specific toward PKC than the inhibitors used by other investigators (see Twomey et al. 1990), was used in this study. Ro 31-8220 has been shown to have a considerably higher selectivity toward PKC than staurosporine or H-7 both of which also inhibit other kinases (Budworth & Gescher 1995) in intact cells (Dieter & Fitzke 1991, Wilkinson & Hallam 1994). It is also noteworthy that cellular differentiation and the decrease in DNA or protein synthesis do not seem to be necessarily linked to each other because Ro 31-8220 attenuated both DNA and protein synthesis without affecting neuronal differentiation, but, at the same time, decreased the number of cells. On the other hand, Heikkilä et al. (1993) have reported that a selective PKC inhibitor, GF 109203X, inhibits PMA-induced morphological and functional differentiation of SH-SY5Y neuroblastoma cells. GF 109203X is a bisindolylmaleimide, and structurally resembles Ro 31-8220 which was used in the present study.

Interestingly, simultaneous incubation of the cells with a PKC activator (PMA) and a PKC inhibitor (Ro 31-8220) caused a marked and rapid differentiation of SH-SY5Y neuroblastoma cells. This phenomenon is in contrast to earlier findings (Jalava et al. 1992, Tint et al. 1992). However, Heikkilä et al. (1989) reported that a non-selective inhibitor of PKC, H-7, enhanced morphological differentiation of SH-SY5Y cells by PMA. This amplification of differentiation was rapid, and in agreement with the present results. In both studies co-incubation of the cells for 72 h with PMA together with H-7 (Heikkilä et al. 1989) or Ro 31-8220 (IV) caused a similar pattern of differentiation of SH-SY5Y neuroblastoma cells. In the present study, the combination of PMA and Ro 31-8220 induced an inhibition similar to that

induced by Ro 31-8220 alone in DNA and protein synthesis. However, the effect of this combination on the total cell number was identical to the effects induced by the agents alone. The time-courses of cell differentiation in these studies (Heikkillä et al. 1989, IV) indicate that the possible differentiating factor could be down-regulation rather than direct activation of PKC. Another possible explanation is that PMA may have effects on enzymes other than PKC, the activity of which would be revealed only when PKC is inhibited. What these other targets may be, remain to be elucidated but they are likely to have an impact on cellular differentiation. It seems that the roles of PKC and PKC inhibition in neuronal differentiation will require further study because of the opposite effects of selective and non-selective PKC inhibitors.

Low lead concentration (10 nM) did not elicit any differentiation of the cells, or decrease the thymide or leucine incorporation, with respect to the control cells. There was, actually, a slight increase in the total cell number after 72 h of incubation of the cells with 10 nM lead. When a PKC inhibitor, Ro 31-8220, was applied together with 10 nM lead, the cell number decreased to a level induced by Ro 31-8220 alone. Thus, PKC may be involved in the modest cellular proliferation induced by low doses of lead (see also Dypbukt et al. 1994).

The present observations agree with proposals of Audesirk et al. (1991) that lead may have bimodal effects on cell number and differentiation, because a high lead concentration (100 μM) caused differentiation of the cells at 72 h, and this effect was associated with decreased DNA and protein synthesis. However, the high lead concentration slightly decreased the total cell number. The positive effects of lead on neuronal differentiation are, however, in contrast to the results of Audesirk and coworkers (1991) who reported inhibitory actions of high lead doses on neurite initiation. The present results suggest that high lead doses activate events different to those activated by low lead doses and that they may be more nonspecific in nature. The PKC inhibitor opposed the differentiation elicited by 100 μM lead indicating that lead-induced differentiation of SH-SY5Y cells is at least under the partial control of PKC. This is consistent with the earlier observation (III) which shows that PKC inhibition may markedly amplify the effect of lead on oxidative stress in neuronal cells. Lead and a PKC inhibitor, Ro 31-8220, may act on different sites in PKC during their interaction. Taken as a whole, low and high lead doses caused markedly different effects on events associated with cellular differentiation.

In the light of the present findings, it seems likely that the differentiation of human neuroblastoma cells is associated with the down-regulation of PKC. There may, in fact, be a PKC-dependent and a PKC-independent component in the differentiation of human SH-SY5Y cells because Ro 31-8220 greatly amplified the PMA-induced neuronal differentiation without affecting DNA or protein synthesis, or the total cell number. The Effects of lead on neuronal differentiation, in turn, seem to be under the partial control of PKC. Lead may have an effect on multiple regulatory processes that influence neuronal survival and differentiation, and these

processes may be variably affected by different doses of lead.

6.5 Muscarinic stimulation induces oxidative response in SH-SY5Y cell line (V)

Cholinergic neuronal stimulation subsequent to excessive activation of brain muscarinic receptors may induce excitotoxicity and neuronal damage *in vivo* (Jope et al. 1986, Hirvonen et al. 1993). This activation may be due to direct binding of muscarinic agonists, e.g. pilocarpine (Jope et al. 1986) to muscarinic receptors, or due to elevated acetylcholine (ACh) levels in the synaptic cleft. Cholinergic-induced seizures also lead to an increase in cerebral ACh levels (Fonnum & Guttmormsen 1969, Shih 1982, Jope et al. 1986) which may lead to excessive stimulation of postsynaptic muscarinic receptors, subsequent seizures and neuronal damage (Jope et al. 1986, 1987, Savolainen et al. 1988). Furthermore, cholinergic stimulation has been shown to induce co-release of both glutamate and aspartate from synaptosomes (Docherty et al. 1987). In neuronal cells, glutamate-induced neuronal excitation is associated with oxidative stress (Bondy 1992, Bondy & Lee 1993, Lafon-Cazal et al. 1993, II). Thus, both cholinergic muscarinic and glutaminergic neuronal stimulation may cause neuronal excitotoxicity, either directly or indirectly. However, to our knowledge, this is the first report that cholinergic stimulation *per se* promoted the production of ROS in neuronal cells *in vitro*. In the present study the concentration of CCh was relatively high (1 mM) but it is likely that the concentrations of ACh are also high in the synaptic cleft after transmitter release due to neuronal stimulation. Moreover, cholinergic neuronal stimulation (V) may be more efficient than glutaminergic receptor activation (II) in inducing ROS production in human neuroblastoma cells.

In this study, ROS production began within 30 min after the beginning of the incubation of the SH-SY5Y neuroblastoma cells with carbachol (CCh). The rapid increase in $[Ca^{2+}]_i$ was typical for CCh-induced stimulation of SH-SY5Y cells (Lambert & Nahorski 1990, Murphy et al. 1991). The complete inhibition of CCh-induced production of ROS by atropine clearly demonstrates that the phenomenon was due to stimulation of cholinergic muscarinic receptors. The QNB binding increased and the binding affinity of QNB decreased within the first hour of incubation but returned back to the control levels after three hours even though ROS production persisted at least for this time period. These data demonstrate that muscarinic receptor stimulation was clearly involved in CCh-induced production of ROS in SH-SY5Y neuroblastoma cells because both binding to receptor occurred and ROS production could be prevented by atropine. The decrease in binding affinity may have been a compensatory response to protect the neurons against excessive cholinergic stimulation. These findings may also point to a possible

triggering role for cholinergic stimulation in the production of ROS in neuroblastoma cells. Persistent ROS production may be due to activation of intracellular signalling events distal to muscarinic receptor stimulation.

CCh-induced muscarinic receptor-mediated stimulation of neuronal cells activates phosphoinositide signalling pathways resulting in the activation of PKC. This activation may contribute to sustained ROS production (Nishizuka 1986, Murphy et al. 1991, Tuomala et al. 1992, Cid & Ortega 1993, Savolainen et al. 1995, II). In this study PKC was translocated to the cell membrane after 20 min of incubation with CCh suggesting that conventional isoforms, i.e. α, βI, βII and γ, of PKC may have the potential to be activated during cholinergic stimulation (Newton 1995). We also demonstrated that a PKC inhibitor completely blocked CCh-induced ROS production. The present study also shows that glutamate-induced production of ROS can be completely blocked by a competitive inhibitor of PKC (II). The present findings indicate that the stimulation of neuronal cells by both muscarinic and glutaminergic agonists is associated with increased production of ROS (II, V). A role for calcium-dependent phospholipase A2 and the arachidonic acid cascade as a source for ROS cannot be excluded at this stage. Moreover, it is possible that phospholipase D is involved in the prolonged PKC activation (Klein et al. 1995).

A modest decrease in cellular glutathione levels occurred after two hours of incubation with carbachol. This finding differs from results on glutamate-induced oxidative stress (II). Glutamate in combination with lead induced a sustained and marked reduction of glutathione in SH-SY5Y neuroblastoma cells (II). These differences may indicate distinct mechanisms of cellular defense towards ROS depending on the receptor system or intracellular signalling cascade initiating the production of ROS.

Increased ROS production by CCh was completely blocked by superoxide dismutase and partially blocked by catalase. These findings indicate that the production of the superoxide anion was the predominant form of ROS induced by CCh. However, some of these species were apparently metabolized to products other than hydrogen peroxide because catalase was only partially active (Halliwell 1992). These findings also support a specific receptor-mediated production of ROS, a process in which PKC is likely to be involved.

It was interesting that CCh stimulation of the cells did not affect the cell viability even though there was a marked and sustained production of ROS. In contrast, when a sustained ROS production was elicited by the incubation of the cells with a combination of glutamate and lead, the cell viability decreased markedly (II). Also this difference between cell viability after cholinergic and glutaminergic neuronal stimulation may be an indication of different mechanisms through which glutamate and carbachol induce oxidative stress in neuronal cells. In addition, differences in early gene expression should not be excluded when exploring mechanisms and consequences of neuronal stimulation by cholinergic and glutaminergic agonists.

These new findings on the role of muscarinic receptors in ROS production may be important for the understanding of cellular signalling and excitotoxic events in neuronal cells. It will be important to explore the role of sites distal to the receptor mediating agonist-induced oxidative stress to reveal which cellular events ultimately are responsible for the production of ROS and subsequent oxidative stress.

7 CONCLUSIONS

The main focus of the present study was on glutaminergic and cholinergic stimulation-induced production of reactive oxygen species and subsequent oxidative stress as well as the modulatory effects of an environmental toxin, lead, on glutamate-induced excitation of the human SH-SY5Y neuroblastoma cell line. The main conclusions are as follows:

1. Ionotropic and metabotropic subtypes of glutamate receptors are present in human SH-SY5Y neuroblastoma cells. The stimulation of these receptors elicits a functional intracellular calcium response. These findings provide a good foundation for the use of this cell line in future studies involving glutaminergic receptor subtypes.

2. Glutaminergic stimulation is capable of promoting the production of ROS in human SH-SY5Y neuroblastoma cells if the cellular glutathione defense system is impaired. Also stimulation of cholinergic muscarinic receptors elicits the production of ROS in this cell line. The present results suggest a triggering role for cholinergic muscarinic receptors in ROS production.

3. The ROS production elicited by glutamate is greatly amplified by lead in human SH-SY5Y neuroblastoma cells. In addition, lead together with a protein kinase C inhibitor, provokes the production of ROS in this cell line. It seems that the phosphorylation status of protein kinase C target proteins is a critical factor in lead neurotoxicity. These results point to a new possible mechanism for lead neurotoxicity, i.e. the amplified production of ROS.

4. The production of ROS by both glutaminergic and cholinergic stimulation

is mediated by protein kinase C. It seems that protein kinase C has an important role in the production of ROS and subsequent oxidative stress in human SH-SY5Y neuroblastoma cells.

5. A PKC inhibitor dramatically increases phorbol myristate acetate-induced differentiation of SH-SY5Y cells. Lead has bimodal actions on differentiation and cell viability depending on the concentration applied to the cells. The effects of lead seem also to be under the partial control of protein kinase C. It seems possible that differentiation of SH-SY5Y cells may have a PKC-dependent and a PKC-independent component.

Proposed mechanisms for glutaminergic- and cholinergic-induced production of ROS and its modulation by lead are depicted in Fig. 1.

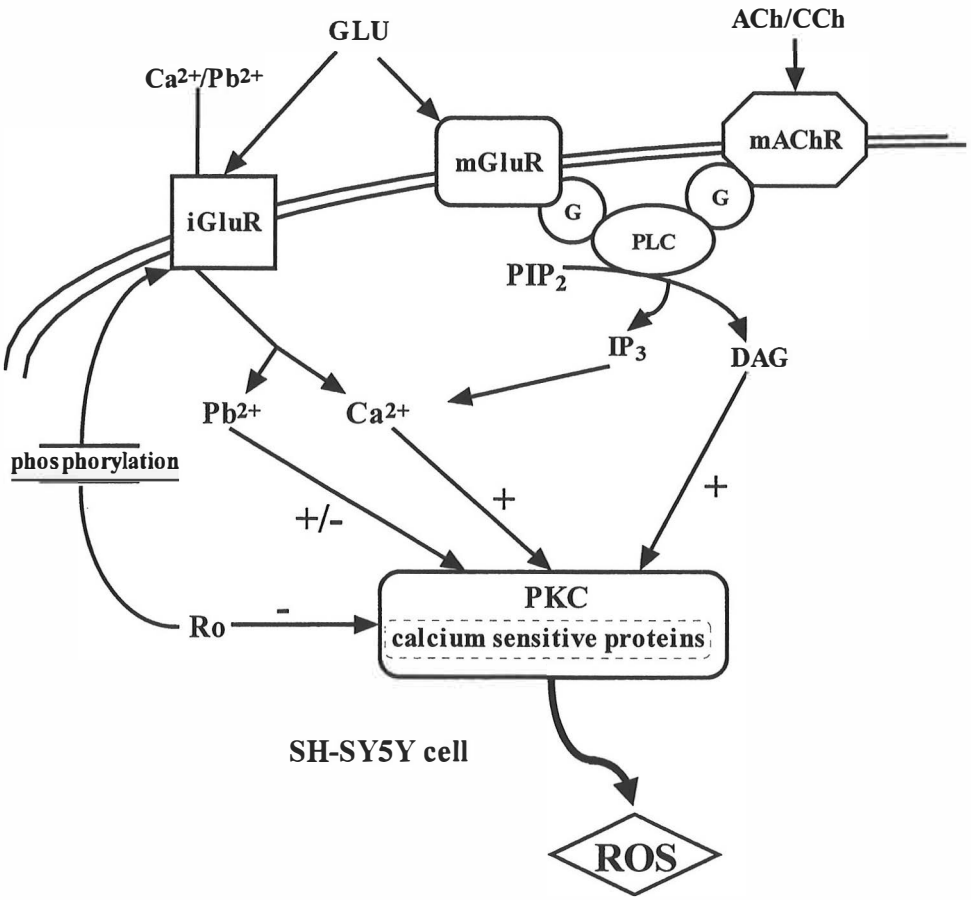

FIGURE 1 Possible mechanisms of glutaminergic- and cholinergic-induced production of ROS and its modulation by lead in human neuroblastoma cells. Glutamate (Glu) activates ionotropic (iGluR) and metabotropic (mGluR) glutaminergic receptors, and acetylcholine (ACh) or carbachol (CCh) activate cholinergic muscarinic receptors (mAChR). Subsequent to opening of a calcium channel in iGluR, calcium, and possibly lead, enter the cell. Calcium has a positive effect on calcium sensitive proteins, including protein kinase C (PKC). Lead may have activating or inhibitory actions on calcium sensitive enzymes. mGluR and mAChR are linked to G proteins and to phospholipase C (PLC) (only one PLC is shown for simplicity). Through activation of PLC, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) are formed. IP_3 releases calcium from intracellular stores and DAG activates PKC. A protein kinase C inhibitor (Ro) directly inhibits PKC and may also block the phosphorylation of a cell surface calcium channel, possibly converting the channels to the open state allowing lead to enter the cell. PKC and other calcium sensitive enzymes may have an important role in the production of reactive oxygen species (ROS), (see text for details).

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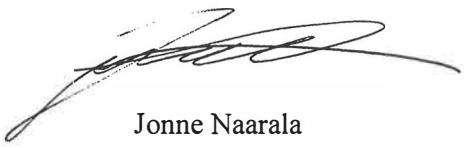
Kuopio 1997  Jonne Naarala

Yhteenveto (Résumé in Finnish)

Tutkimus lyijyn neurotoksisuuden ja oksidatiivisen stressin mekanismeista ihmisen neuroblastoomasoluissa

Glutamaatti ja asetyylikoliini ovat aivojen tärkeimmät kiihottavat välittäjäaineet. Ne osallistuvat stimuloivien hermoimpulssien muodostumiseen hermopäätteissä. Esiintyessään ylimäärin synapsiraoissa ne voivat kuitenkin olla hermosoluille myös myrkyllisiä eli eksitotoksisia. Tällainen reseptorivälitteinen hermosolujen ylikiihottuminen voi olla tärkeä hermosoluvaurioiden aiheuttaja, koska hermosolut ovat uusiutumattomia. Molempien reseptorityppien on todettu osallistuvan happiradikaalien muodostumiseen ja sitä kautta oksidatiivisen stressin käynnistymiseen hermosoluissa. Eksitotoksisuuden ja oksidatiivisen stressin onkin todettu yhdessä liittyvän monien aivosairauksien tai oireiden, kuten epilepsian, Alzheimerin taudin, Huntingtonin taudin ja Parkinsonismin, syntyyn.

Lyijy on klassinen neurotoksinen metalli, jonka myrkyllisyyden solu- ja molekyylibiologisista mekanismeista tiedetään kuitenkin yllättävän vähän. Lyijyn vaarallisuus ei suinkaan ole vähentynyt, vaikka onkin siirrytty käyttämään esimerkiksi lyijytöntä polttoainetta. Lyijyä on kertynyt huomattavia määriä maaperään, josta lyijyllä on pääsy ekosysteemin muihin osiin. Lyijy pystyy läpäisemään veri-aivoesteen lapsilla hyvin ja aikuisillakin kohtalaisesti. Lyijyn vahingolliset vaikutukset ovat moninaisia. Se voi aiheuttaa vähäisempiä neurologisia oireita kuten päänsärkyä ja hyperaktiivisuutta, tai vaikeampia oireita kuten oppimisvaikeuksia tai jopa kouristuksia.

Tässä väitöskirjassa pyrittiin selvittämään oksidatiivisen stressin syntyä ja sen mekanismeja ihmisen neuroblastoomasolulinjassa glutaminergisen tai kolinergisen stimulaation seurauksena. Lisäksi tutkittiin lyijyn muuntelevaa vaikutusta glutamaattistimulaation aiheuttamaan oksidatiiviseen stressiin ja lyijyn vaikutusta neuroblastoomasolujen erilaistumiseen.

Tulosten perusteella ihmisen SH-SY5Y neuroblastoomasolulinja ilmentää sekä glutaminergisia että kolinergisia reseptoreita. Molempien reseptorityyppien stimulointi aiheuttaa soluissa happiradikaalituotannon, kuitenkin siten, että glutaminerginen stimulaatio on tehokas vasta solujen puolustuskyvyn heikentämisen jälkeen. Glutaminergisen ja kolinergisen stimulaation aiheuttama happiradikaalituotanto on ainakin osittain riippuvaista proteiinikinaasi C -entsyymistä. Lyijy vahvistaa voimakkaasti glutamaatin aiheuttamaa oksidatiivista stressiä. Käsiteltäessä soluja yhtäaikaisesti glutamaatilla ja lyijyllä myös solujen glutationitasot laskevat ja solujen elävyys vähenee merkittävästi. Lyijyn aiheuttama happiradikaalituotannon lisääntyminen ja sitä seuraava oksidatiivisen stressin syntyminen hermosoluissa saattaakin olla merkittävä lyijyn hermomyrkyllisyyden mekanismi. Neuroblastoomasolujen erilaistuminen näyttää liittyvän proteiinikinaasi C:n pitkittynyttä aktivaatiota seuraavaan entsyymin aktivaation vähentymiseen. Suuri lyijyannos näyttää aiheuttavan solujen erilaistumista, joka on osittain riippuvaista proteiinikinaasi C:stä.

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ORIGINAL PAPERS

I

Excitatory amino acid-induced slow biphasic responses of free intracellular calcium in human neuroblastoma cells

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Jonne Naarala, Petri Nykvist, Marjo Tuomala & Kai Savolainen

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II

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III

The combination of lead with a protein kinase C inhibitor causes oxidative stress in human neuroblastoma cells

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IV

Effects of lead and protein kinase C inhibition on differentiation of human neuroblastoma cells

by

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V

Cholinergic-induced production of reactive oxygen species in human neuroblastoma cells

by

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