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NADPH oxidase in PC12 cell differentiation and apoptosis

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NADPH OXIDASE IN PC12 CELL DIFFERENTIATION AND APOPTOSIS

BY

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Abstract

Neuronal differentiation is an important process during human development and regenerative medicine. One factor linked to differentiation of neurons *in vivo* as well as *in vitro* is the generation of reactive oxygen species (ROS). In addition, neurodegeneration is the disease of the old age that is currently affecting millions. Although the etiology of each neurodegenerative disease differs, oxidative stress has been the common factor leading to neuronal death. PC12 cells differentiate into sympathetic-like neurons in the presence of nerve growth factor (NGF). Once terminally differentiated, PC12 cells undergo apoptosis following NGF deprivation with similar characteristics of sympathetic neuronal death. These properties make them useful for studying *in vitro* neuronal differentiation and apoptosis. In addition, NADPH oxidase has been implicated in the differentiation and apoptosis of this cell line through production of reactive oxygen species (ROS). However, the subunits involved in both processes have not been identified. A series of studies were designed to examine the effect of physiological activator (angiotensin II) and pharmacological inhibitor (DPI) of NADPH oxidase on PC12 cell differentiation and apoptosis. The differentiation study has revealed that the putative subunits involved in the early phases may be Nox4 and p67-phox, with subsequent recruitment of Nox1 and p47-phox during the later stages of differentiation. NGF withdrawal led to an increase in Nox1, p47-phox and p67-phox suggesting a role for those subunits in PC12 cell apoptosis. Therefore, these data confirm and extend previous results that suggest that neuronal but not phagocytic NADPH oxidase is involved in neurogenesis and apoptosis.

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Abbreviations

AA arachidonic acid
Akt serine/threonine protein kinase
Ang II Angiotensin II
APS Ammonium persulfate
AT1 angiotensin II receptor type I
AT2 angiotensin II receptor type 2
cDNA complementary deoxyribonucleic acid
DAG diacylglycerol
DEPC diethyl pyrocaronate
DMSO dimethylsulfoxide
DNA deoxyribonucleic acid
DNAse deoxyribonuclease
dNTP deoxynucleotide triphosphate
DPI diphenylene iodonium
DTT dithiothreitol
EDTA ethylenediamintetraacetic acid
F-12 K Ham's F-12K
FAD flavin adenine dinucleotide
FBS fetal bovine serum
GDP guanosine diphosphate
GPCR G-protein coupled receptor
GTP guanosine triphosphate
HS horse serum
IP_3 inositol triphosphate
MAP microtubule-associated proteins
MAPK mitogen**-**activated protein kinase
mRNA messenger ribonucleic acid
NADPH oxidase nicotinamide adenine dinucleotide phosphate-oxidase
NFM neurofilament-medium
NS nervous system
NSE neuron-specific enolase
NGF nerve growth factor
OD optical density
Oligo(dT) oligodeoxythymidylic acid
PAGE polyacrylamide gel electrophoresis
PBS phosphate-buffered saline
PC12 cells a pheochromocytoma cell line
PCD programmed cell death
PCR polymerase chain reaction
PD123177 AT_2 receptor antagonist
PKC Protein Kinase C
PI Phospho Inositol

PLC Phospholipase C
PMA phorbol 12-myristate 13-acetate
PRR proline-rich region
RNA ribonucleic acid
RNAse ribonuclease
ROS reactive oxygen species
Rpm rotations per minute
RT-PCR reverse transcription-PCR
SH3 Src Homology Domain 3
SDS sodium dodecylsulfate
SOD superoxide dismutase
TEMED N,N,N'N', tetramethylethylenediamine
Tween-20 polyoxyethylene sorbitan monolaurate
UV ultraviolet

Chapter I

Neurodegeneration and cellular differentiation

An overview

Introduction

Apoptosis in tissue homeostasis and pathogenesis

In 1964, Lokshin and Williams used the expression "programmed cell death" (PCD) to describe the breakdown of silkworm intersegmental muscles during metamorphosis (reviewed in Gusev and Skvortsova, 2003). In 1972, the word "apoptosis" was coined from the Greek words *apo* = from and *ptosis* = falling describing the fall of dead leaves from trees in autumn (Kerr et al., 1972). Subsequently, extensive research has been focusing on the molecular mechanisms underlying cell suicide in development and disease. The motivation was the attempt to exploit programmed cell death for therapeutic purposes (reviewed in Elmore, 2007). During development, the histogenic remodeling ensures optimal nerve innervations of target organs. In addition, it provides an essential mechanism for elimination of transitory organs in tissue remodeling and homeostasis (Levi-Montalcini et al., 1968; Saunders, 1966). According to the neurotrophic theory, growing neurites reaching their targets become dependent on trophic factors for survival and differentiation (Levi-Montalcini et al., 1968). These substances are secreted in limited amount by target cells. Subsequently, neurons receiving sub-threshold amount of neurotrophic factor are destined to die via apoptosis (Levi-Montalcini et al., 1968). Actually, up to 80% of neurons formed during embryogenesis are destined to undergo PCD in the early postnatal development (Oppenheim, 1991). The NGF dependence can be recapitulated *in vitro,* as neonatal sympathetic and sensory neurons require NGF for survival. NGF antibodies administered during the phase of

target field innervations led to the loss of these neurons, whereas exogenous NGF rescued neurons that would have been otherwise lost (Levi-Montalcini, 1987). Although PCD is an integral mechanism during embryogenesis, it has been involved in detrimental neuronal loss in adult life. In fact, PCD activation triggers progressive neurodegeneration as detected in Alzheimer, Parkinson's diseases, amyotrophic lateral sclerosis (ALS) and acute loss of neurons as depicted following a stroke (reviewed in Maurer et al., 1997; Lee et al., 2001; Siderowf and Stern, 2003).

Epidemiology of Neurodegeneration

The incidence of neurodegeneration has been increasing tremendously in the past few decades. It is mainly due to the demographic shift in "aging America." In addition, the increase in life expectancy has put the baby boomers at higher risk of developing brain degenerative diseases (Dychtwald, 1989). Neurodegeneration is characterized by progressive death of neurons in the central and peripheral nervous systems. Clinically, it is manifested by a gradual regression of body functions that are under the control of the diseased areas (reviewed in Siderowf and Stern, 2003; Boksay et al., 2005). A number of environmental and genetic risk factors have been proposed, however none were confirmed to be the definite culprit (Mayeux, 2003). Nevertheless, the oxidative free radical theory has gained common ground in the etiology of neurodegeneration (Coyle and Puttfarcken, 1993). Although a milestone has been reached in understanding this condition, a promising cure to the ailment is still lagging behind.

Oxidative stress in neurodegeneration

Finding a cure for pathological neurodegeneration has been the preoccupation of numerous clinicians and researchers. Many therapeutic substances under clinical studies were used for that purpose, such as NMDA-antagonists, antioxidants and growth factors (Shigeno et al., 1991; Fisher et al., 1995; Li et al., 2002). The results came back disappointing, as most likely *in vitro* success does not necessarily replicate itself *in vivo.* Although each neurological condition exhibits a distinct etiology, there are common grounds to all brain degenerative conditions. Therefore, it is vital to understand the pathological mechanisms and their interactions in order to piece in the enigma behind neurodegeneration. One insight would be to understand the common neuronal intracellular signaling under different pathological conditions. Oxidative stress involvement in the pathophysiology of numerous diseases has created a research emphasis on understanding redox signaling. The latter has been implicated in a wide spectrum of cellular responses ranging from differentiation, to senescence and apoptosis (Thannickel et al., 2000; Droge, 2002).

Reactive oxygen species

Reactive oxygen species (ROS) and free radicals are frequently used interchangeably to refer to species such as superoxide $O_2^{\cdot -}$, hydrogen peroxide H_2O_2, and hydroxyl radical ·OH. Prior to the middle of the 20^{th} century, free radicals were unknown to clinicians and biologists of that time. In 1954, Rebecca Gerschman and her scientific group introduced the concept of reactive oxygen species as toxic elements in aerobes, and explained that oxygen toxicity is due to the formation of free radicals (reviewed in

Gutteridge and Halliwell, 2000). This was followed by the identification of the superoxide dismutase (SOD) by McCord and Fridovich in 1968. Afterward, the emergence of scientific disciplines has contributed further to the understanding of ROS and their involvement in disease pathology (reviewed in Gutteridge and Halliwell, 2000).

Sources of reactive oxygen species (ROS)

The mitochondrial respiratory chain is the major site of cellular ROS production under normal physiological conditions. Electrons produced by the metabolism of glucose, fatty acids and proteins flow through the mitochondrial electron-transport chain leading to ATP production (Lehninger and Kennedy, 1948). Although ROS are created as part of normal respiration, their accumulation beyond physiological levels leads to oxidative stress (Droge, 2002). The mitochondria have limited DNA repair machinery, which makes mitochondrial DNA more prone to oxidant insult than its nuclear counterpart (Croteau and Bohr**,** 1997). Upon oxidative stress, the integrity of the mitochondrial membranes is compromised leading to cytochrome C release and caspase cascade activation (Droge, 2002). NADPH oxidase and xanthine oxidase are non-mitochondrial sources of ROS in various tissues including the vascular system and smooth muscle cells (Tammariello et al., 2000; Hilburger et al., 2005; Galbusera et al., 2006; reviewed in Quinn et al., 2006). The two oxidases are known sources of ROS implicated in many pathological conditions such as bacterial infection and hypertension (Rothfork et al., 2004; Delano et al., 2006). In fact, the oxidative burst generated by the oxidases disturbs cellular structure and functional integrity by oxidative degradation of cellular DNA, proteins, and lipids (Ames et al., 1993).

ROS as second messengers

Superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are produced in many cell types in response to an array of extracellular stimuli. These include peptide growth factors, cytokines, agonists of G protein–coupled receptors, and shear stress (reviewed in Rhee, 1999; Rhee et al., 2000). The incomplete reduction of O_2 produces the superoxide anion ($O_2^{\cdot-}$), which is later enzymatically or non-enzymatically converted to H_2O_2 (reviewed in Rhee, 1999). Since H_2O_2 is a small and diffusible molecule, it is believed to act as a second messenger under subtoxic conditions by targeting various proteins. These include protein kinases, protein phosphatases, transcription factors, phospholipases, and G proteins (reviewed in Rhee et al., 2000). Unlike other second messengers, H_2O_2 exhibit an unconventional protein modification by targeting sulfhydryl groups of cystein residues, which tend to be vulnerable to oxidation (Kim et al., 2000). Subsequently, conformational, structural, and kinetic alterations induce activation or inhibition of the protein (McCubrey and Franklin, 2006).

NADPH oxidase: innate immunity and beyond

The phagocytic NADPH oxidase

The phagocytic NADPH oxidase is a crucial enzyme part of the innate immunity as the phagocytes are the first line of defense against bacterial invasion (Henderson and Chappell, 1996). Individuals with genetic deficiencies or mutations in any of the oxidase subunit genes exhibit recurrent life-threatening infections of chronic granulomatous disease (CGD) (reviewed in El-Banna et al., 2005). CGD is an inherited disorder in

which phagocytes fail to destroy invading pathogens (Bridges et al., 1959). Upon microbial infection, activated phagocytes engulf the pathogen, and eradicate its biomolecules by toxic oxygen molecules causing a complete lysis (Babior, 1999). In fact, the reactive oxygen species are generated through a stimulus-dependent activation of the membrane-bound NADPH oxidase. It involves the reduction of oxygen (O_2) by addition of an unpaired electron to the outermost shell using NADPH as an electron donor producing the superoxide (Gabig and Lefker, 1985). The presence of the orphan electron disturbs the stability of the O_2 ions, which increases its reactivity to pair the electron (Sawyer and Valentine, 1981). The superoxide acts as a precursor for reactive oxygen species (ROS). By itself, it is a weak oxidant; however when it converts to a more potent form it becomes toxic to the organism (Dahl and Richardson, 1978). In fact, hydrogen peroxide causes lipid peroxidation of cell membranes by reacting with reduced transition metals to generate the reactive hydroxyl radicals (Kellogg and Fridovich, 1975). Although hydroxyl radicals are short-lived, they are considered the most injurious radicals (Knight, 2000). As a defense mechanism against hydrogen peroxide, aerobic cells use the enzyme catalase, which decomposes hydrogen peroxide into water and oxygen (Repine et al., 1981; Morehouse et al., 1983).

Cytochrome b_{558} is necessary for the activation of NADPH oxidase

NADPH oxidase is a multi-subunit complex enzyme whose activation is under tight control (Lassegue and Clempus, 2003). At rest, NADPH oxidase subunits are partitioned between the plasma membrane and the cytosol (Lassegue and Clempus, 2003). There are two membrane-bound subunits which include gp91-phox and p22-

phox. The cytosolic complex is composed of p47-phox, p67-phox, p40-phox and a small G-protein Rac1 or Rac2 (Joneson and Bar-Sagi, 1998; Lassegue and Clempus, 2003). Gp91-phox (also known as Nox2) and p22-phox dimerize in a molar ratio of 1:1 to form cytochrome b_{558} the catalytic core of the enzyme (Huang and Kleinberg, 1999) (Fig. 1).

The stability of the cytochrome relies on gp91-phox binding to its adapter protein p22-phox prior to their insertion into the plasma membrane. Mutation in either subunit has resulted in absence of both proteins on the cell membrane (Babior et al., 2002). According to Yu and coworkers (1999), the cytochrome b_{558} contains two heme molecules in its transmembrane domain and their attachment to cytochrome b_{558} occurs by means of four histidines. In addition, the cytochrome b_{558} encloses an NADPH and an FAD binding sites on its cytoplasmic C-terminus, where NADPH is the electron donor and FAD is the electron carrier (Chanock et al., 1994; Krause, 2004). In fact, deletion of FAD causes loss of superoxide generation and restoration of NADPH oxidase activity is detected after FAD replenishment. Moreover, use of flavin antagonist such as diphenylene iodonium (DPI) eliminated superoxide production, which confirmed the presence of a flavin in cytochrome b_{558} (Babior and Peters, 1981). Upon enzyme activation, NADPH releases two electrons to FAD, which are taken by the twin hemes in cytochrome b_{558}. Across the membrane, two O_2 molecules accept the electrons and produce superoxide anions (Lassegue and Clempus, 2003). To counterbalance the negative charges transversely, proton efflux takes place in parallel direction through voltage-gated proton channels. The identity of the channels remains questionable as some argue that it is part of the oxidase, others debate that is coupled to it (Kimball and Saier, 2002; DeCoursey et al, 2002). Beside the plasma membrane, the cytochrome b_{558}

has been localized to cytoplasmic vesicles delivered to the cell membrane by fusion (Lassegue and Clempus, 2003). This led to the hypothesis that superoxides are released inside vesicles and transported out via anion channels; and that membrane-permeable hydrogen peroxide diffuses out easily (Lynch and Fridovich**,** 1978; Forman et al., 2002; Han et al., 2003).

Role of p47-phox-phox in NADPH oxidase activation

The p47-phox subunit is a multi-domain protein that harbors a number of protein-protein interaction elements. The most prominent are the SH3 domains, which play a vital role in NADPH oxidase activation (de Mendez et al., 1997). SH3 homology domains are 60-amino acid tandem repeats which bind to proline-rich regions (PRR) of a target protein and mediate localization of signaling molecules (Finan et al., 1996; Karathanassis et al., 2002). In addition, NADPH oxidase activation engages a series of protein-protein and protein-lipid interactions and involves two key events: hyperphosphorylation of p47-phox subunits and GTP/GDP exchange on Rac1 (Groemping et al., 2003). These two incidents trigger the migration of the cytosolic subunits to the membrane and subsequent binding to the catalytic core at SH3 homology domains of both p47-phox and p67-phox (Leto et al., 1994). Functional and structural studies have demonstrated the existence of p47-phox regulatory domains that direct the activation and inactivation of the enzyme (Ago et al., 2003; Groemping et al., 2003).

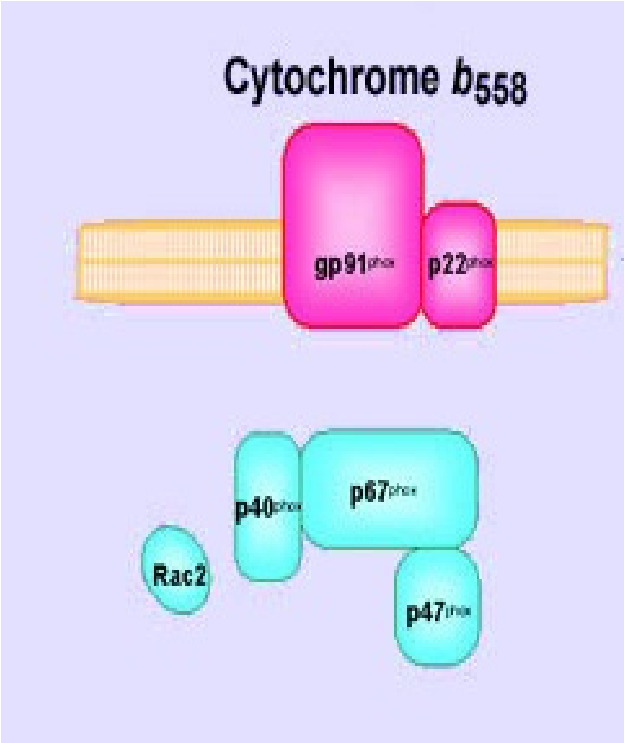


Figure 1. Schematic illustration of NADPH oxidase at rest. Membrane-bound subunits gp-91-phox (also known as Nox 2) and p22-phox form the catalytic core (cytochrome b_{588}). The cytosolic subunits p47-phox, p67-phox and p40-phox reside in the cytoplasm. The small GTPase Rac is part of the enzyme activation. http://www.juntendo.ac.jp

One worth mentioning is the autoinhibitory region (AIR) that folds on itself masking the SH3 domains in unstimulated cells. The phosphorylation of p47-phox C-terminal by protein kinase C (PKC) and serine/threonine protein kinase Akt is critical for inducing a conformational change and subsequent release of the SH3 domains (Ago et al., 2003). Additionally, Yamamoto et al. (2007) showed that arachidonic acid (AA) enhances the SH3-mediated binding of p47-phox to p22-phox. In fact, kinetic studies reported that an anionic amphiphile such as arachidonic acid at high concentrations (50-100 μM) activates p47-phox in cell-free system and induces a conformational change without the hyperphosphorylation by PKC and Akt. At lower concentrations (50-75 μM), AA acts in synergism with PKC to activate p47-phox by the above-described mechanisms (Shiose and Sumimoto, 2000). Another critical domain for the binding of the cytosolic subunits to the membrane counterparts is the phagocytic oxidase homology domain (PX). This is a lipid-binding domain that connects to membrane phosphatidylinositide phosphates by nonspecific electrostatic interactions and subsequent membrane insertion of hydrophobic residues (Stahelin et al., 2003). The conformational change induced by phosphorylation of p47-phox releases PX domain to bind to phosphatidylinositol lipids on cell membrane.

Conflicting roles for p47-phox have been reported. Functional studies have shown that p47-phox activation increased its binding to p67-phox by 100-fold, and subsequently a firmer attachment took place between Rac and p67-phox. These data grant p47-phox a function of a regulator rather than a direct activator of the enzyme (Freeman and Lambeth, 1996). On the same note, in p47-phox-deficient phagocytes translocation of p67-phox failed to occur whereas p47-phox migrated independently to the cell membrane in p67-phox -defective cells (Heyworth et al., 1991; Dusi et al., 1996).

Nevertheless, others depicted partial activation of NADPH oxidase *in vivo* in complete absence of p47-phox and normal physiological levels of p67-phox and Rac (Cross and Curnutte, 1995). Conversely, NADPH oxidase function was independent of p47-phox *in vitro*, granted high concentrations of p67-phox and Rac were provided (Freeman and Lambeth, 1996). On the other hand, p47-phox facilitates electron flow through interacting with p22-phox cytoplasmic tail; however, in the absence of p47-phox subunit NADPH oxidase still produced ROS presumably by different pathways (Cross and Curnutte, 1995). This suggests that p47-phox is necessary but not sufficient for NADPH oxidase activation.

Role of p67-phox-phox in NADPH oxidase activation

The binding of p67-phox to p47-phox is vital for the translocation of the cytoplasmic complex to the membrane (De Leo and Quinn, 1996). The unfolding of p47-phox and the subsequent release of the SH3 domains expose a binding site for p67-phox. Leto et al. (1994) reported that SH3 domain C-terminal of p67-phox is crucial for p47-phox binding. Deletion of the C-terminal, but not of the N-terminal, of SH3 domain abolished the binding of p67-phox to p47-phox. On another note, p67-phox activates NADPH oxidase by attaching to the cytochrome b_{558} and directing the flow of electrons from NADPH to FAD (Cross and Curnutte, 1995; Freeman and Lambeth, 1996). In fact, *in vitro* complementation study of p67-phox-deficient CGD mutants proposed that this subunit is the limiting factor in oxidase assembly and activation as addition of exogenous p67-phox restored cytochrome b_{558} activity (Vergnaud et al., 2000). Miyano and colleagues (2006) showed that p67-phox- requires the small GTPase Rac in order to

activate NADPH oxidase. The same group demonstrated that Rac binds to the N-terminal region of p67-phox at the membrane and subsequently induces a tighter binding between p67-phox and p22-phox. In addition, the small GTPase facilitated membrane localization of p67-phox when interaction between p67-phox and p47-phox was deficient (Miyano et al., 2006).

Role of p40-phox in NADPH oxidase activation

The literature compiles inconsistent information about the precise role of p40-phox in NADPH oxidase activation. P40-phox increases oxidase activity by increasing the binding affinity of p67-phox- for p47-phox and of p40-phox for cytochrome b_{558} (Cross, 2000). However, at higher physiological concentrations it down-regulates the activity of the enzyme by competing with p67-phox for the C-terminal SH3 domain of p47-phox (Sathyamoorthy et al., 1997). This competitive binding between p67-phox and p40-phox is described as the modulator of NADPH oxidase activity (Ito et al., 1996). In addition, deficiencies in either p47-phox or p67-phox detected in autosomal recessive forms of CGD showed decreased translocation of p40-phox to the membrane (Dusi et al., 1996). p40-phox contains a conserved structural stretch of nearly 130 amino acids in the PX domain that binds exclusively phosphatidylinositol 3-phosphate for penetration in the cell membrane with the help of cytochrome b_{558} (Heyworth et al., 1991; Dusi et al, 1996; Stahelin et al., 2003). This suggests that docking requires both binding to the cytochrome b_{558} as well as to the presence of PX domain. It is noteworthy that p40-phox is associated

with NADPH oxidase of phagocytic origin and has not been detected in NADPH oxidase homologs of non-immune cells (Sathyamoorthy et al., 1997).

The small GTPase Rac in NADPH oxidase activation

Rac small GTPases are implicated in cytoskeleton regulation, mitogenesis, and superoxide generation by NADPH oxidase (Abo et al., 1991; Dusi et al., 1996; Kim et al., 1998; Joneson and Bar-Sagi, 1998; Bompard et al., 2005; Cheng et al., 2006). They belong to the Ras family involved in intracellular signaling mainly of the tyrosine receptor kinase. In addition, Rac bears a Ras effector-homolog domain comprised of 26-48 amino acids that interacts with Raf-1 amino-terminal regulatory region of Mitogen-Activated Protein (MAP) kinase-signaling pathway (Warne et al., 1993). Mutations in the homology region of Rac prevented the binding of oxidase cytosolic subunits, suggesting that the homologous residues of Rac are crucial for NADPH oxidase assembly (Freeman et al., 1994). In naïve neutrophils, Rac binds to GDP and dimerizes with Rho-GDI (Guanine nucleotide Dissociation Inhibitor) (Price et al., 2002). Exposure of the cell to pathogens evokes the assembly of NADPH oxidase as well as the activation of guanine nucleotide exchange factors (GEF). GEF releases Rac from its inhibitor Rho-GDI and exchanges GDP for GTP, a prerequisite for Rac activation (Price et al., 2002). In addition, Rac has three critical binding sites necessary for NADPH activation. The insertion, the effector, and the C-terminus prenylated regions bind to the cytochrome b_{558}, p67-phox, and cell membrane, respectively (Freeman et al., 1996). The insertion region is a 12-amino acid stretch (residues 124-136) that is involved in regulating the oxidase kinetics. In fact, point mutations and deletions in this region decreased NADPH oxidase activity tremendously (Freeman et al., 1996). The effector region (residues 26-45),

homolog to Ras effector site, is vital to p67-phox binding. Mutations of amino acids in that region abolished the ability of Rac to support superoxide generation (Freeman et al., 1994).

Non-phagocytic NADPH oxidase

Over the past decade, several studies detected analogues of NADPH oxidase in various non-immune tissues, mainly of mesodermal origin (Babior, 1999). The homologs were shown to be involved in superoxide generation in several organs, including the colon, the kidney, and the vascular system (Lassegue and Clempus, 2003). The discovery of NADPH oxidase in non- phagocytic cells drew question marks around its precise function. Consequently, efforts were invested in unfolding the enigma behind the newly discovered oxidase. The initial work focused on identifying homologs of gp91-phox.

Gp91-phox homolog was cloned and identified by two independent groups. Mox1 (for mitogenic oxidase-1) was implicated in regulating fibroblast growth and transformation (Suh et al., 1999). Parallel investigation led to the discovery of NOH-1 (for NADPH oxidase homolog-1) that acted as a built-in proton channel upon oxidase activation (Banfi et al., 2000). A common classification of Nox (for NADPH oxidase) was adopted afterward to prevent further disparity (Lassegue et al., 2001). Subsequently, four homologs: Nox1, Nox3, Nox4, and Nox5 were identified in non-immune cells (Banfi et al., 2003). Nox homologs exhibit a low constitutive activity that is subject to regulation by different stimuli (Lassegue and Clempus, 2003). In addition, the low levels

of superoxide and H_2O_2 produced in response to cellular growth factors are integral regulators of a wide range of intracellular signaling pathways (Rhee et al., 2000).

NADPH oxidase in neurons

NADPH oxidase subunits were detected in sympathetic neurons at mRNA and protein levels; however the identity of the catalytic subunits was not established (Tammariello et al., 2000; Hilburger et al., 2005). Optical sectioning using laser scanning confocal microscopy confirmed the subcellular localization of NADPH oxidase to resemble the one of resting phagocytic cells. Gp91-phox and p22-phox were membrane-bound while p47-phox and p67-phox were localized to the cytoplasm (Hilburger et al., 2005). Another localization study using immunocytochemistry detected NADPH subunits concentrated in the hippocampus, cortex, amygdala, striatum, and thalamus of a mouse brain (Serrano et al., 2003). Immunocytochemical analysis of cultured hippocampal neurons indicated that all NADPH oxidase proteins were present in the cell body as well as in the dendrites. In addition, stimulation of hippocampal slices with phorbol esters triggered the translocation of the cytoplasmic NADPH oxidase proteins to the membrane concomitant with an increase in superoxide production (Tejada-Simon et al., 2005). Noh and Koh (2000) demonstrated using RT-PCR and western blot analyses that cultured cortical neurons also express subunits of NADPH oxidase at low levels. Upon exposure to toxic amounts of zinc, levels of NADPH oxidase subunits substantially increased. In addition, zinc exposure induced translocation of p47-phox and p67-phox subunits to the membrane which is a characteristic event of NADPH oxidase activation (Noh and Koh, 2000).

Nox1

Nox1 is highly expressed in the colon although additional expression sites were depicted to a lesser degree (Suh et al., 1999; Banfi et al., 2000). In fact, it has been localized to the uterus and the prostate and induced in some cell types by growth factors (Banfi et al., 2000). Nox1 is a 65-kDa protein that is 58% identical to gp91-phox (Lambeth et al., 2000). No precise function has been attributed to Nox1 as it exhibits differential role in various tissues. However, some argued that it is involved in innate immunity of the colon epithelial cells, as they are the first to encounter pathogens and toxins harbored in ingested food (Geiszt et al., 2003). As part of the gut inborn immune system, Nox1 synthesis is upregulated by inflammatory cytokines to increase superoxide production (Krause, 2004). Others have shown that Nox1 is involved in redox signaling leading to tumorgenesis and vascular smooth muscle hypertrophy (Suh et al., 1999; Katsuyama et al., 2005). Conversely, Nox1-induced superoxide generation was shown to be independent of cell stimulants; however it was amplified by cell treatment with protein kinase C (PKC)-activator phorbol 12-myristate 13-acetate PMA (Takeya et al., 2003).

Nox1 has been detected in undifferentiated and differentiated PC12 cells at the mRNA level (Ibi et al., 2006). Following trophic factor addition, *nox1* transcript increased producing a peak at 72 hours. The increase in mRNA levels paralleled the enhancement in neurite outgrowth (Ibi et al., 2006). In addition, superoxide production increased by twofold at 72 hours; however when ROS generation was measured short-term, the fluorescence hit its highest point at 10 minutes after which it declined to the basal level (Ibi et al., 2006). This is in accordance with previous observation, where nerve growth factor (NGF) was shown to increase superoxide generation producing a

climax of 2',7'-dichloro-fluorescein diacetate (DCF) fluorescence at 10 minutes (Suzukawa et al., 2000).

Nox4

Nox4 is a 578-amino acid protein that exhibits 39% homology to gp91-phox (Nox2). It retains the conserved membrane-spanning domains and the binding sites for heme, FAD, and NADPH (Lassegue and Clempus, 2003; Shiose et al., 2001). Renox, a renal Nox homolog, was identified in mouse kidney proximal tubular cells. It was described as an oxygen sensor that controls *Epo* gene expression (Ebert and Bunn, 1999; Geiszt et al., 2000). In 2001, Shiose and coworkers reported the cloning of a human cDNA that encodes Renox, which was given Nox4 nomenclature conforming to the consensus terminology for the Nox family. In fact, these investigators proposed that *nox4* expression is limited to fetal and adult human kidneys. In addition, the protein was predominantly detected in distal tubular cells, which is a novel expression site for Nox4 (Shiose et al., 2001). However, differences in species and experimental methods could have accounted for the inconsistency of these results. The fetal expression of *nox4* transcript in the kidney suggests a role for Nox4 other than oxygen sensing and Epo expression regulation since fetal kidneys produce limited Epo. This implies that Nox4 could be implicated in the regulation of other cellular activities (Rodriguez et al., 1995). Furthermore, Nox4 is involved in vascular pathology of renal diseases. Gorin et al. (2004) reported that Nox4 mediates angiotensin II-induced kidney hypertrophy and increased fibronectin synthesis; two characteristics of early diabetic nephropathy (Gorin et al., 2005).

Unlike Nox1, Nox4 is constitutively active. Consequently, the level of ROS generation via Nox4 might as well be dependent on *nox4* expression and/or post-translational modification such as glycosylation (Lambeth, 2004). Geiszt et al. (2000) showed that Nox4 constitutively produces H_2O_2, which is a by-product of superoxide. The nearly ubiquitous presence of Nox4 and its ROS production suggest a wide involvement in cellular activities ranging from cell division and differentiation to vasodilatation and insulin signaling. In addition, Nox4 contributes to ischemic damage as middle cerebral artery occlusion in mice resulted in a significant increase in cortical Nox4 (Vallet et al., 2005).

NADPH oxidase in cellular differentiation and apoptosis

Recent reports suggest that Nox1 is involved in apoptosis, whereas Nox4 is implicated in differentiation and the later stages of apoptosis (Kobayashi et al., 2004; Wolin, 2004; Griendling, 2006). In fact, Nox1 has been associated with apoptosis and cellular morphogenesis of sinusoidal endothelial cells (SEC) and non-tubulogenic endothelial cell line (NP31), respectively (Kobayashi et al., 2004). However, Nox4 was found to be an inducer of apoptosis in human aortic smooth muscle cells and a necessary element for differentiation of vascular smooth muscle cells (Pedruzzi et al., 2004; Griendling, 2006). The differential activity exhibited by Nox homologs could be related to the amount and rate of reactive oxygen species produced. Indeed, the rate of reactive oxygen species generation in non-phagocytic NADPH oxidase as well as their relative abundance is different from those of phagocytic cells (Droge, 2002). Unlike the cytotoxic effect of gp91-phox that shuttles massive superoxides outside the cell, Nox2-

homologs generate lower intracellular levels of ROS that may act as signaling molecules (Thannickal et al., 2000; Mitsushita et al., 2004).

NADPH oxidase activators and inhibitors significantly modulate enzyme's activity

Angiotensin II is a potent activator of NADPH oxidase. It exerts its action through two types of G-protein coupled receptors AT1 and AT2 (Nakajima et al., 1995). The exact mechanism(s) by which angiotensin II increases NADPH oxidase activity is still unclear. In addition, AT2 receptor activation has been implicated in cellular differentiation and apoptosis and use of AT2 inhibitors have aborted or delayed its effects (Yamada et al., 1996; Meffert et al., 1996). Furthermore, diphenylene iodonium (DPI) is a non-specific NADPH oxidase inhibitor that is widely utilized by researchers. The inhibitory effect of DPI is initiated when electron transport through the flavin moieties of the oxidase causes reduction of DPI to its radical form. This is followed by irreversible phenylation of either the flavin or adjacent amino acid abolishing the enzyme's activity (Pullar and Hampton, 2002). DPI application inhibited ROS production, delayed apoptosis, and neurite outgrowth in neuronal cells (Babior and Peters, 1981; Suzukawa et al., 2000; Tammariello et al., 2000; Matsunaga et al. 2005; Balcerczyk et al., 2005).

Angiotensin II

Historical Background

In 1898, Tigerstedt and Bergman introduced the first notion of blood pressure regulation following the discovery of "rennin," which was isolated from saline rabbit kidney extracts (reviewed by Phillips and Schmidt-Ott, 1999). As subsequent trials failed to confirm Tigerstedt and Bergman's findings, scientists lost interest in the newly discovered substance. In 1934, Harry Goldblatt published a breakthrough study showing that constriction of either one or both renal arteries led to hypertension. In addition, he demonstrated that severe renal ischemia secondary to hypertension was due to increased renin release (Goldblatt et al., 1934). In 1940, two independent investigators isolated a vasoconstrictor substance from the kidney. The group in Argentina named it "hypertensin," while the American group called it "angiotonin" (Braun-Menendez et al., 1940; Page and Helmer, 1940). In 1958, upon discovering of their common finding the two groups agreed on the chimerical term angiotensin (reviewed in de Gasparo et al., 2000). Subsequently, more effort was invested on unfolding the sequential events leading to angiotensin synthesis and activation.

Physiological action of Angiotensin II

Braun-Menendez and coworkers (1940) showed that the enzyme renin enzymatically cleaves a substance called angiotensinogen to release a decapeptide,

discovered later as angiotensin I by Leonard Skeggs. Angiotensin I is then hydrolyzed to form a powerful octapeptide substance known as angiotensin II. The sequential action of this dual hydrolysis system is known as the renin-angiotensin system (RAS). RAS is a major regulator of salt and fluid homeostasis and plays a crucial role in the control of blood pressure and modulation of sympathetic nerve activity (Skeggs et al., 1956).

Angiotensinogen, which is a 453 amino acid protein, circulates constitutively in the blood stream in an inactive form. However, the conversion of angiotensinogen into angiotensin II is under tight regulation to prevent overproduction of the hormone and possible inopportune effects (Thomas, 1999). Angiotensin II exerts its activity through AT1 and AT2 cell-surface receptors, which bear approximately 34% homology (Mukoyama, 1993; Gunther, 1984). Hydropathy plot analysis indicated that AT1 and AT2 receptors are seven-spanning membrane receptors that have been cloned and characterized (reviewed in de Gasparo et al., 2000). Through binding to AT2 receptors, angiotensin II elicits intracellular signaling linked to cellular differentiation and apoptosis through long-term regulation of gene expression (Thomas, 1999).

AT2 receptor and its signaling pathways

AT2 receptor is a 363 amino acid peptide. Studies on distribution of angiotensin II receptors described AT2 receptors being a dominating receptor subtype in fetal and neonatal tissues, and in cell lines of neuronal origin (Millan et al., 1991; Grady et al., 1991). After birth, AT2 receptor expression subsides while AT1 receptor expression increases dramatically (Tsutsumi and Saavedra, 1991; Wolf, 2002). However, a detailed distribution study located AT2 in adult rat brain and depicted a predominant expression

in areas comprising the sensory and motor systems as well as behavior and emotions (Lenkei et al., 1997). In fact, AT2- knockout mice showed anxiety-like symptoms, muscle weakness and reduced drinking response (Hein et al., 1995; Okuyama, 1999). In addition, increased AT2 receptor expression was documented under pathophysiological conditions such as wound, mechanical injury, and brain ischemia (Nio et al., 1995; Steckelings et al., 2005). Moreover, AT2 receptors have been implicated in cell proliferation inhibition, and induction of cellular differentiation and apoptosis (Stoll et al., 1995; Meffert et al., 1996; Wolf, 2002). Conversely, AT2 receptor promoted axonal regeneration after nerve transection (Lenkei et al., 1997). Indeed, elevated AT2 expression was linked to an angiotensin II-induced axonal regeneration by increasing neuronal fibers around the lesion (Lucius et al., 1998). The specific mechanisms of the AT2-induced effects are still unknown; however, antiproliferative and axonal regeneration actions are believed to ensue through signaling leading to cytoskeleton rearrangement during neuronal differentiation (Gendron et al., 2003). Although structurally related to G-protein coupled receptors, activation of AT2 receptor seems to stimulate non-classical signaling cascades. Nouet and Nahmias (2000) have described three major cascades of intracellular signaling: (1) activation of protein phosphatases leading to protein dephosphorylation (2) regulation of the nitric oxide (NO)–cGMP system; and (3) stimulation of phospholipase A_2 (PLA_2) with subsequent release of arachidonic acid.

PC12 cells

PC12 cell characteristics

Greene and Tischler (1976) first established PC12 cells from a transplantable rat adrenal pheochromocytoma. Adrenal chromaffin cells and sympathetic neurons originate from a common neural crest stem cell. This explains the fact that differentiated PC12 cells preserve some neural characteristics such as presence of nicotinic-cholinergic and NGF receptors, synthesis and secretion of catecholamine, and expression of a number of neuropeptide genes (Margioris et al., 1992). These features make this cell line an attractive *in vitro* model for studying differentiation of sympathetic neurons and other neuro-biochemical and -biological modifications such as apoptosis (Guroff, 1985). PC12 cells are round in shape and tend to grow in clusters. They are small in diameter (6-14µm) and slow in growing, which is reflected by their doubling time of 72- 92 hours (Greene and Tischler, 1976). Like primary sympathetic neurons, PC12 cells adhere poorly to plastic tissue-culture dishes; therefore extracellular matrix proteins such as collagen and laminin are used as substrates to enhance anchorage (Greene and Tischler, 1976; Fujita, 1989).

Nerve growth factor signaling

Nerve growth factor (NGF) belongs to the neurotrophin family involved in coordinating the nervous system (NS) development and maintaining neuronal function

(Hoyle, 2003). In fact, NGF promotes the survival and maturation of neurons in both central and developing peripheral NS (Johnson and Yip, 1985; Belliveau et al., 1997; Glebova et al., 2004). Like sympathetic neurons, PC12 cells differentiate in the presence of NGF and become dependent on the trophic factor for survival. Once terminally differentiated, the rat pheochromocytoma cells undergo apoptosis following NGF withdrawal exhibiting similar biochemical characteristics of programmed cell death (PCD) (Lambeng et al., 1999; Vaghefi et al., 2004).

Molecular studies have shown that NGF exerts its effect by binding to two distinct cell-surface receptors, tyrosine kinase A receptor (TrkA) and $p75^{NGF}$ (Johnson et al., 1986; Kaplan et al., 1991). $P75^{NGF}$ is a 75 kDa transmembrane protein that binds NGF with low affinity and belongs to the tumor necrosis factor receptor family. On the other hand, TrkA is a 140 kDa receptor that binds NGF with high affinity (Kaplan et al., 1991). NGF-induced survival is elicited through TrkA receptors, however in the absence of TrkA, $p75^{NGF}$ acts as a neuronal death factor (Deshmukh and Johnson, 1997; Belliveau et al., 1997). On the other hand, Barett and Barlett (1994) demonstrated that the $p75^{NGF}$ activity is dependent on the neuronal stage of development. It is required for NGF-mediated survival in neurons at the stage of target innervations but can mediate an apoptotic signal at a later stage of development (Bamji et al., 1998). Thus, depending on the perinatal stage, $p75^{NGF}$ alternates between function (Chao and Hempstead, 1995). However, other evidence points toward the sufficiency of NGF binding to TrkA to mediate sympathetic neuronal survival and growth. Ligand-mediated activation of TrkA, but not $p75^{NGF}$, was able to maintain growth and survival of sympathetic neurons, which were lost in $TrkA^{-/-}$ mice. Nevertheless, in the absence of Trk receptors or under

conditions of nerve injury and stress, $p75^{NGF}$ is frequently upregulated and capable of independent signaling (Kohn et al., 1999).

Nerve growth factor withdrawal signaling

NGF withdrawal from sympathetic neurons and terminally differentiated PC12 cells elicits a biochemical cascade resulting in PCD activation (Edwards and Tolkovsky, 1994; Lambeng et al., 1999; Vaghefi et al., 2004). In fact, NGF deprivation is followed by an immediate TrkA dephosphorylation, which leads to oxidative stress burst (Kohn et al., 1998). The sudden increase in oxygen species is believed to prompt an intracellular signaling, which activates MEKK, SEK, and JNK, through binding to the adaptor protein POSH (Tapon et al, 1998; Xu et al., 2003). Consequently, stimulation of signaling molecules leads to induction of *c-jun* transcription factor with subsequent expression of pro-apoptotic *Bim* and *Bax* (Papadakis et al., 2006). As a result, cytochrome C is released from the mitochondria and thecaspase cascade is activated (Slee et al., 1999). Chromatin condensation and DNA fragmentation are the final hallmarks of cell death (Lambeng et al. 1999; Gotz et al., 2000).

Effects of NGF on PC12 cell differentiation

As mentioned previously, the PC12 cell line is widely used as a model for studying sympathetic-like neuronal differentiation and other neuro-biochemical and -biological modifications (Guroff, 1985). The description by Greene and Tischler (1976) of PC12 cell differentiation involves cessation of proliferation and neurite outgrowth.

Treatment with nerve growth factor (NGF) (50ng/ml) for 7 days resulted in the termination of cell multiplication with concomitant neuronal-like processes outgrowth (Greene and Tischler, 1976). There are a number of morphological and chemical markers associated with NGF-induced differentiation of PC12 cells. These include growth of neurite processes, enhancement of nicotine-stimulated dopamine release, increase in cAMP levels, and induction of certain proteins and neuropeptides (Margioris et al., 1992).

However, PC12 cell response to NGF withdrawal was dependent on the degree of differentiation. In differentiating cells, NGF withdrawal compromised the cell body integrity and causes loss of neurite extensions while cell division resumed within three days. However, when replated in NGF-containing media, the cells regenerated processes within 24 hours (Greene and Tischler, 1976). When terminally differentiated PC12 cells were NGF deprived, they lost their neurites and failed to regenerate their fibers as they fell short of re-entering the cell cycle and therefore underwent apoptosis (Lambeng et al., 1999). The most rapid changes that occur following NGF addition were detected in the cell membrane and in the growth cones (Connolly et al., 1979; Seeley et al., 1983). Within seconds of NGF addition, the quantity of microvilli decreased and the number of coated pits increased (Connolly et al., 1979). These sudden changes were only transient as the membrane returned to its normal appearance within seven minutes. In addition, PC12 cell nuclei appear to mediate some of NGF activity. In fact, following internalization of NGF a considerable portion of the trophic factor binds to its nuclear receptors (Yankner and Shooter, 1979).

NGF-induced signaling in PC12 cells

PC12 cells have been the primary system used to study neurotrophin signaling *in vitro* (reviewed in Klesse et al., 1999). The activation of TrkA receptors recruits molecules such as the three well characterized effectors PLCγ, Shc and phosphatidylinositol 3-kinase (PI3-K) (Fagan et al., 1996; Klesse et al., 1999). PLCγ splits phophatidylinositol into DAG and IP3 and therefore activates Protein Kinase C (PKC). PKC increases intracellular calcium ions which mediate cytoskeleton remodeling (reviewed in Klesse et al., 1999). Shc activates Ras and downstream MAPK pathway including mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK) (reviewed in Klesse et al., 1999). PI3-K stimulates Akt activation, which has been implicated in survival of different cell types (Datta et al., 1997). In addition, PI3-K effect through Ras/ERK pathway stimulation is essential and adequate signaling for NGF-induced survival in PC12 cells (reviewed in Klesse et al., 1999). PI3-K also stimulates c-Jun NH2-terminal kinases (JNKs), which, through activation of c-Jun, support differentiation or apoptosis, depending on the differentiation status and NGF exposure of the cell. Vaudry et al. (2002) proposed that the duration of Ras/ERK pathway signaling produces distinct outcomes, which include survival, proliferation, and differentiation of PC12 cells. Rapid and transient Ras/ERK activation stimulates survival and proliferation whereas rapid but sustained activation induce differentiation of PC12 cells. The three integrated processes represent obviously a balance between MAPK signaling pathways that depends on the concentration of neurotrophins and the type of first messengers in close proximity (Vaudry et al., 2002).

Effects of AT2 receptor and NGF on PC12 cell differentiation and apoptosis

The high expression of AT2 receptor during fetal and neonatal development has suggested the implication of this receptor in tissue growth and/or differentiation (Nakajima et al., 1995). *In vitro*, there is a restricted number of primary cell cultures and cell lines that express AT2 receptors in culture, usually only under certain culture conditions (reviewed in de Gasparo et al., 2000). PC12 cells and the subclone PC12W are among the cell lines that express AT2 receptors (reviewed in de Gasparo et al., 2000; Wolf et al., 2002). In PC12W cells, stimulation of AT2 receptor via angiotensin II led to cell differentiation and enhanced neurite outgrowth by increasing polymerization of microtubules (Stroth et al., 1998). When compared to the differentiation effect of NGF on PC12W cells, AT2 receptors were found to have a differential upregulation of microtubule-associated proteins (MAP). While NGF increased expression of β-tubulin, MAP1 and MAP2, angiotensin II increased polymerization of β-tubulin but down-regulated MAP1 protein levels in naïve and differentiated PC12W cells. In addition, AT2 receptor antagonist PD123177, but not AT1 receptor antagonist losartan, abolished all the above-mentioned effects of angiotensin II (Stroth et al., 1998). These findings suggest that AT2 receptors play a critical role in cell differentiation via regulation of the cytoskeleton.

Consequently, AT2 and NGF receptors induce differentiation via differential cytoskeleton reorganization (Stroth et al., 1998). One possible mechanism for this disparity is the activation of phosphatases by AT2 receptors. Growth factors mediate

their growth promoting actions via TrK receptors and downstream kinase-driven phosphorylation. Extracellular signal-regulated kinases 1 and 2 (ERK 1/2) and mitogen activated protein kinases (MAPK) appear to play a key role in these phosphorylation cascades (Nakajima et al., 1995; Yamada et al., 1996). Angiotensin II via AT2 receptor transiently increases ERK phosphorylation; however its action is reversed through phosphatase activation (Steckelings et al., 2005). Conversely, angiotensin II-dependent and –independent AT2 receptor activation has been linked to PC12W cell apoptosis (Yamada et al., 1996; Miura et al., 2000). In fact, apoptosis was shown to occur through ERK inactivation by two separate phosphatases: MAPK phosphatase-1(MKP-1), which dephosphorylates Bcl-2 and SH2 domain-containing phosphatase-1 (SHP-1) (Horiuchi et al., 1997; Lehtonen et al., 1999).

Taken together, this suggests that NADPH oxidase may be involved in PC12 cell differentiation and apoptosis by possible differential production of ROS. In addition, angiotensin II, the activator of the enzyme, may increase both processes through activation of AT2 receptors.

Chapter II

NADPH oxidase expression in PC12 cell differentiation

Abstract

PC12 cells differentiate into sympathetic-like neurons in the presence of nerve growth factor (NGF). The trigger for this process is a burst of reactive oxygen species (ROS) that is necessary but not sufficient for this phenomenon. A wealth of data points to the mitochondria as the major source of ROS, however new data suggest that the non-mitochondrial enzyme NADPH oxidase may be directly involved in this process. However, the identity of the subunits involved in PC12 cell differentiation has not been established. We report that co-incubation of NGF and angiotensin II (NADPH oxidase activator) produces a more pronounced burst of ROS and accelerates the rate of PC12 cell differentiation in comparison to NGF alone. In this study, we measured cellular ROS generation in the presence of different pharmacological agents. Cellular differentiation and ROS production significantly decreased upon addition of DPI (an NADPH oxidase inhibitor) or PD123177 (angiotensin II- type 2 receptor inhibitor). Using western blot analysis, we demonstrated that the addition of NGF increases Nox1, Nox4, and p47-phox levels. Concurrent administration of NGF and angiotensin II produced a similar increase with an earlier synthesis of those subunits. Taken together, NADPH oxidase appears to be involved in PC12 cell differentiation following NGF treatment.

Introduction

Neuronal differentiation during development

Neurons are generated from repeated divisions of progenitor cells during embryogenesis (Gotlieb, 2002). In addition, neuritogenesis is a vital process that accompanies neuronal growth. It includes three major phases: neurite protrusion, elongation, and network formation (Sebok et al., 1999; Lindwall et al., 2007). The premature neuron begins as a flat, perisomatic lamellipodium before short neurites emerge. At this early point of development, the early neurites are identical in morphology and molecular composition. One set of neurites becomes axons, while others develop into dendrites (Arimura and Kaibuchi, 2005; Jiang et al., 2005; Wiggin et al., 2005). Axons find their targets through long- and short-range guidance cues and chemoattractants (Mueller, 1999; Liu and Snider, 2001; Lindwall et al., 2007). Once at the target, axons conclude their maturation process by a series of events including the formation of terminal branches and synapses. Further, as neurons develop they exhibit an increase in neurite thickness and in cell-body size, both of which are controlled at transcriptional and translational levels (Meinertzhagen, 1994; Stocker and Hafen, 2000).

NADPH oxidase in cellular differentiation

While the majority of published reports focus on the role of ROS in neurodegeneration, oxidative intermediates may also function as essential mediators of cellular differentiation (Su et al., 2001; Suzukawa et al., 2000). In fact, sub-toxic amounts of ROS are able to act as intracellular second messengers by activating signaling

cascades involved in growth and differentiation in several cell types (reviewed in Rhee, 1999; Laloi et al., 2004). *In vivo*, neuronal differentiation is regulated by the action of growth factors such as NGF (Barde, 1989). *In vitro*, NGF stimulates growth arrest and induces neurite development in PC12 cells (Barde, 1989), and recent reports link the generation of ROS to NGF-mediated neurite outgrowth in these cells (Suzukawa et al., 2000; Ibi et al., 2006). Independent use of N-acetylcysteine (an antioxidant) and of diphenylene iodonium (a flavoprotein antagonist) abolished the increase in ROS production with concomitant inhibition of NGF-induced neurite outgrowth. This suggests that ROS-stimulated PC12 cell differentiation occurs through the regulation of a flavin-containing enzyme similar to NADPH oxidase (Suzukawa et al., 2000). NADPH oxidase is necessary for cellular differentiation of various tissues through redox signaling (Suzukawa et al., 2000; Geiszt et al., 2003). Nox4, a catalytic subunit of NADPH oxidase, is involved in cellular differentiation of cardiac, vascular smooth muscle and renal cells (Wolf, 2005; Griendling, 2006; Li et al., 2006). Despite these latest reports, the precise mechanisms involving ROS, and specifically the role of NADPH oxidase, in neuronal differentiation requires further investigation.

Effect of angiotensin II-induced AT2 receptor activation on cellular differentiation

Angiotensin II type 2 receptor (AT2) has been found to be directly involved in growth control and cell differentiation in many cell types (Millan et al., 1991; Nakajima et al., 1995). Indeed, the activation of AT2 receptors has been linked to growth arrest and cell differentiation in PC12W cells (Meffert et al., 1996; Gallinat et al., 1997; Stroth et al., 1998). In fact, stimulation of AT2 receptors via angiotensin II led to PC12W cell

differentiation and enhanced neurite outgrowth by increasing microtubule polymerization. When compared to the effect of NGF through TrkA activation, AT2 receptors produced a differential upregulation of microtubule-associated proteins (MAP) (Gallinat et al., 1997). MAP1 increases axonal stability and MAP2 induces dendritic maturation. NGF increased protein levels of MAP1 whereas angiotensin II down-regulated MAP1 protein levels in naïve and differentiated PC12W cells (Gallinat et al., 1997). Administration of PD123177, an AT2 receptor antagonist, reversed the observed angiotensin II effects (Stroth et al., 1998). All together, this is suggestive that angiotensin II acts through AT2 receptors to modulate the cytoskeleton. In addition, distinct intracellular pathways are involved in NGF- and angiotensin II-induced regulation of the cytoskeletal proteins, as AT2 receptor activation resulted in a transient increase in ERK activity (Stroth et al., 2000). We hypothesized that NADPH oxidase is involved in PC12 cell differentiation, which might be linked to an early burst of ROS production. In addition, angiotensin II as the enzyme activator may increase or speed-up the process.

Here we report that the combination of NGF and angiotensin II results in earlier and more pronounced ROS than is observed after adding NGF alone, suggesting NADPH oxidase may be important in this process. Western blot analysis indicates that NGF treatment alone increases the levels of specific NADPH oxidase subunits, and the addition of angiotensin II with NGF results in an earlier protein synthesis of NADPH oxidase subunits. Overall, these results confirm and extend our hypothesis that NADPH oxidase is involved in PC12 cell differentiation.

Materials and Methods

PC12 cell culture

Rat pheochromocytoma (PC12) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) at an unknown passage. Cultures were maintained at 37°C, 5% CO_2 / 95% air in Ham's F-12K growth media supplemented with 2 mM L-glutamine 15% horse serum; 2.5% fetal bovine serum (Cellgro, Herndon, VA). Cultures were grown in collagen-treated 10-cm culture dishes with media exchange every other day (Falcon, San Jose, CA). For experimental purposes, 6-well, 24-well, or 96-well tissue were used (Falcon). For expression studies, the cells were treated with NGF (50 ng/ml) as was suggested by Greene and Tischler (1976) or NGF (50 ng/ml) + angiotensin II (10 μM) (NGF/AngII) (Yamada et al., 1996) for different experimental time points (2, 4, 6, 8, 18, 24 and 48h).

Cell passage

Cells were analyzed before passaging using a light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, New York). Old media was removed by power suction followed by addition of 2 ul of trypsin/EDTA (Sigma). The cells were incubated at 37°C for 5 minutes. To stop the proteolytic action of trypsin, 3 ml of culture media was added and cells were dispersed by repeated forceful pipetting. The suspension was transferred

into a 50-ml BD Falcon Conical tube and the cells were centrifuged at 400 x g for 10 minutes. The supernatant was discarded and the cells were resuspended in fresh medium.

Differentiation Cell Count

PC12 cells were seeded as single cells at a concentration of 0.5 x 10^4 cell/well. Twenty-four hours after seeding, the old media was removed and fresh experimental media was added. The experimental groups included: NGF (50 ng/ml), NGF (50 ng/ml) + angiotensin II (10 μM) (NGF/AngII), NGF (50 ng/ml) + angiotensin II (10 μM) + PD123177 (1 μM) (NGF/AngII /PD), NGF (50 ng/ml) + angiotensin II (10 μM) + DPI (5 μM) (NGF/AngII /DPI), or NGF (50 ng/ml) + PD123177 (1 μM) (N/PD). The frequency of cells manifesting differentiating morphology (neurites) was scored by an observer "blinded" as to the experimental treatment. Each condition was evaluated at least three times.

Oxidative stress analysis

2', 7'-dichlorofluorescein diacetate (DCFHDA) assay is a widely used method for measuring intracellular reactive oxygen species (ROS). DCFHDA is converted to DCFH by cleavage of its ester bond by means of intracellular esterases. Non-fluorescent DCFH is oxidized to DCF (fluorescent dichlorofluorescein) in the presence of H_2O_2, which is the immediate byproduct of ROS. Thus, it is a valuable tool to measure intracellular ROS generation. PC12 cells were cultured onto collagen-treated 96-well plates at a concentration of 3 x 10^3 cell/well. The control groups comprised cells cultured in the presence of F-12K, NGF and angiotensin II. Experimental groups included the addition

of NGF + angiotensin II (N/A), NGF + DPI (5 μM) (N/DPI), NGF+ angiotensin II + PD123177 (N/A/PD), NGF+ angiotensin II + DPI (5 μM) (N/A/DPI). The cells were treated with different treatments at different time points and preloaded with DCFHDA in DMSO (5 μg/ml, Molecular Probes) for 30 minutes at 37 ^{o}C. All DCFHDA solutions were prepared fresh instantly before the assay. A multi-channel pipette was used to supply 100 μl/well. The DCF fluorescence was measured using a CytoFluor II Fluorescence Plate reader (Applied Biosystems, Foster City, CA) with an excitation wavelength of 485/20 nm and an emission wavelength of 530/35 nm.

RNA isolation procedure

PC12 cells were seeded at a concentration of 0.25 x 10^6 cells/well onto 24-well plates. Each experimental group comprised four wells. RNA extraction was carried out following the manufacturer's protocol. Briefly, RNA was isolated using 250 μl of Trizol® reagent (Gibco Invitrogen, Carlsbad, CA, USA) per well, incubated at room temperature for 10 min, and collected in RNAse-free 1.5 ml Eppendorf tubes. Chloroform (1/5 volume) was added per 1ml of Trizol®-cell solution. The tubes were manually inverted and allowed to incubate for 5 minutes at room temperature. This was followed by centrifugation at 4°C at 7558 x g for 5 min. The aqueous phase, which contains RNA, was transferred into separate 1.5 ml Eppendorf tubes (Westbury, NY). Isopropyl alcohol (100%) 1/2 volume was added and the sample was left for 10 minutes at room temperature. Another cold centrifugation at 7558 x g for 15 minutes forms the RNA the pellet, which was washed with 250 μl of 75% ethanol and cold centrifuged at 2516 x g for 5 minutes. The ethanol was removed by careful pipetting to avoid disruption of the

precipitated RNA, and the pellet was allowed to dry for 10 min at room temperature and resuspended in RNAse-free DEPC water (0.1%). Quantification of the RNA was performed by measuring optical density at 260 nm using a spectrophotometer. RNA samples were kept frozen at -80 °C until used.

Reverse Transcription Reaction

Five µg of total RNA was mixed with oligo (dT) primers (50mM) and 10mM dNTP in PCR tubes. The combination was heated at 70°C for 5 minutes and immediately placed on ice for 2 minutes. A stock solution of the following was prepared: 6 µl 5 x reverse transcription buffer, 1 µl of RNAseOut® (40 U/µl), 1 µl of SuperScript RT III (200 U/µl) and 1 µl DTT (0.1M). Negative controls were prepared with no addition of RT to detect any DNA contamination. Gentle pipetting and a quick spin were performed to collect the content. The stock solution was added to the RNA/oligo (dT) mixture and incubated at 50°C for 50 minutes. The reaction was inactivated at 85°C for 5 minutes. To destroy the RNA template, 1 µl of RNAse H was added to the mixture and followed by incubation at 37°C for 20 minutes. The cDNA mixture was stored at -20°C until use for polymerase chain reaction.

Polymerase Chain Reaction (PCR)

Platinum® Taq DNA Polymerase (Sigma, St. Louis, MO) is a recombinant DNA polymerase attached to an inhibitory antibody to prevent unnecessary activity at ambient temperatures. Heating the complex to 94°C causes the denaturation of the antibody and

subsequent activation of the Platinum® Taq DNA Polymerase. The mixture provides the free nucleotides needed for the elongation of the product. All reagents were mixed and centrifuged briefly before addition and were kept on ice. The reaction mixture was prepared in 0.5 ml RNAse-free PCR tubes as follows: 2.0 unit of Taq Polymerase**,** 0.6 µl (5pmole) of each primer (+/-), and 5 µg of template in 15 µl reaction **.** The reaction mix was briefly centrifuged to ensure homogeneous blending. All PCR reactions were performed using a PCRexpress thermocycler ® (Hybaid Omnigene Thermocycler, UK). Briefly, the mixture was heated to 94°C for 2 minutes to activate Taq polymerase known as the "hot start" and denature the DNA. The first step was followed by an annealing for 30 seconds (the temperature used depended on the primer utilized) and an extension step at 72°C for 30 sec- 1 minute depending on the size of the target product. The annealing temperatures were optimized by doing a temperature gradient PCR starting 5°C below the calculated T_m going up to 12°C until the most stringent conditions were found. Subsequent cycling heated the mix at 94°C for only 30 seconds. The cycle of melting, annealing, and extending was repeated between 25-30 times determined by cycle bracketing. Final extension was at 72°C for 10 minutes. All primers were generated at Integrated DNA Technology (Coralville, IA) and are listed in Table 1.

Table 1. Primer sequences used in the differentiation study

Primer	Sequence
nox1 sense	5'-AACCACACGCTGAGACCATTCGA-3'
nox1 antisense	5'-TCAGCTAAGGCAGGTTTCCCAGA-3'
nox4 sense	5'-AGGTGTCTGCATGGTGGTGGTATT-3
nox4 antisense	5'-AAAGAGGGCTGTGGCTATCAGCTT-3'
p47-phox sense	5'-CAGCCAGCACTATGTGTACAT-3'
p47-phox antisense	5'-GAACTCGTAGATATAGGTGAA-3'
p67-phox sense	5'-GGGAACCAGCTGATAGACTACAT-3'
p67-phox antisense	5'-TCCATTCCTCTTTCTTGGCAATT-3'
NSE sense	5'-ACGCTGGACTCGCTGGGCAA-3'
NSE antisense	5'-GCGAGCGCGCTGCGCTTGTA-3'

DNA gel electrophoresis

PCR products were separated on 1-2% agarose gels in 1X TAE (0.04 M Tris-Acetate, 0.002 M EDTA, pH 8.0). The electrophoresis apparatus was set at 100 V for 45 min and 0.5 x SYBER Gold (Molecular Probes, Eugene, OR) stained the gel for at least 30 minutes. The PCR products were visualized under ultraviolet light.

DNA sequencing

Identification of PCR products was confirmed by direct DNA sequencing. cDNA sequencing was performed using DYEnamic ET terminator Cycle Sequencing kit® (GE Healthcare, formerly Amersham Bioscience, Piscataway, NJ) according to the manufacturer protocol using ABI Prism® 310 genetic analyzer (PE Applied Biosystems, PE Applied Biosystems, Foster City,. CA).

Protein extraction procedure

In order to obtain samples for western blot analysis, PC12 cells were seeded at a concentration of 0.25 x 10^6 cells/well onto 24-well plates. Each experimental group comprised four wells. Protein extraction was carried out on ice to prevent further protein modification. Cells were washed with cold sterile phosphate-buffer saline (PBS) (500 µl/well) to remove traces of proteins from the media. Cell lysates were collected in lysis buffer (1 X 10mM Tris-HCl, 1mM EDTA, pH 7.4) + Protease Inhibitor Cocktail (PIC) (Roche, Mannheim, Germany) + 0.1 % Triton X+ phosphatase inhibitor). PIC contains a mixture of proteases that inhibit serine, cysteine, and metalloprotease activity. The composition of the PIC is as follows: Chymotrypsin, 1.5 µg/ml, Thermolysin, 0.8 µg/ml, Papain, 1 mg/ml, Pronase, 1.5 µg/ml, Pancreatic extract, 1.5 µg/ml, Trypsin, 0.002 µg/ml. Samples were sonicated for cell disruption and centrifuged at 4° C 7558 x g for 10 minutes. The supernatant containing the protein extract was collected and stored at -80°C until further analysis.

Western Blot analysis

Protein concentrations were quantified using serial dilutions of BSA as standards for Bradford protein assay (Bio-Rad, Hercules, CA). The BSA solution was added in increments of 2µg and assayed in triplicate. Equal amounts of protein were boiled in SDS electrophoresis buffer (containing 2-mercaptoethanol) at 90^{o}C for 10 minutes. Protein separation was performed by a 12% SDS-PAGE using Xcell SureLock® (Invitrogen). The proteins were transferred to a polyvinylidene fluoride (PVDF)

microporous membrane (Millipore Immobion-P #IPVH 000 10, St. Louis, MO) pre-soaked in 100% methanol and transfer buffer (200mM glycine, 25mM Tris, 20% methanol) . Transfer of the blot onto the membrane was performed using Xcell II Blot Module® (Invitrogen). The blot was blocked with 5% non-fat milk in Tris-Buffered Saline (27mM Tris, 57 mM NaCl, pH 7.5) for 1 hour at room temperature. The primary antibody was diluted at a concentration of 1:800 in blocking solution and incubated for 60 minutes at room temperature on a rocker. The following antibodies were used in this study: anti-Nox1 (cat. 25545, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Nox4 (cat. 5514, Santa Cruz), anti-α-tubulin (cat. 2144, Cell Signaling Technology, Inc., Danvers, MA), anti-p47-phox and anti-p67-phox were a generous gift from Dr. Mark Quinn (Montana State University). The membrane was washed three times with 1xTBS, 0.05% Tween 20). A horseradish peroxidase (HRP)-conjugated secondary antibody (cat.7074, Cell Signaling Technology, Inc.) diluted in TBS-t (1:1000) was added for 45 minutes. Amersham ECL kit (RPN 2106) (GE healthcare, Piscataway, NJ) was prepared following the manufacturer protocol. Band detection was achieved using Kodak X-Ray film (Eastman Kodak, Rochester, NY) and quantified by QuantOne (BioRad).

Statistics

The results are expressed as the mean $\pm$ SEM of three or more independent experiments. Significant differences in measured values were evaluated with an analysis of a Student's *t*-test or ANOVA. Statistical significance was set at $p < 0.05$. Statistical analysis and graphing were performed using Microsoft Excel.

Results

Differentiation of PC12 cells in response to different treatments

Since PC12 cells differentiate in the presence of nerve growth factor, NGF-treated cells were used as a control group in this experiment (Greene and Tischler, 1976). NGF/AngII treatment significantly increased the percentage of differentiated cells up to day 4 after NGF addition after which NGF-treated PC12 cells became statistically comparable (Fig. 2). In contrast, DPI- and PD123177- treated cells generated a significant delay in differentiation when compared to the averaged values from cells treated with NGF alone. However, DPI-treated cells showed the slowest rate of differentiation among all as both DPI groups lagged behind other treatments, suggesting that DPI significantly delays but does not block differentiation (Fig. 2).

Qualitative observations revealed that NGF/AngII-treated PC12 cells had longer and neurite extensions as soon as day 3 post-treatment (data not shown) and more neurite branching when compared to cells treated with NGF alone on day 7 (Fig. 3). DPI-treated cells exhibited a lesser degree of differentiation (Fig. 3). Interestingly, the size of the DPI-treated cell body appeared comparable to the NGF-treated group on day 7 and day 8, however the network of neurite outgrowth was poorly developed as no branching of neurites was observed (Fig. 3). In contrast, PD123177 slowed down the differentiation process without affecting the morphology of the neurites. In fact, differentiated PD123177-treated cells developed a neurite meshwork similar to that detected in NGF-counterparts but fewer cells differentiated.

Co-administration of NGF and angiotensin II produces an earlier and higher burst of ROS

Previous investigators revealed that NGF-dependent neurite outgrowth in PC12 cells is subsequent to a transient increase in ROS concentration peaking at 10 minutes following NGF addition. The same group suggested that ROS acts as an intracellular signal mediator for NGF-induced neuronal differentiation (Suzukawa et al., 2000). Our finding regarding NGF/AngII accelerating differentiation of PC12 cells prompted us to examine the effect of this particular combination on ROS production. We hypothesized that this increase in the rate of PC12 cell differentiation was due to an earlier and/or higher level of ROS production. NGF addition produced a peak of ROS at 10 minutes followed by a gradual decrease to basal levels by 60 minutes confirming previous reports. On the other hand, NGF/AngII addition created significantly higher values when compared to NGF administration alone up to 20 minutes post-treatment, supporting our theory (Fig. 4). Interestingly, NGF/AngII at 5 minutes produced statistically similar values to those of NGF at 10 minutes. These data suggest that an earlier and a higher burst of ROS may play a role in the accelerated rate of PC12 cell differentiation.

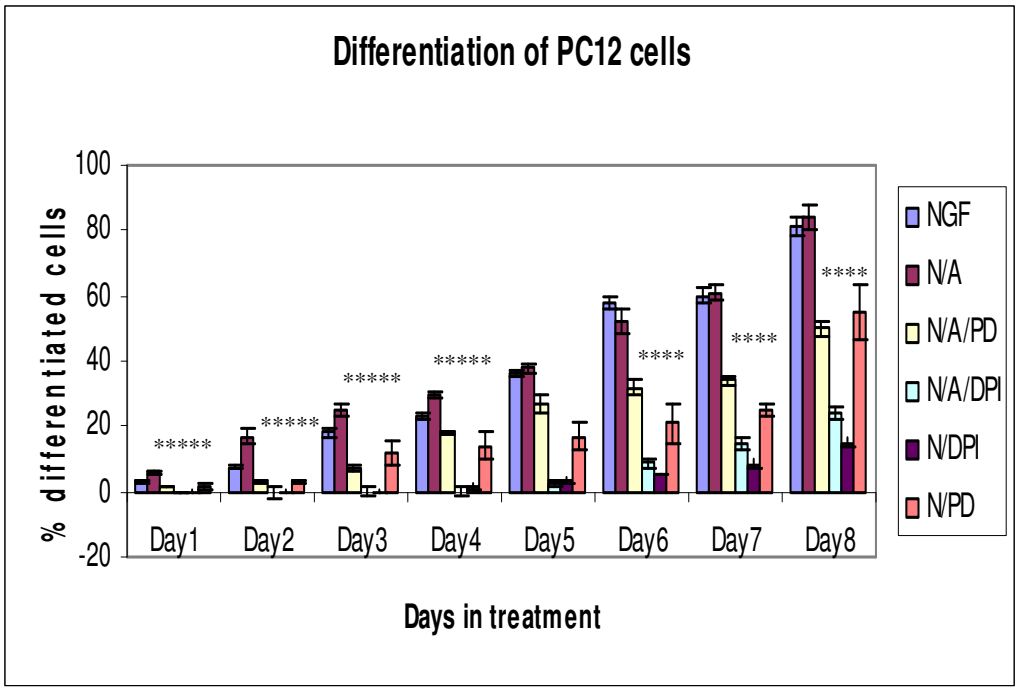
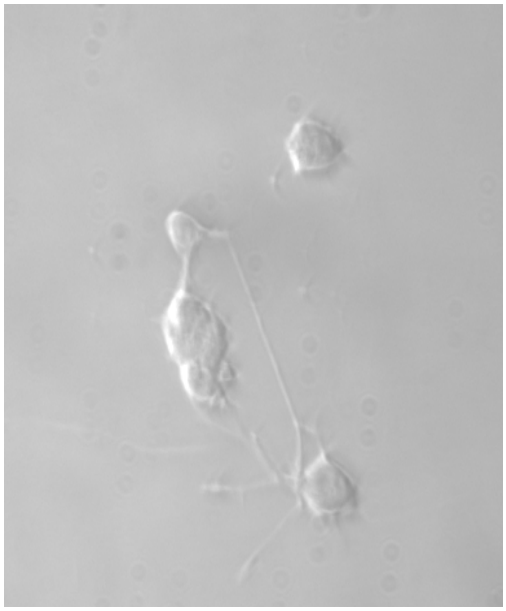
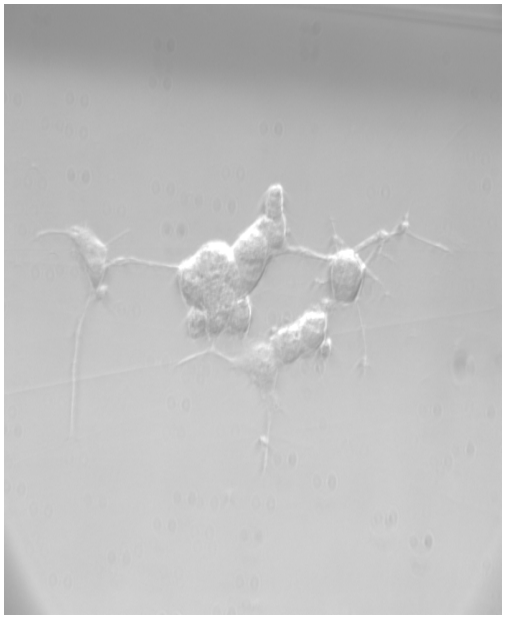


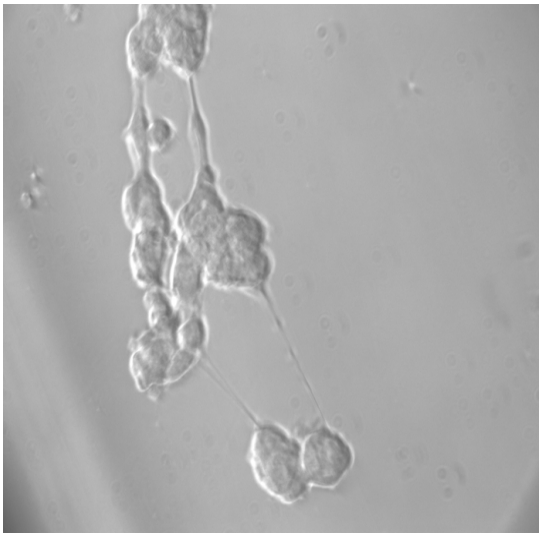
Figure 2. Differentiation of PC12 cells for 8 days using different pharmacological treatments. Differentiated cells were defined as cells with protruding processes regardless of size. Counting was blinded and performed three times to account for any preconceived conclusion. NGF, N/A= NGF + angiotensin II, N/A/PD = NGF + angiotensin II + PD123177, N/A/DPI = NGF + angiotensin II + DPI, N/DPI = NGF+ DPI; N/PD = NGF + PD1231 77. Each bar represents the average of three independent readings. The asterisk represents significant difference from the control (NGF) at each timepoint. The data represent the mean $\pm$ SEM of three blinded and independent cell counting ($p<0.05$).



NGF-7days



NGF/Ag II- 7days



NGF/DPI-7days

Figure 3. PC12 differentiation in the presence of NGF, NGF/AngII and NGF/DPI DPI on day 7. N/A-treated PC12 cells showed a higher degree of differentiation, larger cell body and more elaborate neurite network. The DPI-treated PC12 cells lack the mesh of network detected in the NGF and N/A treated PC12 cells.

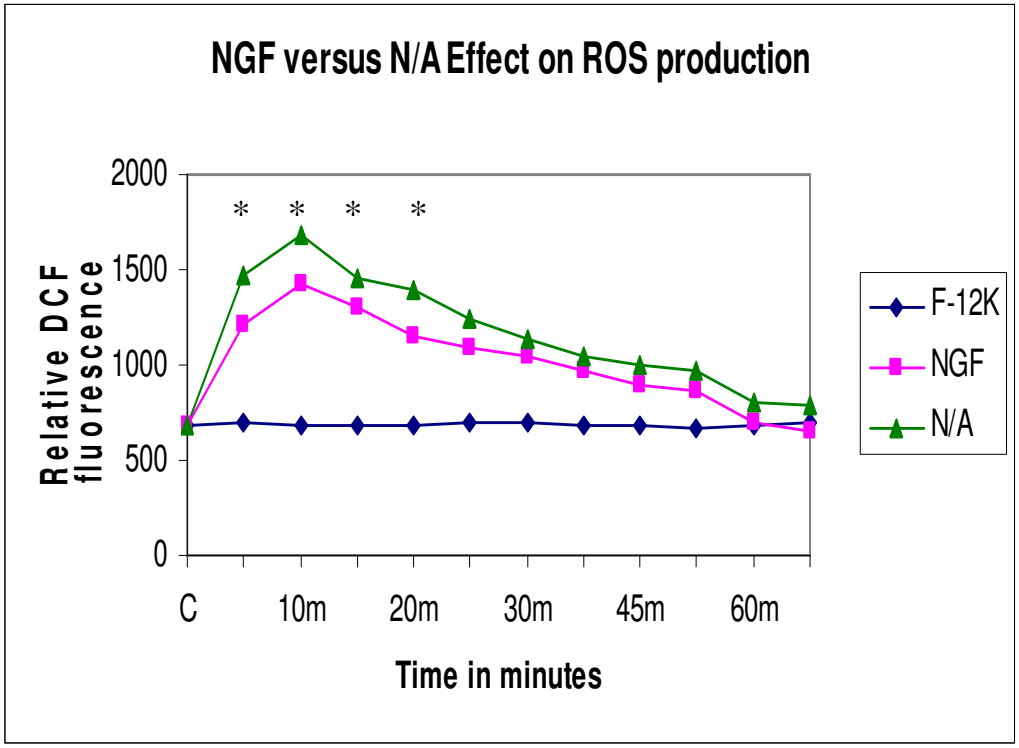
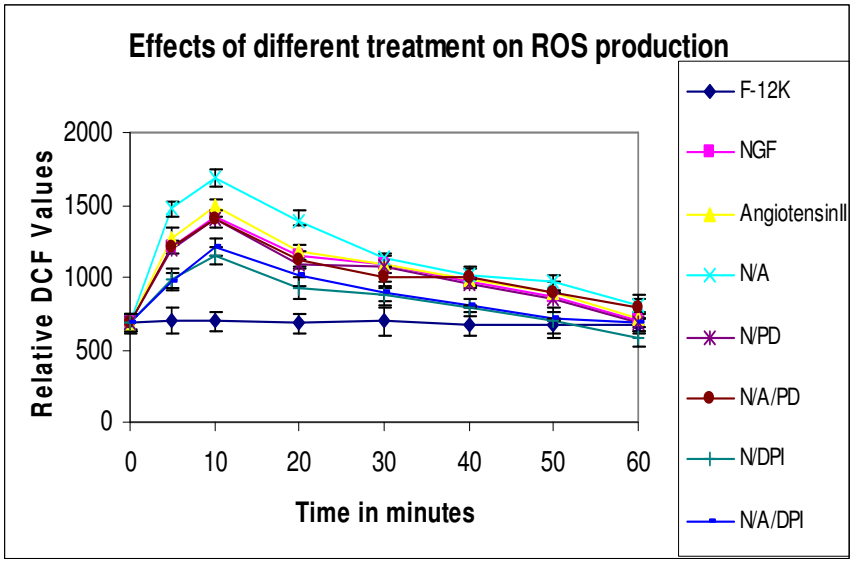


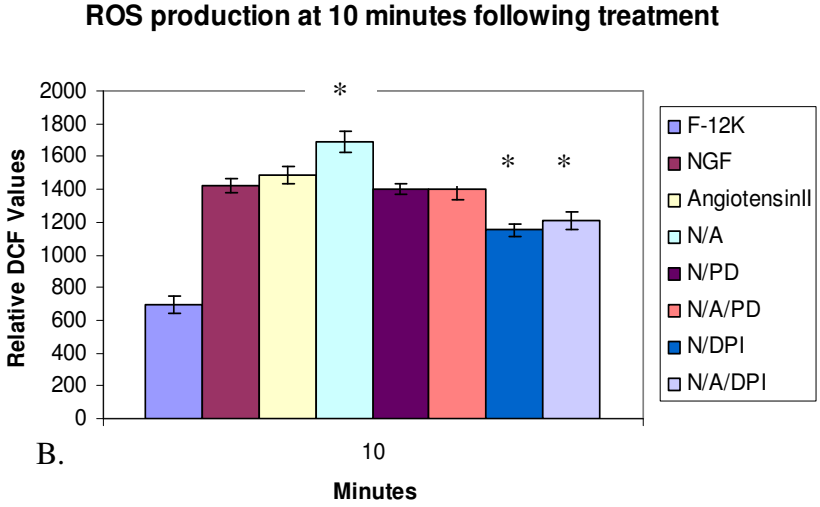
Figure 4. NGF/AngII produces an earlier and more pronounced burst of ROS compared to NGF treatment. PC12 cells were seeded as described in Materials and Methods. Twenty-four hours after seeding, PC12 cells were treated with either NGF (50 ng/ml) or N/A (NGF + angiotensin II (10 μM) at different experimental time points starting 75 minutes before fluorescence reading. The DCFHDA was preloaded for 30 minutes prior to fluorescence quantification. Relative DCF fluorescence intensity shown is from triplicate experiments. The asterisk denotes significant difference between both experimental groups ($p < 0.05$).

ROS production varies in response to NGF, angiotensin II, PDI and PD123177 treatments

Since NADPH oxidase inhibitor DPI significantly delayed NGF-mediated differentiation in PC12 cells, we examined the effect of this compound on ROS production following NGF addition. Enhanced generation of ROS was observed in the first 20 minutes of NGF/AngII exposure as compared to NGF or angiotensin II alone (Fig. 5) suggesting that the trophic factor and NADPH oxidase activator exhibit a synergistic activity on ROS production. PD123177 or DPI (5 μM) concurrent administration with NGF/AngII significantly decreased DCF fluorescence compared to the NGF/AngII values. In addition, the fact that AT2- antagonist, PD123177, significantly reduced ROS production when administered with NGF/AngII suggests that angiotensin II may act through AT2 receptor stimulation to generate ROS. DPI (1 μM), (5 μM) or PD123177 produced basal levels of DCF fluorescence. In addition, concurrent addition of DPI (1 μM) and NGF did not significantly decrease ROS production when compared to NGF values (data not shown). DCF readings were carried up to 8h post-treatment; however no significant differences were detected among the experimental groups beyond 20 minutes of treatment administration.



A.



B.

Figure 5. Effects of different treatments on ROS production in PC12 cells. (A)To examine the effect of angiotensin II, DPI and PD123177 on intracellular ROS production, thirteen 96-well plates were seeded as previously described. The control groups comprised cells cultured in the presence of F-12K, NGF and angiotensin II. Experimental groups included the addition of NGF + angiotensin II (N/A), NGF + PD123177(N/PD), NGF + angiotensin II +PD123177 , (N/A/PD), NGF + DPI (N/DPI), NGF + angiotensin II + DPI (N/A/DPI). Treatment started starting 60 minutes before fluorescence reading. The DCFHDA was preloaded for 30 minutes prior to fluorescence quantification. ROS generation was detected by DCF fluorescence using CytoFluor II plate reader. The data represent the mean of three independent DCF measurements. (B) Graphical representation of the 10-minute time point. (* $P < 0.05$ significance from NGF treatment).

Expression of NADPH oxidase subunits in PC12 cells following short-term NGF and NGF/AngII addition.

Previous data from our laboratory pointed toward the presence of NADPH oxidase in sympathetic neurons and PC12 cells (Tammariello et al., 2000; Hilburger et al., 2005). We theorized that NADPH oxidase is a source of the ROS-mediated PC12 differentiation. We next looked at the transcript and protein levels of NADPH oxidase subunits following NGF addition to determine which subunits are putatively involved in differentiation of PC12 cells. No changes were detected at the level of transcription following NGF addition alone except for p47-phox which was downregulated 4 hours post-treatment (Fig 6A). On the other hand, addition of NGF + angiotensin II resulted in *nox4* upregulation (approximately two-fold increase) by 2h post-treatment, and *nox1* and *p47-phox* by 4h following NGF/angII addition (Fig 6B). At the protein level Nox1 and p47-phox proteins were almost undetectable and were not altered by the addition of NGF. Nox4 and p67-phox protein levels remained constantly high regardless of differentiation status (Fig. 7A). However, the addition of AngII coincident with NGF caused a significant proportional increase in the protein levels of Nox1 and p47-phox, and to a lesser extent Nox4 (Fig. 7B).

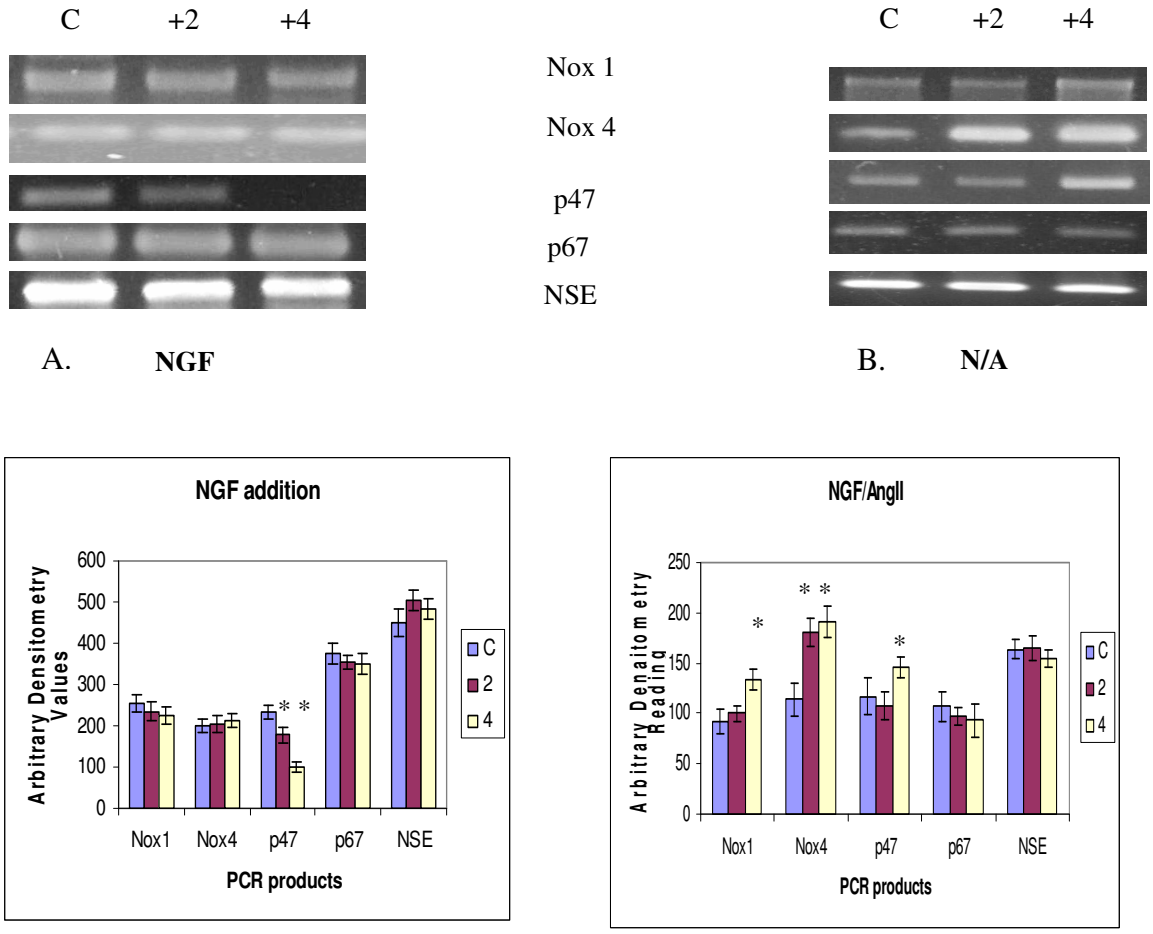


Figure 6. RT-PCR analysis of NADPH oxidase subunit expression following NGF and NGF/AngII treatments with corresponding densitometry analysis. PC12 cells were cultured as described in Material and Methods. Twenty-four hours after seeding, the cells were kept in regular F-12 K media (C, control) or treated with either NGF-supplemented F-12K (50 ng/ml) (A) or NGF (50 ng/ml) and angiotensin II (10 µM) (NGF/AngII) (B) for 2 and 4 hours (+2h, +4h, respectively). Equal loading control is Neuron Specific Enolase (NSE). Densitometry values are means of three to four gels (* $p < 0.05$).

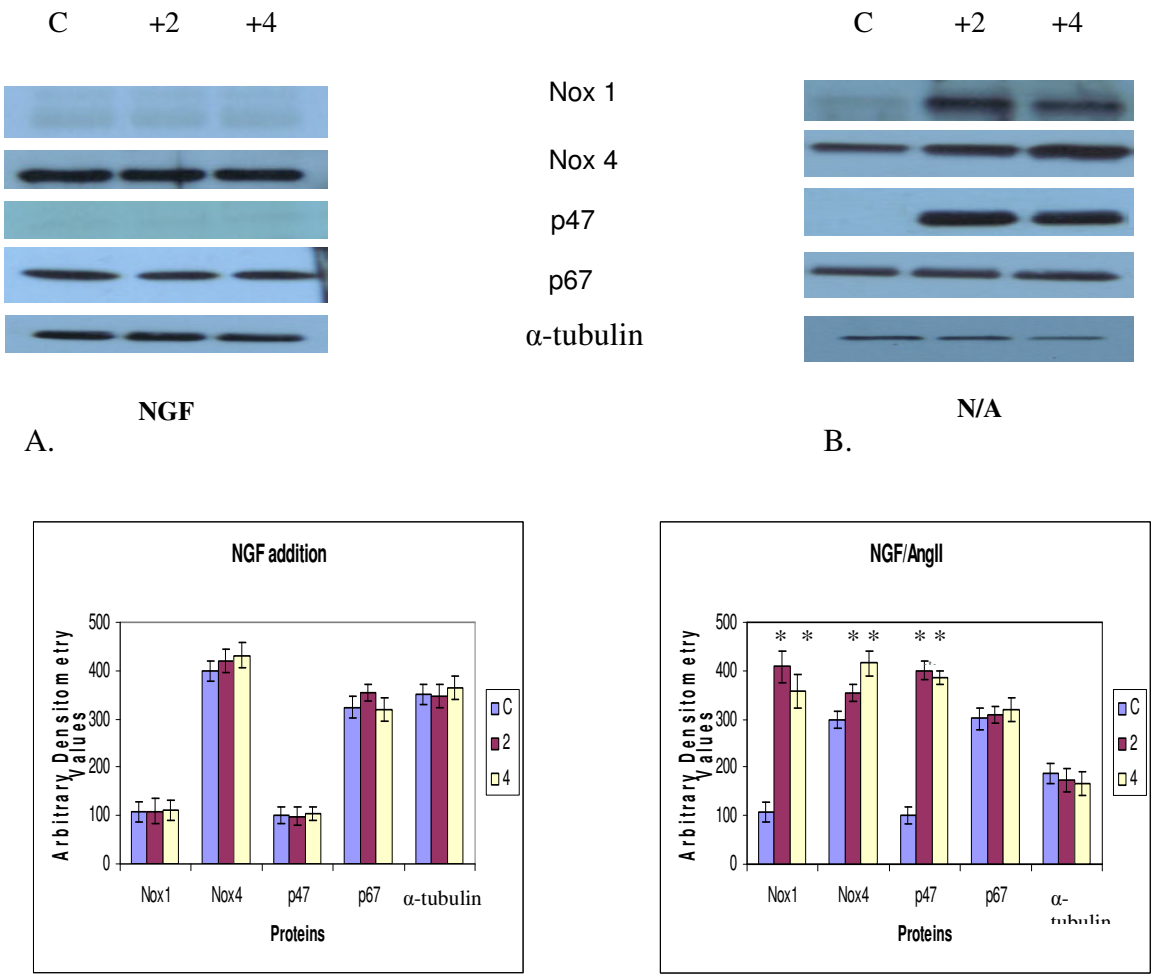


Figure 7. Western blot analysis of NADPH oxidase subunit synthesis following NGF and NGF/AngII treatments with corresponding densitometry. PC12 cells were cultured as described in Material and Methods. Twenty-four hours after seeding, the cells were kept in regular F-12 K media (C, control) or treated with either NGF-supplemented F-12K (50 ng/ml) (A) or NGF (50 ng/ml) and angiotensin II (10 uM) (NGF/AngII) (B) for 2 and 4 hours (+2h, +4h, respectively). Equal loading control is α-tubulin. Densitometry values are means of three independent experiments (* $p < 0.05$).

Effects of long-term NGF and NGF/AngII addition on NADPH oxidase subunit synthesis

While differences at the mRNA and protein levels were minimal at early time points after the addition of NGF, other investigators have reported that Nox1 levels increase significantly at the protein level in PC12 cells 72 hours following NGF addition (Ibi et al., 2006). We therefore studied time-points between two and 48 hours after NGF addition in order to examine mRNA and protein profiles of the NADPH oxidase subunits. The addition of NGF did not alter the expression of the subunits except for the aforementioned decrease in *p47-phox* at four hours post-treatment. By eight hours post-NGF administration the *p47-phox* transcript levels were re-established and significantly increased between 18 and 48 hours after NGF addition (Fig. 8A). When NGF and angiotensin II were administered concomitantly, *nox1*, *nox4* and *p47-phox* transcripts were significantly increased by the 6h time-point and remained high for the duration of the experiment (Fig. 8B). At the protein level Nox1, Nox4 and p47-phox exhibited significant increases by eight hours following NGF addition (Fig. 9A). Interestingly, the addition of angiotensin II concurrent with NGF led to the increase of these subunits at the protein level by six hours after NGF/AngII addition (Fig. 9B).

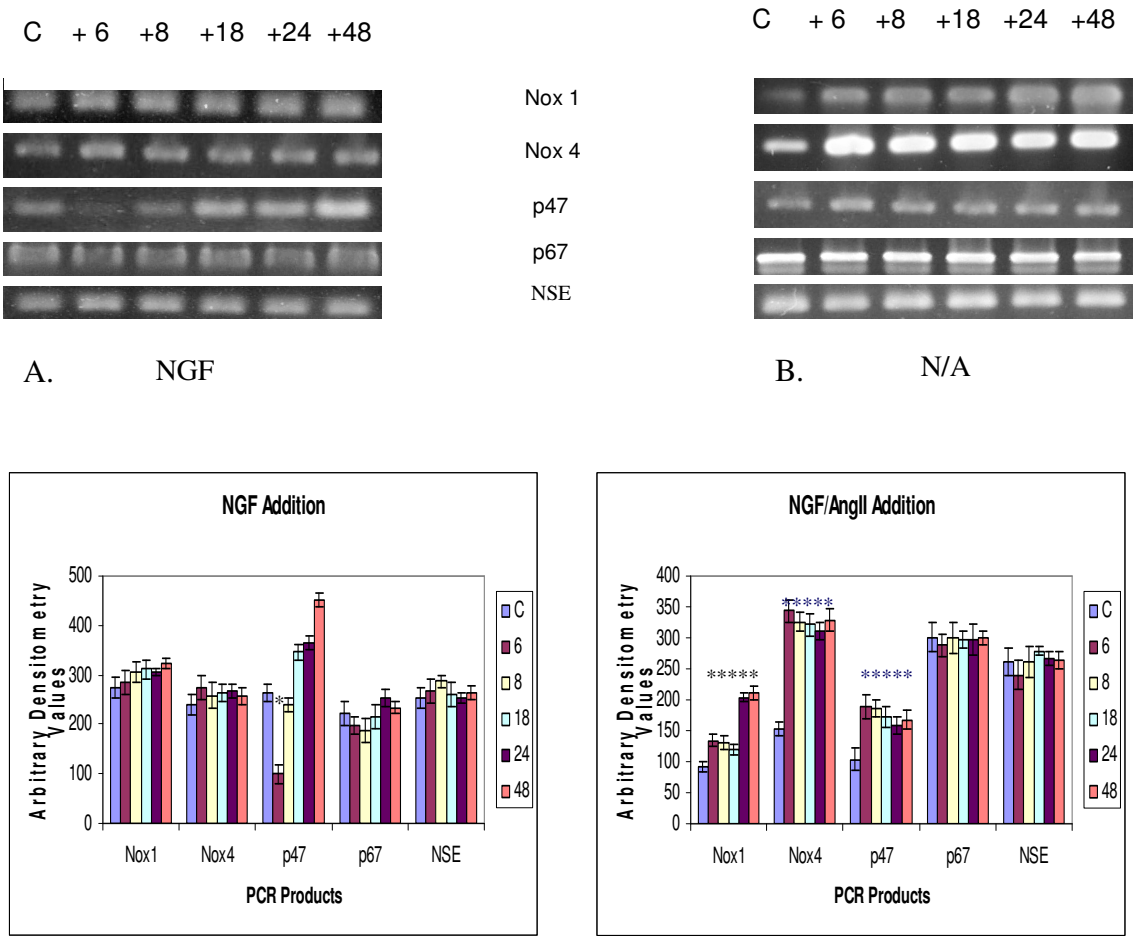


Figure 8. RT-PCR analysis of NADPH oxidase subunit expression following long-term addition of NGF and NGF/AngII treatments and corresponding densitometry. PC12 cells were cultured as described in Material and Methods. Twenty-four hours after seeding, the cells were either kept in regular F-12 K media (C, control) or treated with either NGF-supplemented F-12K (50 ng/ml) (A) or NGF (50 ng/ml) and angiotensin II (10 uM) (NGF/AngII) (B) for 6, 8, 18, 24, and 48 hours. Neuron Specific Enolase (NSE) is a loading control. Three independent experiments were carried out (* $p < 0.05$).

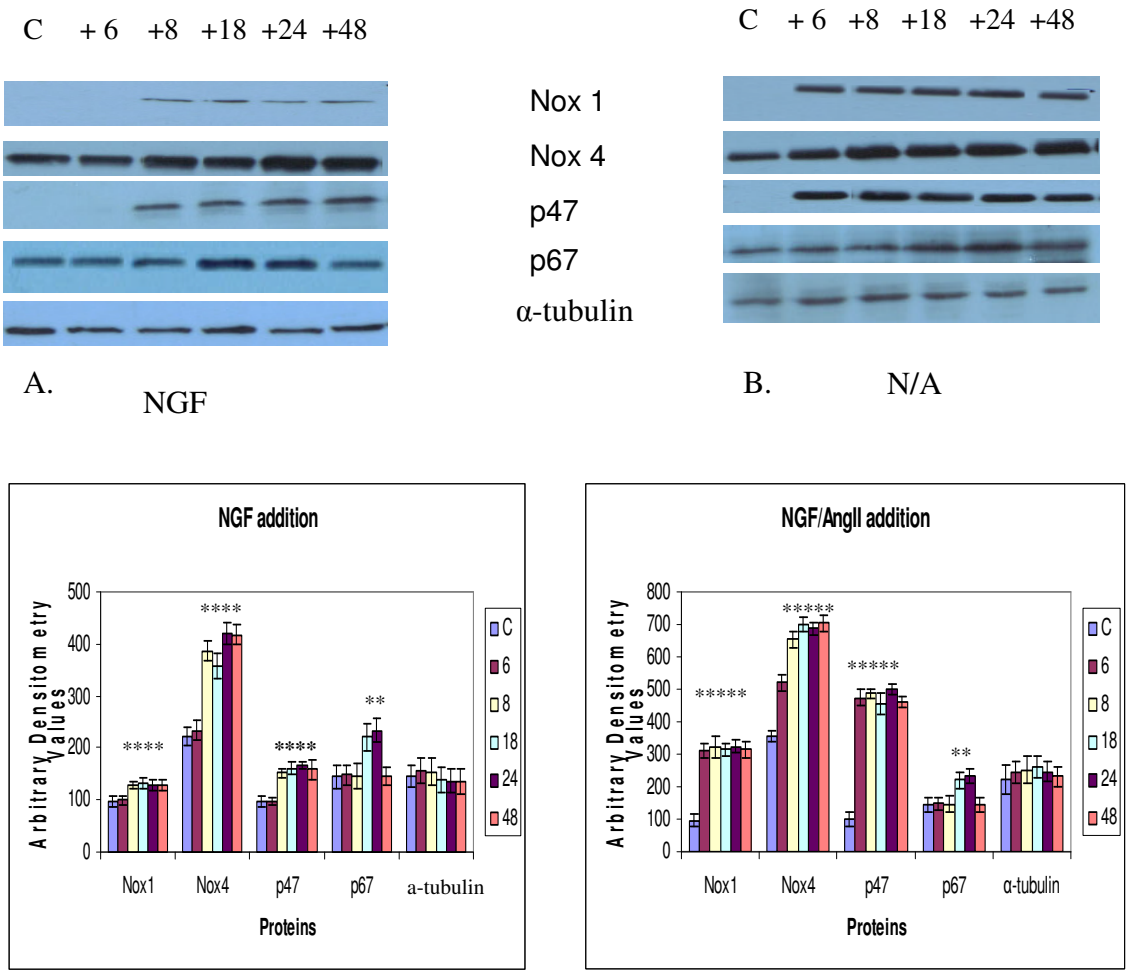


Figure 9. Western blot analysis of NADPH oxidase subunit synthesis following long-term addition of NGF and NGF/AngII treatments with corresponding densitometry. PC12 cells were cultured as described in Material and Methods. Twenty-four hours after seeding, the cells were kept in regular F-12 K media (C, control) or treated with either NGF-supplemented F-12K (50 ng/ml) (A) or NGF (50 ng/ml) and angiotensin II (10 uM) (NGF/AngII) (B) for 6, 8, 18, 24, and 48 hours. Densitometry values are means of three independent experiments (* $p < 0.05$).

Discussion

Rate of PC12 cells differentiation varies with different physiological and pharmacological treatments

Various pharmacological agents have the ability to alter the differentiation process in eukaryotic cells by influencing the level of ROS production (Wolf, 2005). Therefore, by using NADPH oxidase activator (angiotensin II) and inhibitor (DPI) we examined ROS production during PC12 cell differentiation. Diphenylene iodonium (DPI) inhibits activity of NADPH oxidase through direct addition of a phenyl group to the flavin molecule (Pullar and Hampton, 2002). Angiotensin II acts through AT2 receptors to induce cellular differentiation (Meffert et al., 1996). Therefore, PD123177 was used to limit AT2 receptor signaling involved in this process. The results of our study provide insight into the synergism of NGF and angiotensin II as illustrated by enhanced PC12 cell differentiation. However, their molecular mechanisms were reported to be dissimilar when it comes to cytoskeletal rearrangement (Gallinat et al., 1997). According to Stroth et al. (1998), NGF treatment of PC12W cells increased synthesis of cytoskeletal proteins involved in axonal stability such as MAP1, whereas angiotensin II increases levels of MAP2 proteins needed for dendritic maturation. Nevertheless, both treatments were reported to stimulate microtubule polymerization. This suggests that axons and dendrites are formed and stabilized simultaneously, which could substantiate in part that NGF/AngII-treated cells differentiated faster and produced a more extensive neurite network. Compared to the NGF/AngII group, the NGF/AngII /PD showed a significant reduction in the number of differentiated PC12 cells. However, the morphology of these cells detected on day 7 and day 8 exhibited phenotypic characteristics of a mature neuron;

however only a few cells were differentiated compared to the NGF-treated group. This does not fully agree with a published report stating that PD123177 completely abolished angiotensin II-enhanced differentiation of PC12W cells (Stroth et al., 1998). However, the discrepancy probably lies in the concentration of angiotensin II and PD123177 administered. While we used 10 μM of angiotensin II and 1 μM of PD123177, these investigators used 0.1 μM and 10 μM, respectively. Another divergence was noted in the days of differentiation. While we carried out PC12 differentiation up to eight days, their study only looked at molecular changes up to day 4. According to our results, NGF/AngII /PD123177 produced little differentiation by day 4, but neurite growth was observed by day 5.

DPI-treated cells exhibited a reduced neurite outgrowth confirming previous published reports (Suzukawa et al., 2000). Surprisingly, the cell body size of DPI-treated cells exhibited a morphology similar to the one detected in NGF-treated cells on day 7 and day 8 post-treatment, suggesting that PC12 cell differentiation involves cross-talk between two or more signaling pathways.

The rate and amount of ROS produced corresponded to the degree of PC12 cell differentiation

We examined ROS production in PC12 cells following NGF and NGF/AngII addition. NGF administration produced a peak of ROS at 10 min post-treatment, which corroborated other reports (Suzukawa et al., 2000; Ibi et al., 2006). This ROS burst is necessary to trigger differentiation in PC12 cells (Suzukawa et al., 2000). The increase in ROS and the higher degree of differentiation observed following NGF/AngII treatment may explain in part the fact that neurons produce an elaborate network of axons and

dendrites through altering the kinetics of the signaling pathways involved (Vaudry et al., 2002). In fact, developing neurons *in vivo* produce cycles of high ROS as part of their differentiation process and these ROS were confirmed to be developmentally regulated (Tsatmali et al., 2006). Administration of DPI (5 μM) significantly decreased the levels of ROS below that observed with NGF addition and halted the maturity of the neurite network observed in the previous study. This is in accordance with an *in vivo* study which demonstrated that administration of antioxidant treatment to cortical progenitor cells led to a dramatic increase in the number of poorly differentiated neurons (Tsatmali et al., 2006). Addition of PD123177 eliminated the peak increase produced by NGF/AngII treatment and brought ROS levels down to NGF values. Overall, NADPH oxidase appears to be involved in ROS-driven differentiation of PC12 cells and angiotensin II seems to induce ROS generation through AT2 receptor activation.

Expression of NADPH oxidase subunits in PC12 cells following short-term NGF addition.

A recently published report demonstrated that Nox1 mRNA was detected in NGF-stimulated and -unstimulated PC12 cells in accordance with our results, which depicted a constant level of expression in naïve PC12 cells and post-NGF addition (Ibi et al., 2006). In addition, the same group reported that the peak elevation of Nox1 levels paralleled the increase in neurite outgrowth at 72 hours (Ibi et al., 2006). Although we did not extend our examination up to 72 hours, it substantiated our finding that Nox1 protein levels were gradually increasing with NGF addition. Moreover, concomitant NGF and angiotensin II addition accelerates PC12 cell differentiation and an earlier Nox1 level increase. However, for unknown reasons the Nox transcripts did not always reflect the protein

synthesis profile. However, Nox4 transcript and protein levels remained constant following NGF addition. This is in accordance with previous reports stating that Nox4 is ubiquitous and produces constitutive levels of ROS, except in certain instances in which Nox 4 can be regulated at the transcript level (Lassegue and Clempus, 2003; Lambeth, 2004). Interestingly, p47-phox proteins were absent in untreated and NGF-treated cells, which paralleled Nox1 protein response. This is suggestive of a possible collaboration between Nox1 and p47-phox for ROS modulation, which was demonstrated in colon epithelial and endothelial cells (Banfi et al., 2003; Laufs et al., 2005). If this holds true, it could further the hypothesis that Nox1 requires p47-phox for an optimum activity as was reported previously (Banfi et al., 2003; Laufs et al., 2005). In fact, independent reports revealed that Nox1 and p47-phox activation is under the control of Protein Kinase C (PKC) (Ago et al., 2003; Geiszt et al., 2003; Wolin, 2004; Sadok et al., 2008). It is likely that PKC produces its effect on both subunits through control of protein translation and/or mRNA stability. Obviously, supplementary analyses are needed to support this notion in PC12 cells. Conversely, p67-phox proteins exist in undifferentiated PC12 cells and their levels remain constant after NGF treatment. This is in accordance with the constitutive presence of p67-phox in rabbit aortic adventitia, which was linked to a steady generation of ROS (Pagano et al., 1997).

Expression of NADPH oxidase subunits in PC12 cells following short-term NGF/AngII addition.

NGF/AngII addition increased Nox1 and Nox4 at the transcript and protein levels. The increase in the catalytic subunits may be linked to higher ROS production. In fact, angiotensin II- induced upregulation of NADPH oxidase subunits was linked to increased

oxidative stress in tissues such as vascular smooth muscle cell and cardiac cells (Lassegue et al., 2001; Chabrashvili et al., 2003; Guzik et al., 2006). The concurrent addition of NGF and angiotensin II increased p47-phox levels as opposed to merely absent levels post-NGF administration. Several studies showed that the increase in p47-phox level was crucial to angiotensin II-induced ROS generation in microvascular endothelial, vascular smooth muscle and aortic cells (Murphy et al., 2000; Lavigne et al., 2001; Li et al., 2002). These findings suggest that angiotensin II acts as an NADPH oxidase activator through gene expression regulation. On the other hand, the action of angiotensin II on NADPH oxidase activity could also stem from a direct involvement in upregulation of early-response transcription factors such as c-jun. Indeed, angiotensin II increases the expression of seven immediate early gene-encoded transcription factors among which is c-jun (Lebrun et al., 1995). Zohn et al. (1995) showed that angiotensin II produced an approximate four-to six-fold increase in JNK (c-Jun N-Terminal kinase) activation when compared to growth factors. In addition, potent activation of JNK was detected following addition of ROS to cell culture and use of diphenyleneiodonium (DPI) inhibited JNK activity (Lo et al., 1996).

Effect of long-term treatment with NGF on NADPH oxidase subunits in PC12 cells

Although long-term addition of NGF did not produce a change at the transcript level in *nox1* and *nox4,* their protein levels increased at 8 hours post-NGF treatment. The increase in Nox4 levels was detected either concurrent with or following an increase in Nox1 levels. Vallet et al. (2005) suggested that Nox4 synthesis is redox-sensitive as protein levels increased was ROS-mediated following brain ischemia. In addition, the

increase in the protein levels of p47-phox and Nox1 corroborates the fact that Nox1 and p47-phox might work in concert (Banfi et al., 2003; Laufs et al., 2005).

Effect of long-term treatment with NGF and angiotensin II (NGF/AngII) on NADPH oxidase subunits in PC12 cells.

The addition of angiotensin II increased the transcript level of *nox1* and *nox4* and reversed the decrease in *p47-phox* transcript level detected with NGF addition. In addition, angiotensin II with NGF produced an earlier increase in the protein levels of Nox1, Nox4 and p47-phox subunits. The same experiment was performed using angiotensin II alone and neither the RT-PCR nor the western blot analyses showed any change at the transcriptional and translational levels of NADPH oxidase (data not shown). This suggests that angiotensin II and NGF act in synergy to upregulate gene expression and increase protein levels of the enzyme. Taken all together, the fact that angiotensin II and DPI significantly increased and decreased, respectively, ROS production and differentiation rate suggests a role for NADPH oxidase in PC12 cells differentiation. The increase in protein levels of Nox 1 and p47-phox at 8 hours after NGF addition suggests that both subunits are needed to maintain a certain level of ROS or possibly produce a second burst.

Chapter III

NADPH oxidase expression in PC12 cell apoptosis

Abstract

Neurodegeneration could be due to activation of programmed cell death pathways targeting specific neurons in the nervous system. A body of evidence points to oxidative stress as a mediating factor in neurodegenerative disease. In addition, the multi-subunit NADPH oxidase is a prominent source of reactive oxygen species (ROS) in different tissues including the brain and other cells of the central and peripheral nervous system. Preliminary reports from our lab have linked the oxidase to PC12 cell apoptosis; however, the subunits involved have not been elucidated. Using NADPH oxidase activator (angiotensin II) and inhibitor (diphenylene iodonium, DPI), we demonstrate by TUNEL labeling that angiotensin II addition to NGF-deprived PC12 cells accelerates apoptosis while DPI significantly reduces cell death. In addition, angiotensin II causes a higher burst of ROS as compared to cells deprived of NGF, while DPI attenuates the increase. Withdrawal of NGF produces a significant increase in Nox1, p47-phox, and p67-phox protein levels which might indicate their involvement in the machinery of PC12 cell apoptotic cascade. Our results demonstrate that NADPH oxidase may be involved in PC12 cell apoptosis and that angiotensin II accelerates this process through increasing ROS production.

Introduction

Nerve Growth Factor receptors

NGF acts through two structurally unrelated classes of cell surface receptors, the Tyrosine-Receptor Kinase A (TrkA) and $p75^{NGF}$ receptors (Zapf-Colby et al., 1998). TrkA is the high-affinity NGF receptor, which is involved in cell survival and differentiation (Zapf-Colby et al., 1998). $p75^{NGF}$, a transmembrane glycoprotein low-affinity receptor, exhibits a dual function as a mediator of cell survival and apoptosis (Chao and Hempstead, 1995). In fact, the mode of action of $p75^{NGF}$ is still unclear, however it forms a heteromultimeric complex with Trk receptors which increases the affinity of TrkA for NGF (Lodish, 2000). In the absence of TrkA activation, $p75^{NGF}$ acts as a neuronal death factor (Deshmukh and Johnson, 1997; Belliveau et al., 1997). Indeed, withdrawal of trophic factor activates $p75^{NGF}$ and induces sympathetic neuronal death (Bamji et al., 1998). The precise ligand for $p75^{NGF}$ apoptotic signaling is still unclear. However, stimulation of this receptor results in c-Jun N-terminal Kinase (JNK) activation with subsequent stimulation of the caspase cascade (Bakhar et al., 2003).

Role of Oxidative Stress in neurodegeneration

Programmed cell death (PCD) is a highly regulated process. It is under the control of intrinsic and extrinsic factors of which oxidative stress has been prominently implicated (Putcha et al., 2002; Tsatmali et al., 2005). Although neurodegenerative diseases have different etiologies, at the molecular level they all converge into a disadvantageous activation of PCD (reviewed by Kanazawa, 2001). In fact, reactive

oxygen species, nerve growth factor deprivation, and excessive levels of neurotransmitters are involved in the pathology of both acute and progressive neurodegeneration (Duke and Ojcius, 1996). Studies done on toxic models revealed that cellular disturbances such as oxidative stress trigger neuronal death via activation of PCD pathways that lead to apoptosis (reviewed by Halliwell and Whiteman 2004; Halliwell and Gutteridge 2006). In addition, the brain is a susceptible target to oxidative stress as it consumes high levels of oxygen and contains high transition metals (Tsay et al., 2000). Reduction of these metals by hydrogen peroxide leads to the deleterious hydroxyl free radical generation by Fenton-type reactions (Obata, 2003). Although cells possess an intricate network of defense mechanisms to neutralize excess ROS, the brain is much more vulnerable to oxidative stress due to restricted antioxidant capacity (Babcock, 1999). In addition, the brain has limited access to most antioxidants mainly due to the blood-brain barrier. As a consequence, neurons are the first cells to be affected by accumulation of ROS (Perry et al., 2004).

NADPH oxidase contributes to neuronal apoptosis through oxidative stress

NADPH oxidase has been implicated in apoptosis of sympathetic, cortical and hippocampus neurons through production of ROS (Tammariello et al., 2000; Noh and Koh, 2000; Tejada-Simon et al., 2005; reviewed by Bedard and Krause, 2007). Induction of neuronal apoptosis linked to NGF withdrawal is mediated by NADPH oxidase. The identity of the subunits involved in PC12 cell apoptosis have not been identified (Tammariello et al., 2000; Kim et al., 2002). PC12 cells represent a viable *in vitro* model widely used to study neurodegeneration as these cells become dependent on nerve growth

factor (NGF) for survival once they are terminally differentiated (Lambeng et al., 1999). This post-mitotic condition mirrors the state of the mature neurons in the brain, which makes terminally differentiated PC12 cells a valuable tool to recapitulate neuronal apoptosis (Rukenstein et al., 1991). In fact, upon NGF withdrawal, terminally differentiated PC12 cells undergo apoptosis with biochemical and physical characteristics similar to neuronal apoptosis (Batistatou and Greene, 1993; reviewed by Valavanis et al., 2001). To investigate the putative subunits associated with apoptosis, we analyzed the expression and synthesis of NADPH oxidase subunits in terminally differentiated and NGF-deprived PC12 cells. Using a TUNEL assay, we compared cell death following treatment with an activator and a pharmacological inhibitor of NADPH oxidase. We also examined ROS production post-treatment with the identical agents. Our results revealed that angiotensin II coupled with NGF deprivation produced a higher burst of ROS and increased TUNEL-positive cells, which were attenuated by use of DPI and PD123177. In addition, NGF withdrawal increased Nox1, p47-phox and p67-phox proteins, which suggest a possible role for these subunits in PC12 cell apoptosis**.**

Materials and Methods

PC12 cell culture

Rat pheochromocytoma (PC12) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) at an unknown passage. Cultures were maintained at 37°C, 5% CO_2/95% air in Ham's F-12K growth media supplemented with 2 mM L-glutamine 15% horse serum; 2.5% fetal bovine serum (Cellgro, Herndon, VA).

Cultures were grown in collagen-treated 10-cm culture dishes with media exchange every other day (Falcon, San Jose, CA). For experimental purposes, 6-well, 24-well, or 96-well tissue were used (Falcon). For expression studies, the cells were terminally differentiated with NGF (50 ng/ml) for ten days. On day 11, NGF deprivation was performed for 2, 4, 6, 15, 18, 24 and 48 hours.

Cell passage

Cells were analyzed before passaging using a light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, New York). Old media was removed by power suction followed by addition of 2 µl of trypsin/EDTA (Sigma). The cells were incubated at 37°C for 5 minutes. To stop the proteolytic action of trypsin, 3 ml of culture media were added and cells were dispersed by repeated forceful pipetting. The suspension was transferred into a 50-ml BD Falcon Conical tube and the cells were centrifuged at 400 x g for 10 minutes. The supernatant was discarded and the cells were resuspended in fresh medium.

TUNEL assay

TUNEL (Terminal Deoxynucleotide **T**ransferase d**U**TP **N**ick **E**nd **L**abeling) assay is a widely used method to label breaks in the DNA, and is accepted as a hallmark of apoptosis. PC12 cells were terminally differentiated in the presence of NGF for 10 days. NGF was removed for 12 and 16 hours and an anti-NGF was added at a concentration of 1/1000 diluted in F-12K media as described previously. The different experimental

groups included: NGF withdrawal (-NGF), -NGF + angiotensin II (10 μM), -NGF + Angiotensin II (10 μM) +PD 123177 (1 μM), and -NGF + Angiotensin II (10 μM) + DPI (5 μM). The protocol provided by the manufacturer (APO-BrdU™ TUNEL Assay Kit (Invitrogen, Carlsbad, CA) was closely followed. Briefly, the cells were fixed with 1% paraformaldehyde in PBS, subjected to ice-cold 70% (volume/volume) ethanol and stored at -20º C for 18 hours. The fixed cells were resuspended and 1 ml aliquots were added to 12 x 75 mm flow cytometry tubes. After centrifugation, the pellet was washed and resuspended in 50 μl DNA-labeling solution. After rinsing, 100 μl of antibody staining solution was added per tube. Iodide/RNAse (0.5 ml) staining buffer addition followed. The samples were incubated in this solution for 30 minutes in the dark at room temperature and analyzed by flow cytometry (Quest Diagnostics, Chantilly, VA).

Oxidative stress analysis

2', 7'-dichlorofluorescein diacetate (DCFHDA) assay is a widely used method for measuring intracellular reactive oxygen species (ROS). DCFHDA is converted to DCFH by cleavage of its ester bond by means of intracellular esterases. Non-fluorescent DCFH is oxidized to DCF (fluorescent dichlorofluorescein) in the presence of H_2O_2, which is the immediate byproduct of ROS. Thus, it is a valuable tool to measure intracellular ROS generation. PC12 cells were cultured onto collagen-treated 96-well plates at a concentration of 3 x 10^3 cell/well. PC12 cells were either maintained in the presence of F-12K for ten days (NGF) or deprived of NGF (-NGF) in the presence or absence of angiotensin II (10 μM)(-NGF/AngII), angiotensin II + PD123177 (1 μM) (-NGF/AngII/PD), DPI (5 μM) (-NGF/DPI), angiotensin II + DPI (5 μM) (-NGF/A/DPI).

The experimental time points included 1, 2, 3, 4, 5, 6, 7, 8, 15, 18, 24 and 48 hours post-NGF withdrawal. NGF-deprivation started 48 hours prior to DCF reading. Cells were preloaded with DCFHDA in DMSO (5 μg/ml, Molecular Probes) for 30 minutes at 37 ^{o}C. All DCFHDA solutions were prepared fresh instantly before the assay. A multi-channel pipette was used to supply 100 μl/well. The DCF fluorescence was measured using a CytoFluor II Fluorescence Plate reader (Applied Biosystems) with an excitation wavelength of 485/20 nm and an emission wavelength of 530/35 nm.

RNA isolation procedure

PC12 cells were seeded at a concentration of 0.25 x 10^6 cells/well onto 24-well plates. Each experimental group comprised four wells. RNA extraction was carried out following the manufacturer's protocol. Briefly, RNA was isolated using 250 μl of Trizol® reagent (Gibco Invitrogen, Carlsbad, CA, USA) per well, incubated at room temperature for 10 min, and collected in RNAse-free 1.5 ml Eppendorf tubes. Chloroform (1/5 volume) was added per 1ml of Trizol®-cell solution. The tubes were manually inverted and allowed to incubate for 5 minutes at room temperature. This was followed by centrifugation at 4°C at 7558 x g for 5 min. The aqueous phase, which contains RNA, was transferred into separate 1.5 ml Eppendorf tubes (Westbury, NY). Isopropyl alcohol (100%) 1/2 volume was added and the sample was left for 10 minutes at room temperature. Another cold centrifugation at 7558 x g for 15 minutes precipitates the RNA pellet, which was washed with 250 μl of 75% ethanol and cold centrifuged at 2516 x g for 5 minutes. The ethanol was removed by careful pipetting to avoid disruption of

the precipitated RNA, and the pellet was allowed to dry for 10 minutes at room temperature and resuspended in RNAse-free DEPC water (0.1%). Quantification of the RNA was performed by measuring optical density at 260 nm using a spectrophotometer. RNA samples were kept frozen at -80 °C until used.

Reverse Transcription Reaction

Five µg of total RNA was mixed with oligo (dT) primers (50mM) and 10mM dNTP in PCR tubes. The combination was heated at 70°C for 5 minutes and immediately placed on ice for 2 minutes. A stock solution of the following was prepared: 6 µl 5 x reverse transcription buffer, 1 µl of RNAseOut® (40 U/µl), 1 µl of SuperScript RT III (200 U/µl) and 1 µl DTT (0.1M). Negative controls were prepared with no addition of RT to detect any DNA contamination. Gentle pipetting and a quick spin were performed to collect the content. The stock solution was added to the RNA/oligo (dT) mixture and incubated at 50°C for 50 minutes. The reaction was inactivated at 85°C for 5 minutes. To destroy the RNA template, 1 µl of RNAse H (2 units/µl) was added to the mixture and followed by incubation at 37°C for 20 minutes. The cDNA mixture was stored at -20°C until use for polymerase chain reaction.

Polymerase Chain Reaction (PCR)

Platinum® Taq DNA Polymerase (Sigma, St. Louis, MO) is a recombinant DNA polymerase attached to an inhibitory antibody to prevent unnecessary activity at ambient temperatures. Heating the complex to 94°C causes the denaturation of the antibody and subsequent activation of the Platinum® Taq DNA Polymerase. The mixture provides the free nucleotides needed for the elongation of the product. All reagents were mixed and centrifuged briefly before addition and were kept on ice. The reaction mixture was prepared in 0.5 ml RNAse-free PCR tubes as follows: 2 unit of Taq Polymerase, 0.6 µl (5 pmol/µl) of each primer (+/-), and 5 µg of template**.** The reaction mix was briefly centrifuged to ensure homogeneous blending. All PCR reactions were performed using a PCRexpress thermocycler ® (Hybaid Omnigene Thermocycler, UK). Briefly, the mixture was heated to 94°C for 2 minutes to activate Taq polymerase known as the "hot start" and denature the DNA. The first step was followed by an annealing for 30 seconds (the temperature used depended on the primer utilized) and an extension step at 72°C for 30 sec- 1 minute depending on the size of the target product. The annealing temperatures were optimized by doing a temperature gradient PCR starting 5°C below the calculated T_m going up to 12°C until the most stringent conditions were found. Subsequent cycling heated the mix at 94°C for only 30 seconds. The cycle of melting, annealing, and extending was repeated between 25-30 times. Final extension was at 72°C for 10 minutes. All primers were generated at Integrated DNA Technology (Coralville, IA) and are listed in Table 2.

Table 2. Primer sequences used in the apoptosis study

Primer	Sequence
nox1 sense	5'-AACCACACGCTGAGACCATTCGA-3'
nox1 antisense	5'-TCAGCTAAGGCAGGTTTCCCAGA-3'
nox4 sense	5'-AGGTGTCTGCATGGTGGTGGTATT-3
nox4 antisense	5'-AAAGAGGGCTGTGGCTATCAGCTT-3'
p47-phox sense	5'-CAGCCAGCACTATGTGTACAT-3'
p47-phox antisense	5'-GAACTCGTAGATATAGGTGAA-3'
p67-phox sense	5'-GGGAACCAGCTGATAGACTACAT-3'
p67-phox antisense	5'-TCCATTCCTCTTTCTTGGCAATT-3'
NSE sense	5'-ACGCTGGACTCGCTGGGCAA-3'
NSE antisense	5'-GCGAGCGCGCTGCGCTTGTA-3'

DNA gel electrophoresis

PCR products were separated on 1-2% agarose gels in 1X TAE (0.04 M Tris-Acetate, 0.002 M EDTA, pH 8.0). The electrophoresis apparatus was set at 100 V for 45 min and 0.5 x SyberGold (Molecular Probes, Eugene, OR) stained the gel for at least 30 minutes. The DNA fragments were visualized under ultraviolet light.

DNA sequencing

Identification of PCR products was confirmed by direct DNA sequencing. cDNA sequencing was performed using DYEnamic ET terminator Cycle Sequencing kit® (Amersham Biosciences) according to the manufacturer protocol using ABI Prism® 310 genetic analyzer (PE Applied Biosystems).

Protein extraction procedure

PC12 cells were seeded at a concentration of 0.25 x 10^6 cells/well onto 24-well plates. Each experimental group comprised four wells. Protein extraction was performed on ice to prevent further protein modification. Cells were washed with cold sterile phosphate-buffer saline (PBS) (500 µl/well) to remove traces of proteins from the media. Cell lysates were collected by scrapping in lysis buffer (1 X 10mM Tris-HCl, 1mM EDTA, pH 7.4) + Protease Inhibitor Cocktail (PIC) (Roche, Mannheim, Germany) + 0.1 % Triton X+ phosphatase inhibitor). PIC contains a mixture of proteases that inhibit serine, cysteine, and metalloprotease activity. The composition of the PIC is as follows: Chymotrypsin, 1.5 µg/ml, Thermolysin, 0.8 µg/ml, Papain, 1 mg/ml, Pronase, 1.5 µg/ml, Pancreatic extract, 1.5 µg/ml, Trypsin, 0.002 µg/ml. Samples were sonicated for cell disruption and centrifuged at 4° C 7558 x g for 10 minutes. The supernatant containing the protein extract was collected and stored in -80°C until further analysis.

Western Blot analysis

Protein concentrations were quantified using serial dilutions of BSA as standards for Bradford protein assay (Bio-Rad, Hercules, CA). The BSA solution was added in increments of 2µg and assayed in triplicate. Equal amounts of protein were boiled in SDS electrophoresis buffer (containing 2-mercaptoethanol) at 90^{o}C for 10 minutes. Protein separation was performed by a 12% SDS-PAGE using Xcell SureLock® (Invitrogen). The proteins were transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore Immobion-P #IPVH 000 10, St. Louis, MO) pre-soaked in 100% methanol and transfer buffer (200mM glycine, 25mM Tris, 20% methanol) . Transfer of the blot onto the membrane was performed using Xcell II Blot Module® (Invitrogen). The blot was blocked with 5% non-fat milk in Tris-Buffered Saline (27mM Tris, 57 mM NaCl, pH 7.5) for 1 hour at room temperature. The primary antibody was diluted at a concentration of 1:800 in blocking solution and incubated for 60 minutes at room temperature on a rocker. The following antibodies were used in this study: anti-Nox 1 (cat. 25545, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Nox 4 (cat. 5514, Santa Cruz), anti-α-tubulin (cat. 2144, Cell Signaling Technology, Inc., Danvers, MA), anti-p47-phox and anti-p67-phox were a generous gift from Dr. Mark Quinn (Montana State University). The membrane was washed three times with 1xTBS, 0.05% Tween 20). A horseradish peroxidase (HRP)-conjugated secondary antibody (cat.7074, Cell Signaling Technology, Inc.) diluted in TBS-t (1:1000) was added for 45 minutes. Amersham ECL kit (RPN 2106) (GE healthcare, Piscataway, NJ) was prepared

following the manufacturer protocol. Band detection was achieved using Kodak X-Ray film (Eastman Kodak, Rochester, NY).

Statistics

The results are expressed as the mean ± SEM of three or more independent experiments. Significant differences in measured values were evaluated with an analysis of a Student's *t*-test or ANOVA. Statistical significance was set at $p < 0.05$. Statistical analysis and graphing were performed using Microsoft Excel.

Results

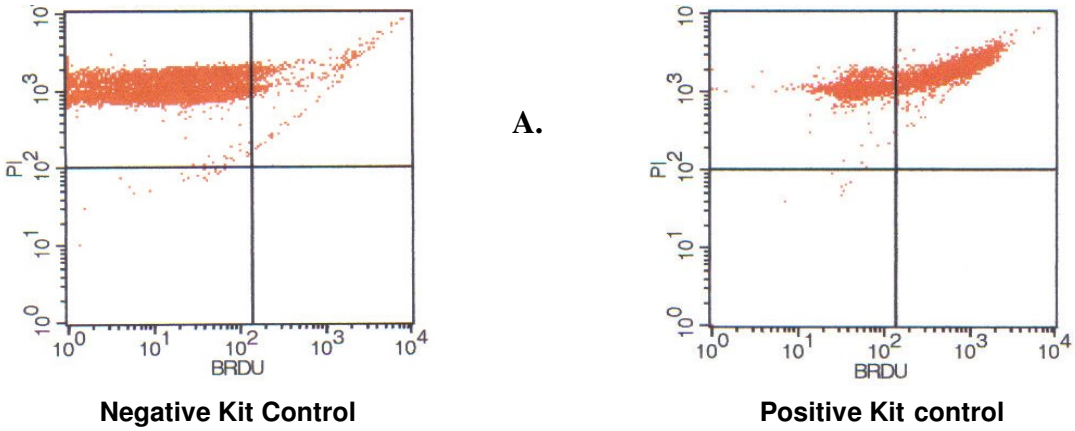
Angiotensin II accelerates apoptosis following NGF withdrawal

A TUNEL assay was performed to examine apoptosis in PC12 cells following NGF withdrawal and addition of different pharmacological treatments for 12h and 16h. Positive and negative BrdU lymphoma cells (provided by the manufacturer) and NGF-treated and NGF-deprived PC12 cells were used as controls (Fig.10). Withdrawal of NGF (-NGF) produced a significant increase in TUNEL-positive cells compared to cells maintained in NGF (Fig. 11). Addition of angiotensin II post-NGF withdrawal significantly increased the number of TUNEL-positive cells in comparison to NGF-deprived PC12 cells at 12h while PD123177 significantly reduced the percentage (Table 3). Furthermore, addition of DPI significantly reduced the percentage of TUNEL positive cells in comparison to NGF-deprived PC12 cells at 12h and 16h following NGF removal

(Fig. 12). This suggests that NADPH oxidase may be involved in NGF-deprivation induced apoptosis in PC12 cells.

Table 3. Average value of TUNEL-positive cells in different treatment groups. The numbers represent readings from 3 independent experiments. -NGF: NGF-deprived cells; ang II :angiotensin II. * $p < 0.05$ significant difference from negative control. ** $p<0.05$ significant difference from α-NGF

	12h	16h
Negative Control (+NGF)	4 ± 2%	6 ± 1%
-NGF	53 ± 6 %*	86 ± 7 %*
-NGF + ang II	85 ± 5 %**	92 ± 6 %
-NGF + ang II + PD123177	22 ± 2 %**	91 ± 4 %
-NGF + ang II + DPI	27 ± 8 %**	32 ± 10 %**



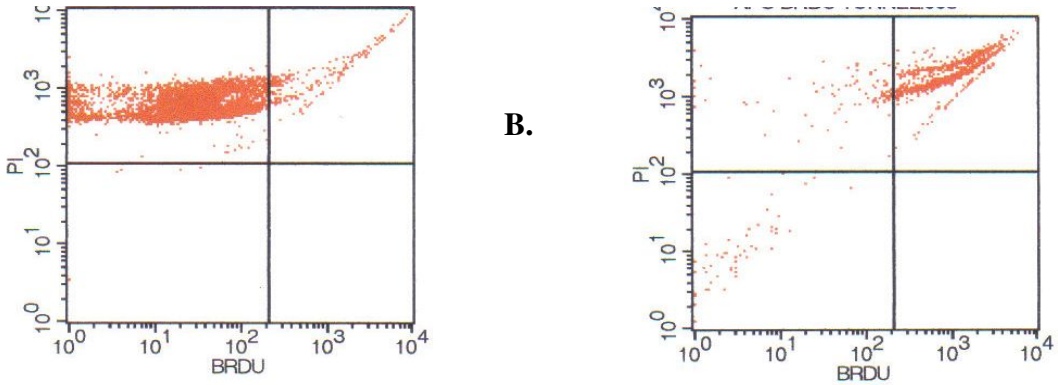
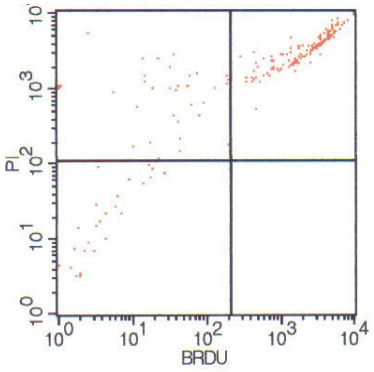
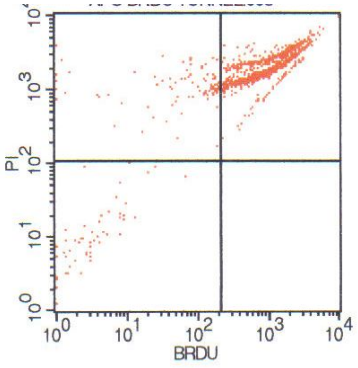


Figure 10. TUNEL assay controls. A) Negative and positive BrdU control lymphoma cells were provided by the manufacturer of the APO-BrdU™ TUNEL Assay Kit. **B)** Negative (+NGF) and positive (-NGF) control PC12 cells as controls for this experiment.

-NGF

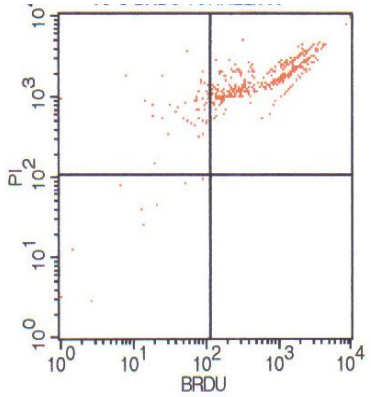


12 hours

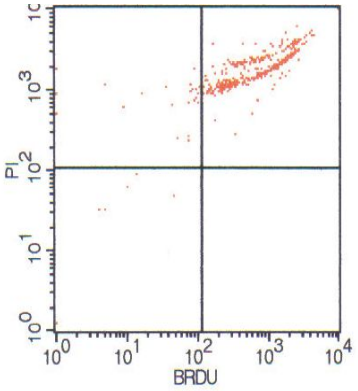


16 hours

-NGF
+ Angiotensin II



12 hours



16 hours

Figure 11. TUNEL assay following NGF withdrawal and addition of angiotensin II in PC12 cells. Cells were fixed with 1% paraformaldehyde in PBS and incubated in DNA-labeling solution. This was followed by addition of an antibody staining solution and iodide/RNAse staining buffer. The samples were incubated in this solution for 30 minutes in the dark at room temperature and analyzed by flow cytometry (Quest Diagnostics - Chantilly, VA) -NGF: NGF-deprivation.

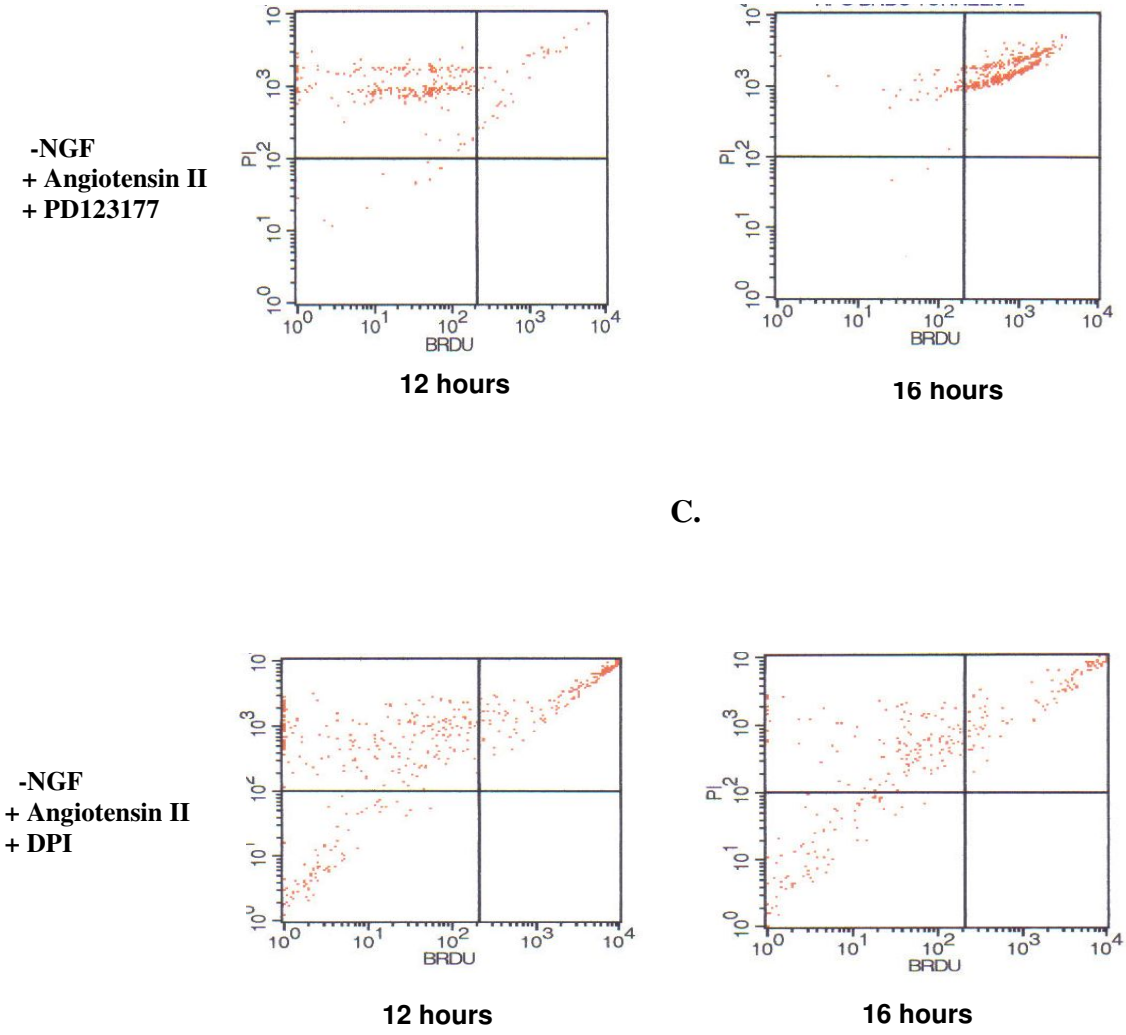
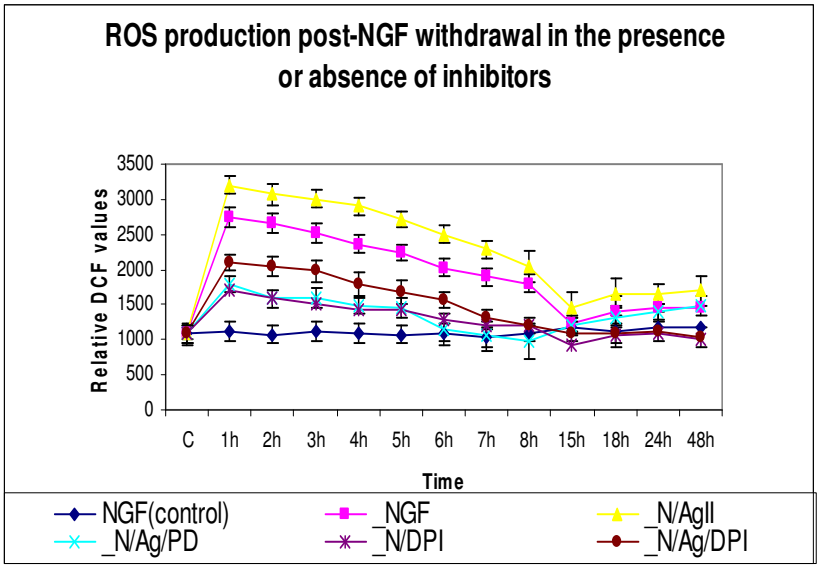


Figure 12. TUNEL assay following NGF withdrawal and co-addition of angiotensin II and DPI or PD123177. Cells were fixed with 1% paraformaldehyde in PBS and incubated in DNA-labeling solution. This was followed by addition of an antibody staining solution and iodide/RNAse staining buffer. The samples were incubated in this solution for 30 minutes in the dark at room temperature and analyzed by flow cytometry (Quest Diagnostics - Chantilly, VA) -NGF: NGF-deprivation

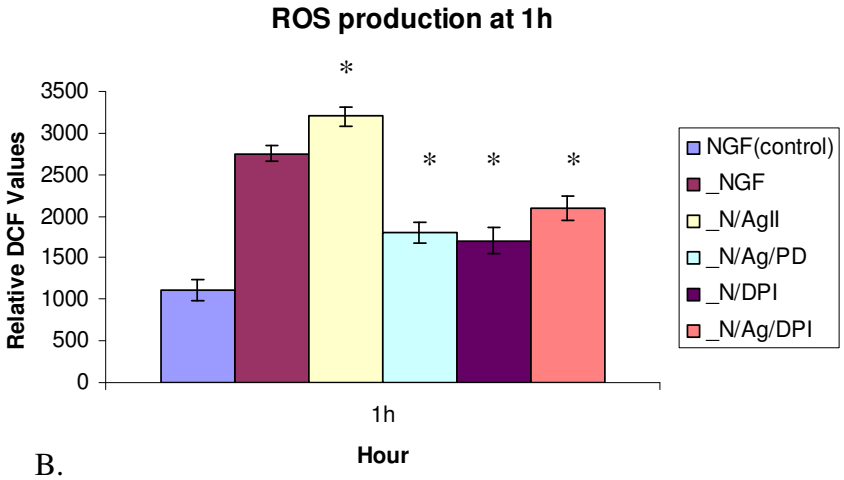
Angiotensin II increases and DPI blocks NGF deprivation induced oxidative stress

The DCF assay was performed to examine the effect of NGF withdrawal on ROS production in the presence or absence of angiotensin II. NGF deprivation alone and NGF deprivation coupled with angiotensin II (-NGF +AngII) produced a peak of ROS at 1 hour and decreased thereafter (Fig. 13). However, the angiotensin II group produced significantly higher fluorescence (except between 8h and 15h post-treatment). As noticed in the DCF study involving differentiation, angiotensin II addition produced an early increase in ROS, as the 45-minute values were statistically similar to the 1h values of α-NGF post-trophic factor withdrawal. These data suggest that the increase in ROS could play an important role in accelerating apoptosis of PC12 cells detected in the TUNEL experiment.

The decrease in TUNEL-positive cells following use of PD123177 and DPI experiment led us to examine ROS generation following NGF removal and addition of those inhibitors. DPI in NGF-deprived PC12 cells resulted in a statistically significant decrease in ROS production compared to -NGF cells. DPI produced a similar effect when co-administered with angiotensin II following NGF deprivation. Furthermore, concurrent addition of angiotensin II and PD123177 in NGF-deprived PC12 cells led to significant lower DCF values when compared to -NGF + AngII values (Fig 13).



A



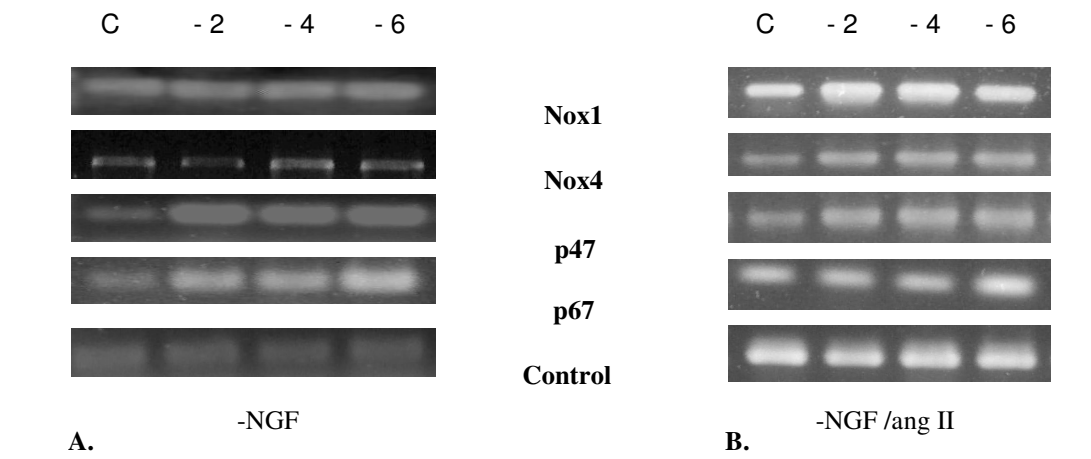
B.

Figure 13. Angiotensin II increases and DPI attenuates oxidative stress. (A) PC12 cells were terminally differentiated in NGF for 10 days. Cells were either left in NGF (NGF) or NGF-deprived (-NGF) in the presence or absence of angiotensin II (-NGF/AgII), angiotensin II +PD123177 (-NGF/Ag/PD), DPI, or angiotensin II +DPI (-NGF/ Ag/DPI). Treatment started 48h prior to DCF fluorescence reading. Cells were pre-loaded with DCFHDA for 30 minutes at 37°C before fluorescence quantification. (B) Graphic representation of ROS peak at 1h post-NGF withdrawal (* $p < 0.05$ significance from –NGF).

NGF withdrawal produces a parallel increase in Nox1, p47-phox, and p67-phox subunits

To identify subunits that may be involved in PC12 apoptosis, we examined the expression of NADPH oxidase at the transcript and protein levels. The withdrawal of NGF resulted in approximate two-fold increases of *p47-phox* and *p67-phox* transcripts (Fig. 14A). However, when angiotensin II was administered following NGF withdrawal, all subunit transcripts increased significantly suggesting that angiotensin II may have a regulatory effect on NADPH oxidase subunit expression (Fig 14B). At the protein level, removal of NGF increased Nox1, p47-phox and p67-phox in a similar manner (Fig. 15A). Nox4 levels at 4h and 6h post-NGF removal (Fig. 15B). Cells treated with angiotensin II concurrent with NGF withdrawal exhibited a similar protein pattern to those cells deprived of only NGF, except for Nox 4 which is modestly, but significantly, increased. Thus angiotensin II appears to affect the level of transcription, but not translation, in this model (Fig. 15B).

To further examine the long-term effect of NGF withdrawal on the expression and synthesis of NADPH oxidase subunits in PC12 cells, we performed RT-PCR and western blot analyses on cells 15-48 hours after NGF deprivation. NGF removal significantly increased *nox1* and *nox4* transcripts, however NGF deprivation resulted in a transient downregulation of *p47-phox* and *p67-phox* transcripts (Fig. 16A). At the protein level, NGF removal significantly increased Nox1, p47-phox and p-67-phox by 15h after NGF withdrawal, when compared to the control cells that were maintained in NGF (Fig. 16B)



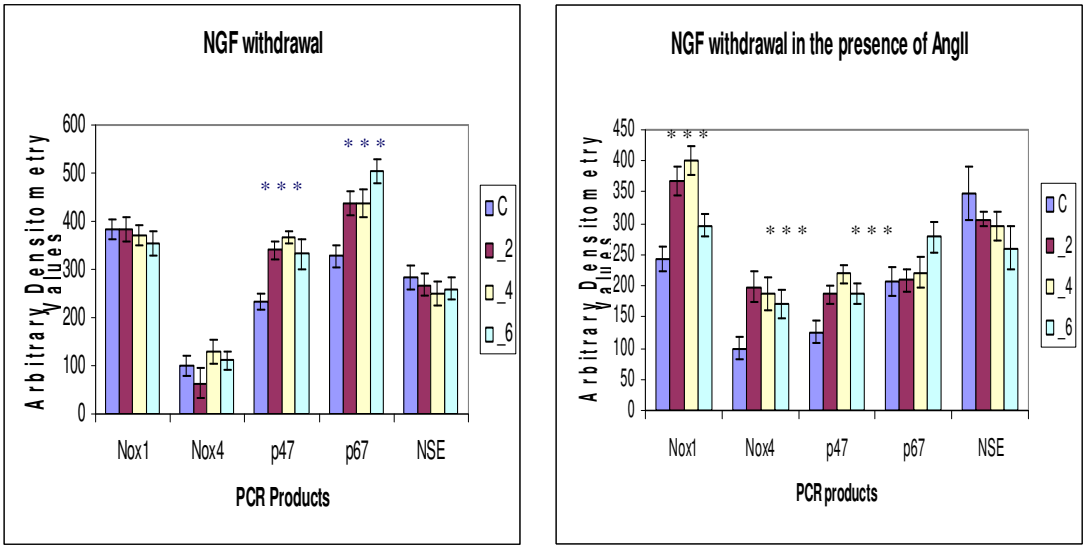


Figure 14. RT-PCR analysis of NADPH oxidase subunits following NGF removal and angiotensin II addition with corresponding densitometry analysis. PC12 cells were seeded as described in Materials and Methods. Cells were kept in the presence of NGF (Control C) or absence of NGF for 2, 4, and 6 hours (A). Angiotensin II (10 µM) was added at same time-points following NGF removal (B). Neuron-specific Enolase is a loading control. Minus sign represents withdrawal of NGF.

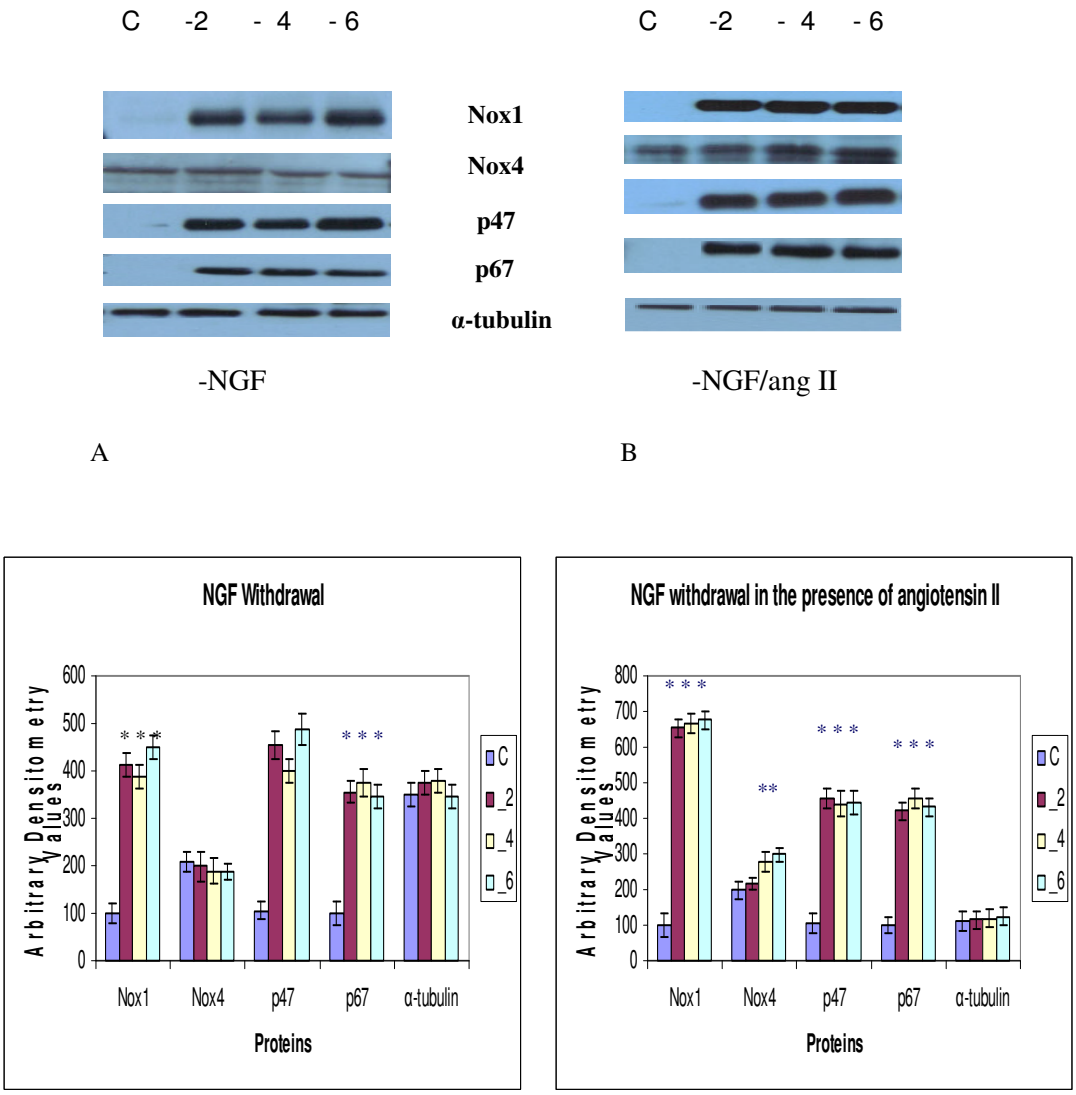


Figure 15. Western blot analysis of NADPH oxidase subunit expression following NGF removal and addition of angiotensin II with corresponding densitometry analysis. PC12 cells were seeded as described in Materials and Methods. Cells were kept in the presence of NGF (Control C) or absence of NGF for 2, 4, and 6 hours (A). Angiotensin II (10 μM) was added at same time-points following NGF removal (B). α-tubulin is a loading control. Minus sign represents withdrawal of NGF.

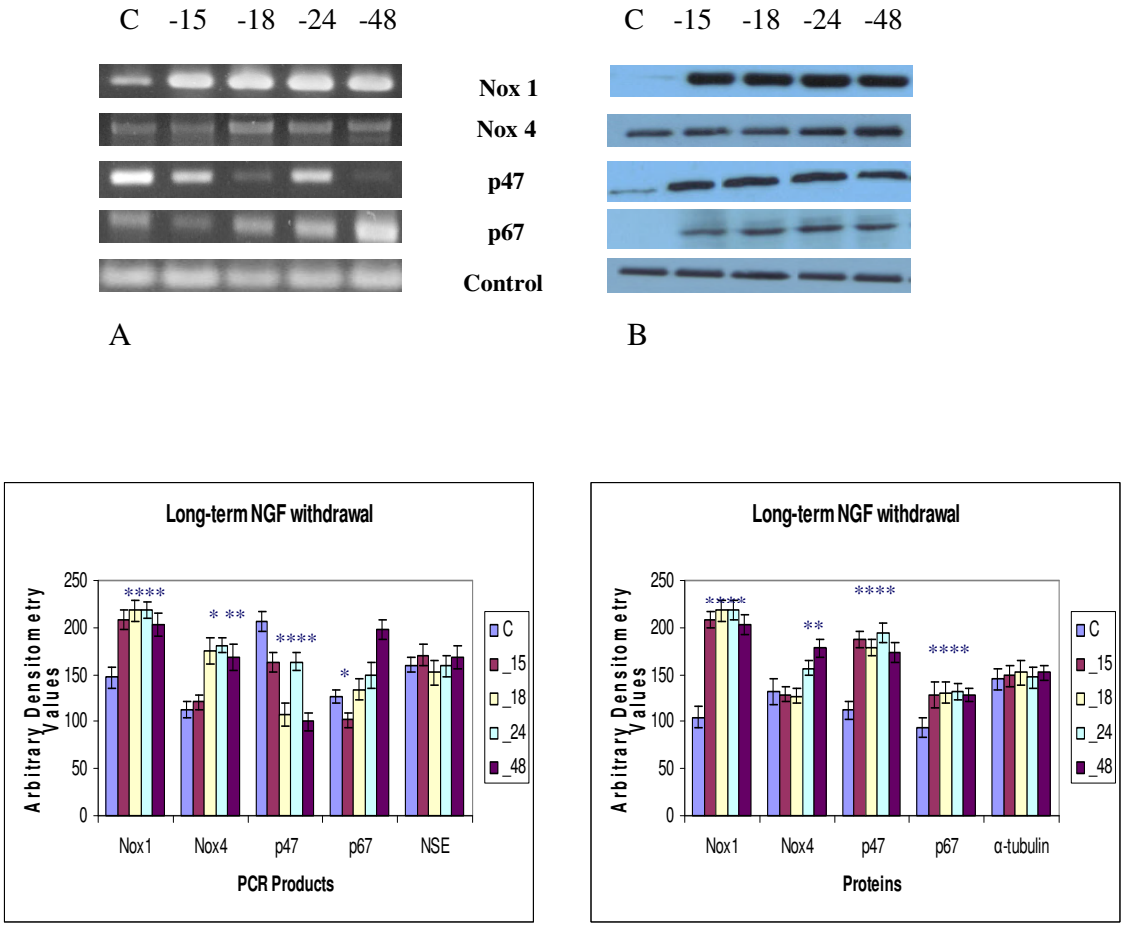


Figure 16. RT-PCR and western blot analyses of NADPH oxidase subunit following long-term NGF deprivation of PC12 cells. Cells were seeded as previously described in Materials and Methods. Cells were kept in the presence of NGF (Control C), or in absence of NGF for 15, 18, 24 and 48 hours. Loading controls: NSE and α-tubulin are the loading controls for the RT-PCR and western blot analyses, respectively. Minus sign represents withdrawal of NGF.

Discussion

Angiotensin II accelerates apoptosis while DPI and PD123177 slow down the rate of PC12 cell death

NGF withdrawal produced about a 50% increase in TUNEL positive staining when compared to the negative control at 12h post-NGF removal. This is in agreement with Vaghefi et al. (2004) who reported that half of the terminally differentiated PC12 cells were no longer viable at 12h following NGF-deprivation. Furthermore, cells treated with angiotensin II coincident with NGF withdrawal exhibited a significantly higher percentage of TUNEL positive cells 12h after NGF withdrawal. This substantiates previous data from our laboratory, which showed that angiotensin II produced an accelerated apoptosis in NGF-deprived sympathetic neurons (Hilburger, 2004). The AT2 antagonist, PD123177, counteracted the angiotensin II effect for up to 12 hours, which substantiates other reports that angiotensin II exerts its apoptotic action mainly through AT2 receptor stimulation (Yamada et al., 1996; Dimmeler et al., 1997; Wang et al., 1999). However, the percentage of TUNEL positive PD123177-treated cells was statistically similar to -NGF values by 16h post-NGF withdrawal. This suggests that an increase in AT2 receptor synthesis may have occurred and/or receptor activation took place between 12 and 16 hours. When DPI was co-administered with angiotensin II to NGF-deprived cells, the percentage of TUNEL positive cells dropped significantly at both times substantiating previous reports from our laboratory that DPI decreased apoptosis in NGF-deprived sympathetic neurons, even when treated with angiotensin II (Hilburger, 2004).

Angiotensin II produces a higher burst of oxidative stress following NGF withdrawal from PC12 cells

Angiotensin II-induced cellular apoptosis has been established in epithelial and endothelial cells (Dimmeler et al., 1997; Wanget al., 1999). The results from the TUNEL study point toward a comparable involvement in PC12 cells. In fact, most of the documented angiotensin II–mediated cellular action including apoptosis was linked to oxidative stress (Griendling et al., 1994; Imanishi et al., 2005; Mehta and Griendling, 2007). DCF analysis of –NGF/AngII-treated cells revealed that apoptosis was preceded by a significant elevation in ROS when compared to the burst observed in PC12 cells deprived of NGF alone. In fact, the -NGF and - NGF/AngII groups produced a ROS peak at 1h post-NGF withdrawal. However, - NGF/AngII values were significantly higher at all times except for 8h-15h time-points. - NGF/AngII addition generated an earlier increase in ROS as the 45- minute values were statistically similar to the 1h values of the –NGF cells . Our laboratory has detected a similar result in sympathetic neurons (Hilburger, 2004). The early increase in angiotensin II-mediated ROS could be due in part to enhanced expression of NADPH oxidase subunits or enzyme activation. Interestingly, a second moderate increase in ROS was depicted at 18h, which could be NADPH oxidase related and/or mitochondrial-mediated. Lee et al (2006) proposed that Nox1 and mitochondria work jointly to extend the production of ROS as the mitochondrial reactive oxygen species are not enough to promote cell death, which is a process that requires a sustained production of ROS. However, since higher values were produced with angiotensin II this might further indicate that a higher number of AT2 receptors are synthesized and /or activated as apoptosis progresses.

NADPH oxidase and AT2 inhibitors decrease ROS production in NGF-deprived PC12 cells

DPI treatment produced a significant decrease in DCF values as compared to -NGF which suggests that NADPH oxidase may be a source of ROS in PC12 cells as reported by others (Tammariello et al., 2000; Beddard et al., 2007). Interestingly, co-administration of PD123177 with angiotensin II in NGF deprived cells completely abolished the angiotensin II-induced ROS production and resulted in DCF values significantly lower from the values observed in NGF deprived cells. This proposes a possible role for the AT2 receptor in PC12 cell apoptosis as suggested by other investigators (Yamada et al., 1996; Horiuchi et al., 1997). AT2 receptor involvement in PC12 cell apoptosis may be crucial as these receptors were reported to have a ligand-independent constitutive apoptotic function (Miura and Karmic, 2000).

Nox1, p47-phox, and p67-phox proteins increase following withdrawal of NGF

NGF-withdrawal resulted in differential expression of *p47-phox* and *p67-phox* transcripts with no effect on *nox1* and *nox4* mRNAs following short-term NGF withdrawal. Nox1, p47-phox and p67-phox also increased significantly at the protein level post-NGF removal. Previous reports indicate that p47-phox and p67-phox are crucial for optimal Nox 1 activity and are required to sustain certain level of ROS production (Geiszt et al., 2003; Bedard and Heinz-Krause, 2007). When the same experiment was repeated with the addition of angiotensin II, *nox1* and *nox4* transcripts were upregulated. No increase however was detected for p47-phox and p67-phox. We

observed a similar pattern of increase for Nox1, p47-phox and p67-phox at the level of translation. The addition of angiotensin II had no significant effect at the protein level. Following long-term NGF withdrawal, nox1 and nox4 transcripts were upregulated. p47-phox and p67-phox transcripts, however exhibited a transient downregulation. This is suggestive that the regulation of these two transcripts could be linked to the overall activity of the enzyme. This proposition is supported by a body of evidence pointing toward the existence of regulatory factors unique to oxidase genes in phagocytic NADPH oxidase (Li et al., 2001). In fact, Skalnik et al. (1991) demonstrated that there are repressor proteins that bind the positive promoters of NADPH oxidase genes to repress transcription when needed. Although, this report showed that the repressor protein was tissue-specific, similar regulatory factors could exist in PC12 cells. Taken together, NADPH oxidase may play a role in PC12 cell apoptosis as suggested by the increase in the rate of TUNEL positive cells following enzyme activator addition, angiotensin II. Similarly, the decrease in the TUNEL positive cell number following DPI addition, the enzyme inhibitor furthers this proposition. Nox1, p47-phox and p-67-phox may play a role in the apoptotic process as their protein levels were elevated following NGF withdrawal.

Chapter IV

c-Jun and AT2 receptors expression and synthesis in PC12 cells differentiation and apoptosis

Abstract

PC12 cell differentiation and apoptosis are processes that require the activation of c-Jun by the c-Jun N-terminal kinase (JNK). Similarly, angiotensin II-type 2 (AT2) receptors are involved in cellular differentiation and apoptosis of PC12 cells. In addition, c-Jun and AT2 receptors independently were shown to induce neuronal regeneration. We investigated the effect of angiotensin II on c-jun expression, synthesis, and phosphorylation as possible mediators of cellular apoptosis and differentiation. A parallel study examined the expression of AT2 receptors, which are necessary for angiotensin II-induced cellular differentiation and apoptosis. Here we report that NGF treatment of PC12 cells upregulates c-jun expression and increases protein levels and phosphorylation of c-Jun proteins. Concurrent addition of angiotensin II with NGF induces higher levels of total and phosphorylated c-Jun leading to enhanced transcriptional activation of c-jun. Further, NGF treatment increases AT2 receptors at the transcriptional and translational levels. However, NGF co-administered with angiotensin II leads to an earlier expression of AT2 receptor transcript and protein synthesis that are abolished by use of DPI and PD123177. c-jun expression and phosphorylation decrease following NGF withdrawal from terminally differentiated PC12 cells. This suggests that signaling pathways may be switching from survival/ differentiation to death signaling. Furthermore, AT2 receptors exhibit an angiotensin II-independent upregulation following NGF deprivation. Overall, these results indicate that angiotensin II plays a critical role in c-Jun regulation in differentiation and apoptosis of PC12 cells. In addition, AT2 receptors are involved in these two processes; however their implication in apoptosis is angiotensin II-independent as well.

Introduction

The c-Jun transcription factor is upregulated during PC12 cell differentiation

NGF-induced Trk phosphorylation stimulates Ras through recruitment of adaptor proteins such as Grb-2 and SOS-1 (Obermeier et al., 1993; 1994). The MAP kinase cascade, downstream of Ras, results in the activation of c-Fos and c-Jun transcription factors which have been linked to neurite outgrowth and axonal regeneration (Smeal et al., 1991; Pulverer et al., 1991; Dragunow et al., 1993). c-Jun is an inducible transcription factor capable of gearing PC12 cell gene expression toward cellular differentiation and apoptosis depending on the extracellular stimuli and subsequent signaling pathway activation (Angel and Karin, 1991). In addition, c-Jun activity is controlled largely at the protein level. Regulatory phosphorylations take place on four N-terminal sites which are Ser63, Ser73, and Thr91 and /or and Thr93 residues (Pulverer et al., 1991). The posttranslational modifications confer stabilization and enhanced c-Jun/ DNA binding interaction (Papavassiliou et al., 1995). These regulatory phosphorylations are linked to MAP kinases, ERK1 and ERK2, which receive their signals from activated Ras subsequent to Trk receptor stimulation (Smeal et al., 1991; Pulverer et al., 1991). In addition, in vitro and in vivo studies suggest that c-jun expression in neurons might be involved in axonal regeneration after axotomy (Jenkins and Hunt, 1991; Dragunow et al., 1993). In fact, damage to peripheral axons in rat produced a long-term c-jun expression in neuronal cell bodies and all c-Jun-positive peripheral neurons were able to project into

the spinal cord. However, c-jun expression returned to basal levels after full axonal recovery (Jenkins et al., 1993).

NGF removal activates the N-terminal Jun Kinase (JNK) pathway

JNK is a member of the mitogen-activated protein kinase superfamily. It is activated by a variety of extracellular stimuli such as some growth factors, G protein-coupled receptors and ultraviolet light (Kyriakis et al., 1994; Derijard et al., 1994). JNK phosphorylates the N-terminal of c-Jun transcription factor that has been correlated with cellular stress and neuronal death. Phosphorylated c-Jun homodimerizes or forms heterodimers with members of the c-Fos superfamily, and acquires an activator protein-1 (AP-1) property that controls the expression of many different genes including c-jun itself (Angel and Karin, 1991). Studies have reported c-Jun kinase to be an obligate component of the cell death pathway in neuronal and PC12 cells (Xia et al., 1995; Park et al., 1996). Inhibition of JNK activity prevented death of motor, sympathetic neurons and PC12 cells (Maroney et al., 1997). Furthermore, N-terminal phosphorylation of c-Jun has been found to be involved in programmed cell death in the adult brain. After ischemia-reperfusion, c-Jun expression is bilaterally induced in the cortical hemispheres. N-terminal phosphorylation, however was only detectable in neurons of infarcted areas (Herdegen et al., 1998). JNK activation occurs by a post-translational mechanism. In fact, addition of a transcriptional terminator (actinomycin D) concomitant with trophic factor deprivation led to an increase in JNK stimulation (Ham et al., 2000). This implies that JNK activation is independent of gene upregulation in response to trophic factor

withdrawal. In addition, Bcl-2 the anti-apoptotic protein appears to block death of NGF-deprived PC12 cells by acting upstream of JNK stimulation (Park et al., 1996). JNK signaling is particularly implicated in ischemic stroke and Parkinson's disease, which makes it an attractive target for therapeutic intervention (Kuan and Burke, 2005). There are three known isoforms of JNK: JNK1, 2 and 3 which are all encoded by 3 independent genes. JNK1 and JNK2 have a less specific expression profile, whereas JNK3 expression is believed to be restricted to the central nervous system. JNK3 deficiency proved neuroprotective in NGF-deprived sympathetic neurons and gene JNK3- knockout prevented excitotoxic cell death of hippocampal neurons suggesting that JNK3 mediates neuronal apoptosis (Yang et al., 1997; Bruckner et al., 2001). However, JNK3 does not contribute to oxidative stress following NGF withdrawal, which implies that it is not involved in NADPH oxidase activation (Bruckner et al., 2001).

AT2 receptors are important in cellular differentiation

The high expression of AT2 receptor during fetal and neonatal development has suggested that this receptor may be involved in tissue growth and/or differentiation (Nakajima et al., 1995). In healthy adult, the AT2 receptor is only expressed in restricted tissues such as parts of the brain, vascular endothelial cells, the adrenal and cardiac structures, myometrium and ovaries (de Gasparo et al., 2000). However, the AT2 receptor is upregulated in response to certain pathophysiological conditions in adults (Gallinat et al., 1998; Nio et al, 1995). In addition, AT2 receptor activation induces neuronal regeneration. Axotomy of both dorsal root ganglia, sciatic and optic nerves

significantly upregulated AT2 expression (Lucius et al., 1998; Gallinat et al., 1998). The specific mechanism(s) of the AT2-induced effects are still unknown. It is believed, however to ensue through the same signaling that leads to the cytoskeleton rearrangement during neuronal differentiation (Gendron et al., 2003). One of the prominent roles attributed to the AT2 receptor is induction of neurite outgrowth and elongation by increasing the polymerization of microtubules (Meffert et al., 1996; Stroth et al., 1998).

Data on the effect of trophic factors on AT2 expression are limited. Shanmugam and colleagues (1995) showed that AT2 mRNA was elevated during development of rat kidney as long as the trophic factor was in good supply during embryogenesis. A subsequent decrease in AT2 expression was concomitant with the completion of nephrogenesis (Shanmugam et al., 1995).

AT2 receptors induce apoptosis in terminally differentiated PC12cells

The AT2 receptor is also known to play an important role in cellular apoptosis (Yamada et al., 1996; Horiuchi et al., 1997). Serum starvation-induced apoptosis can be salvaged by the addition of trophic agents such as nerve growth factor (Pittman et al., 1993). However, concurrent angiotensin II administration with trophic factor nullified the protective effect of NGF and induced apoptosis by potential upregulation of AT2 expression (Yamada et al., 1996). In addition, angiotensin II increased AT2 receptor mRNA level in rat cortical cells in a dose-dependent manner, which led eventually to apoptosis (Shibata et al., 1997; Makino et al., 1998).

The dual effect of AT2 receptor on cellular differentiation and apoptosis provides evidence that the cellular response of this receptor is dictated by the presence or absence of serum and trophic growth factors such as NGF (Gendron et al., 2003). During cellular proliferation or differentiation, the AT2 receptor is hypothesized to cross-talk with signaling proteins that gear its effect toward these two processes (Gendron et al., 2003). In the absence of serum or NGF, thus of those signaling proteins, the activation of AT2 receptor induces apoptosis. In fact, Miura and Karnik (2000) proposed that the induction of apoptosis is a constitutive function of the AT2 receptor. The latter produces low angiotensin II-independent apoptosis signals that activate p38 MAPK and caspase-3. In addition, the same group demonstrated that a specific conformation of the AT2 receptor is required for those low signals to be produced (Miura et al., 2000). Moreover, mutations in the AT2 receptor or use of pharmacological agents that interrupt apoptotic signals have abolished AT2-mediated cell death of R3T3 cells and vascular smooth muscle cells (Miura et al., 2000).

There is a possible cross-talk between serum-deprivation induced AT2 receptor activation and JNK signaling in PC12 cell apoptosis (Xia et al., 1995). Activation of the p38 MAPK was reported independently to induce apoptosis via JNK and AT2 receptor stimulation (Xia et al., 1995; Yamada et al., 1996). In addition, activation of AT2 receptor increases the expression of MAPK phosphatase-1 (MKP-1), which dephosphorylates ERK1/2 and Bcl-2 leading to cell death (Yamada et al., 1996; Horiuchi et al., 1997).

AT2 signaling in cellular differentiation and apoptosis

Although this receptor belongs to the seven-transmembrane domain receptor family, it does not exhibit G protein-coupled receptor characteristics regarding agonist affinity and ability to activate second-messengers (Kambayashi et al., 1993). In fact, its mode of action is believed to be independent of adenylyl cyclase and PKA, the classical pathways for the family of G protein-linked membrane receptors (Bottari et al., 1993). However, a growing body of evidence points to its involvement with Gαi and subsequent activation of cGMP in neurons (Sumners et al., 1991). Signal transduction cascades following AT2 receptor activation appear to involve protein phosphatases acting either on tyrosine or serine/threonine residues (Tsuzuki et al., 1996; Yamada et al., 1996). Angiotensin II-induced cellular differentiation in the presence of trophic factors has been linked to ERK1/2 activity. As mentioned above, serum-deprivation induced apoptosis has been associated with p38 MAPK pathway activation (Wang et al., 1998; Gendron et al., 1999).

Angiotensin II has shown to accelerate the rate of PC12 cell differentiation and apoptosis. We wished to examine the effect of angiotensin II on c-Jun expression, synthesis, and phosphorylation during these two cellular events. In addition, AT2 receptors proved to play a crucial role in both PC12 cell differentiation and apoptosis. Use of PD123177 during ROS production differentiation and cell death consistently antagonized the effect of angiotensin II. The PD123177 response was as effective as the cellular effect of DPI. Therefore, a parallel examination of AT2 receptor expression and synthesis was performed during PC12 cell differentiation and apoptosis. We report that

angiotensin II addition concurrent with NGF addition significantly increases total and phosphorylated c-JUN and AT2 expression and protein levels as compared to NGF addition alone. Withdrawal of NGF from terminally differentiated PC12 cells transiently downregulates c-Jun expression and phosphorylated proteins and increases transcript and protein levels of AT2 receptors. Taken together, c-Jun and AT2 receptors may play a role in PC12 cell differentiation and apoptosis and that the combined effect of NGF and angiotensin II addition to PC12 cells may be to enhance transcription and synthesis of these two molecules.

Materials and Methods

PC12 cell culture

Rat pheochromocytoma (PC12) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) at an unknown passage. Cultures were maintained at 37°C, 5% CO_2 / 95% air in Ham's F-12K growth media supplemented with 2 mM L-glutamine 15% horse serum; 2.5% fetal bovine serum (Cellgro, Herndon, VA). Cultures were grown in collagen-treated 10-cm culture dishes with media exchange every other day (Falcon, San Jose, CA). For experimental purposes, 6-well, 24-well, or 96-well tissue were used (Falcon). For c-jun and AT2 expression analyses following NGF addition, the cells were treated with NGF (50 ng/ml) or NGF (50 ng/ml) + angiotensin II (10 μM) (NGF/AngII) for different experimental time points (2, 4, 6, 8, 18, 24 and 48h). To study the effect of DPI and PD123177, PC12 cells with were either treated with NGF

(50 ng/ml) + angiotensin II (10 μM) + DPI (5 μM) or with NGF(50 ng/ml) + angiotensin II (10 μM) + PD123177 (1 μM). Terminally differentiated PC12 cells were in the presence of NGF for 10 days. NGF withdrawal was performed at 2, 4, 6 15, 18 24 and 48 hours prior to RNA and protein collection. All controls were cells left in F12-K media.

Cell passage

Cells were analyzed before passaging using a light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, New York). Old media was removed by power suction followed by addition of 2 ul of trypsin/EDTA (Sigma). The cells were incubated at 37°C for 5 minutes. To stop the proteolytic action of trypsin, 3 ml of culture media was added and cells were dispersed by repeated forceful pipetting. The suspension was transferred into a 50-ml BD Falcon Conical tube and the cells were centrifuged at 400 x g for 10 minutes. The supernatant was discarded and the cells were resuspended in fresh medium.

RNA isolation procedure

PC12 cells were seeded at a concentration of 0.25 x 10^6 cells/well onto 24-well plates. Each experimental group comprised four wells. RNA extraction was carried out following the manufacturer's protocol. Briefly, RNA was isolated using 250 μl of Trizol® reagent (Gibco Invitrogen, Carlsbad, CA, USA) per well, incubated at room temperature for 10 min, and collected in RNAse-free 1.5 ml Eppendorf tubes. Chloroform (1/5 volume) was added per 1ml of Trizol®-cell solution. The tubes were manually inverted

and allowed to incubate for 5 minutes at room temperature. This was followed by centrifugation at 4°C at 7558 x g for 5 min. The aqueous phase, which contains RNA, was transferred into separate 1.5 ml Eppendorf tubes (Westbury, NY). Isopropyl alcohol (100%) 1/2 volume was added and the sample was left for 10 minutes at room temperature. Another cold centrifugation at 7558 x g for 15 minutes forms the RNA pellet, which was washed with 250 µl of 75% ethanol and cold centrifuged at 2516 x g for 5 minutes. The ethanol was removed by careful pipetting to avoid disruption of the precipitated RNA, and the pellet was allowed to dry for 10 min at room temperature and resuspended in RNAse-free DEPC water (0.1%). Quantification of the RNA was performed by measuring optical density at 260 nm using a spectrophotometer. RNA samples were kept frozen at -80 °C until used.

Reverse Transcription Reaction

Five µg of total RNA was mixed with oligo (dT) primers (50mM) and 10mM dNTP in PCR tubes. The combination was heated at 70°C for 5 minutes and immediately placed on ice for 2 minutes. A stock solution of the following was prepared: 6 µl 5 x reverse transcription buffer, 1 µl of RNAseOut® (40 U/µl), 1 µl of SuperScript RT III (200 U/µl) and 1 µl DTT (0.1M). Negative controls were prepared with no addition of RT to detect any DNA contamination. Gentle pipetting and a quick spin were performed to collect the content. The stock solution was added to the RNA/oligo (dT) mixture and incubated at 50°C for 50 minutes. The reaction was inactivated at 85°C for 5 minutes. To destroy the RNA template, 1 µl of RNAse H (2 units/µl) was added to the mixture and

followed by incubation at 37°C for 20 minutes. The cDNA mixture was stored at -20°C until use for polymerase chain reaction.

Polymerase Chain Reaction (PCR)

Platinum® Taq DNA Polymerase (Sigma, St. Louis, MO) is a recombinant DNA polymerase attached to an inhibitory antibody to prevent unnecessary activity at ambient temperatures. Heating the complex to 94°C causes the denaturation of the antibody and subsequent activation of the Platinum® Taq DNA Polymerase. The mixture provides the free nucleotides needed for the elongation of the product. All reagents were mixed and centrifuged briefly before addition and were kept on ice. The reaction mixture was prepared in 0.5 ml RNAse-free PCR tubes as follows: 2.0 unit of Taq Polymerase**,** 0.6 µl (5pmole) of each primer (+/-), and 5 µg of template in 15 µl reaction **.** The reaction mix was briefly centrifuged to ensure homogeneous blending. All PCR reactions were performed using a PCRexpress thermocycler ® (Hybaid Omnigene Thermocycler, UK). Briefly, the mixture was heated to 94°C for 2 minutes to activate Taq polymerase known as the "hot start" and denature the DNA. The first step was followed by an annealing for 30 seconds (the temperature used depended on the primer utilized) and an extension step at 72°C for 30 sec- 1 minute depending on the size of the target product. The annealing temperatures were optimized by doing a temperature gradient PCR starting 5°C below the calculated T_m going up to 12°C until the most stringent conditions were found. Subsequent cycling heated the mix at 94°C for only 30 seconds. The cycle of melting, annealing, and extending was repeated between 25-30 times. Final extension was at

72°C for 10 minutes. All primers were generated at Integrated DNA Technology (Coralville, IA) and are listed in Table 4.

Table 4. Primer sequences used in this study

Primer	**Sequence**
NSE sense	5'-ACGCTGGACTCGCTGGGCAA-3'
NSE antisense	5'-GCGAGCGCGCTGCGCTTGTA-3'
c-jun sense	5'-CTGAAGCAGAGCATGACCTTGAAC-3'
c-jun anti-sense	5'-TTCTGGCTAAGTTCAGCTAGG-3'
AT2 sense	5'-TTGCTGCCACCAGCAGAAACT-3'
AT2 anti-sense	5'-GTGTGGGCCTCCAAACCATTGCTA-3'

DNA gel electrophoresis

PCR products were separated on 1-2% agarose gels in 1X TAE (0.04 M Tris-Acetate, 0.002 M EDTA, pH 8.0). The electrophoresis apparatus was set at 100 V for 45 min and 0.5 x SyberGold (Molecular Probes, Eugene, OR) stained the gel for at least 30 minutes. The DNA fragments were visualized under ultraviolet light.

DNA sequencing

Identification of PCR products was confirmed by direct DNA sequencing. cDNA sequencing was performed using DYEnamic ET terminator Cycle Sequencing kit®

(GE Healthcare, formerly Amersham Bioscience, Piscataway, NJ) according to the manufacturer protocol using ABI Prism® 310 genetic analyzer (PE Applied Biosystems, PE Applied Biosystems, Foster City,. CA).

Protein extraction procedure

In order to obtain samples for western blot analysis, PC12 cells were seeded at a concentration of 0.25 x 10^6 cells/well onto 24-well plates. Each experimental group comprised four wells. Protein extraction was carried on ice to prevent further protein modification. Cells were washed with cold sterile phosphate-buffer saline (PBS) (500 µl/well) to remove traces of proteins from the media. Cell lysates were collected by scrapping in lysis buffer (1 X 10mM Tris-HCl, 1mM EDTA, pH 7.4) + Protease Inhibitor Cocktail (PIC) (Roche, Mannheim, Germany) + 0.1 % Triton X+ phosphatase inhibitor). PIC contains a mixture of proteases that inhibit serine, cysteine, and metalloprotease activity. The composition of the PIC is as follows: Chymotrypsin, 1.5 µg/ml, Thermolysin, 0.8 µg/ml, Papain, 1 mg/ml, Pronase, 1.5 µg/ml, Pancreatic extract, 1.5 µg/ml, Trypsin, 0.002 µg/ml. Samples were sonicated for cell disruption and centrifuged at 4° C 7558 x g for 10 minutes. The supernatant containing the protein extract was collected and stored in -80°C until further analysis

Western Blot analysis

Protein concentrations were quantified using serial dilutions of BSA as standards for Bradford protein assay (Bio-Rad, Hercules, CA). The BSA solution was added in increments of 2µg and assayed in triplicate. Equal amounts of protein were boiled in SDS electrophoresis buffer (containing 2-mercaptoethanol) at 90^{o}C for 10 minutes. Protein separation was performed by a 12% SDS-PAGE using Xcell SureLock® (Invitrogen). The proteins were transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore Immobion-P #IPVH 000 10, St. Louis, MO) pre-soaked in 100% methanol and transfer buffer (200mM glycine, 25mM Tris, 20% methanol) . Transfer of the blot onto the membrane was performed using Xcell II Blot Module® (Invitrogen). The blot was blocked with 5% non-fat milk in Tris-Buffered Saline (27mM Tris, 57 mM NaCl, pH 7.5) for 1 hour at room temperature. The primary antibody was diluted at a concentration of 1:800 in blocking solution and incubated for 60 minutes at room temperature on a rocker. The following antibodies were used in this study: anti-c-Jun (cat. 9165, Cell Signaling Technology, Inc., Danvers, MA), anti-Ser63-c-Jun (cat. 9261, Cell Signaling Technology, Inc.), anti-AT2 (cat. 9040, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-α-tubulin (cat. 2144, Cell Signaling Technology, Inc., Danvers, MA). The membrane was washed three times with 1xTBS, 0.05% Tween 20). A horseradish peroxidase (HRP)-conjugated secondary antibody (cat.7074, Cell Signaling Technology, Inc.) diluted in TBS-t (1:1000) was added for 45 minutes.

Amersham ECL kit (RPN 2106) (GE healthcare, Piscataway, NJ) was prepared following the manufacturer protocol. Band detection was achieved using Kodak X-Ray film (Eastman Kodak, Rochester, NY)

Statistics

The results are expressed as the mean ± SEM of three or more independent experiments. Significant differences in measured values were evaluated with an analysis of a Student's *t*-test or ANOVA. Statistical significance was set at $p < 0.05$. Statistical analysis and graphing were performed using Microsoft Excel.

Results

Angiotensin II increases *c-jun* expression and phosphorylation in NGF-treated PC12 cells

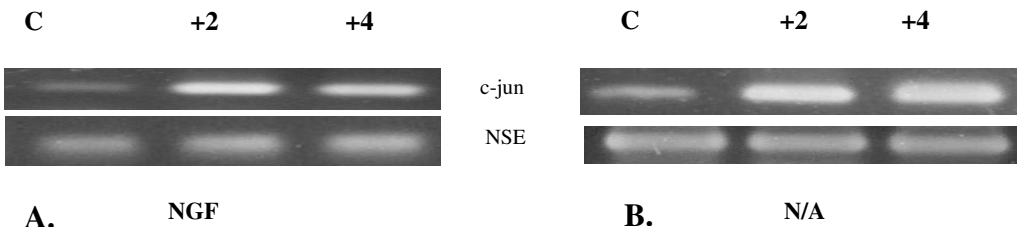
c-jun expression was examined at the mRNA level by RT-PCR analysis. Exposure to NGF substantially increased the mRNA level at 2h with a slight reduction observed by 4h after NGF addition (Fig. 17A). On the other hand, angiotensin II co-incubation with NGF (NGF/AngII) produced a gradual increase in *c-jun* transcript at 2h and 4h and produced approximately a 1.5 fold increase in *c-jun* transcript levels. In addition, angiotensin II not only prevented the decrease of *c-jun* at 4h detected with NGF addition but also maintained a progressive elevation in *c-jun* expression (Fig. 17B). Western blot analysis revealed that the addition of NGF to undifferentiated PC12 cells increased total and phosphorylated c-Jun protein (the activated form) paralleling the transcript elevation (Fig. 18A). Moreover, NGF/AngII produced a gradual increase in

total and Ser63-phospho c-Jun (Fig. 18B). The increase in c-Jun expression and phosphorylation following NGF/AngII addition might in part explain the increased level of PC12 cell differentiation depicted previously with addition of NGF/Ang II.

c-Jun expression and phosphorylation at Ser63, but not total c-Jun, produce a parallel response following NGF withdrawal

c-jun expression following NGF withdrawal from differentiated PC12 cells was examined by RT-PCR. *c-jun* transcript levels significantly decreased (by approximately two-fold) at 4h and reappeared at 6h (Fig. 19A). This finding was enigmatic since it was anticipated that *c-jun* transcript levels increase and remain high during the early stages of apoptosis. When long-term time points were examined, *c-jun* expression was high and constitutive during the later stage of apoptosis (Fig. 19B). Western blot analysis revealed a gradual decrease in total c-Jun levels during the first 6 hours of NGF deprivation. Surprisingly, Ser63-phospho-c-Jun exhibited a decrease that paralleled the transcript levels. The phosphorylated c-Jun levels were up at 2h, completely down by 4h and re-appeared at 6h after NGF withdrawal (Fig. 20A). This led us to examine the phosphorylation state of c-Jun at Ser63 more closely by performing a western blot analysis including earlier time points. The immunoblotting depicted a robust Ser63 phosphorylation at 30 and 60 minutes. By 2h, the levels started to subside with complete disappearance at 3h and 4h and reappearance at 5h (data not shown). It is unclear what caused the transient elimination of phosphorylated c-Jun. However, protein levels of total c-Jun and phospho-c-Jun show similar increasing patterns following long-term NGF

withdrawal (Fig. 20B). This data shows that the phosphorylation status of c-Jun mimics the pattern of transcription of the *c-jun* gene after short-term NGF withdrawal.



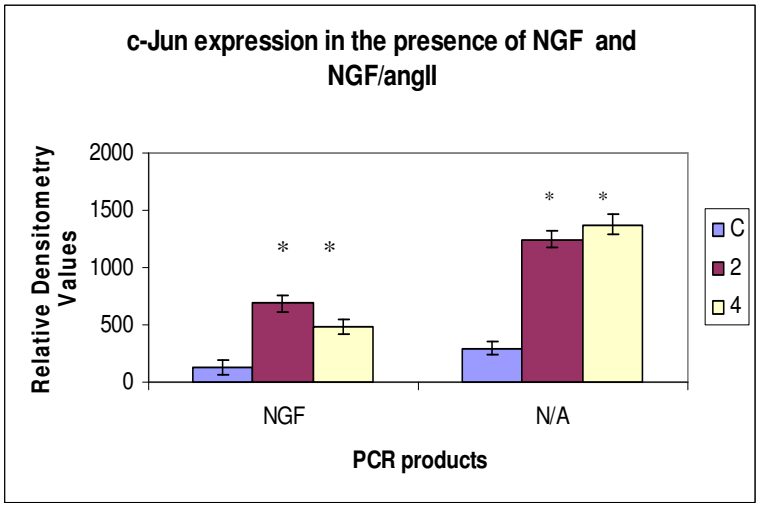


Figure 17. RT-PCR analysis of *c-Jun* expression in the presence of NGF or NGF and angiotensin II with corresponding densitometry. PC12 cells were cultured as described in Materials and Methods. Twenty-four hours after seeding, cells were kept in regular F-12 K media (C, control) or treated with either NGF-supplemented F-12K (50 ng/ml) (A) or NGF (50 ng/ml) and angiotensin II (10 uM) (NGF/AngII) (B) for 2 and 4 hours (+2h, +4h, respectively). Densitometry values are means of three independent experiments and represent values corrected for loading control (* $p < 0.05$). Equal loading control is Neuron Specific Enolase (NSE).

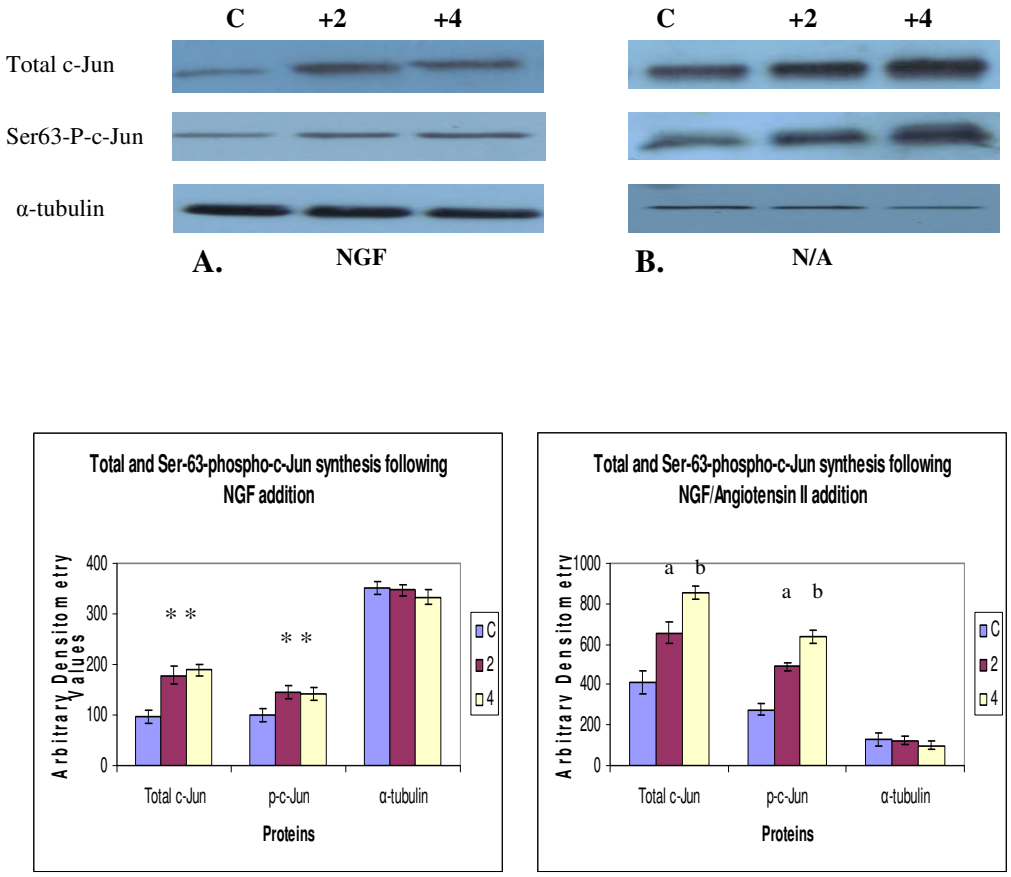


Figure 18. Western blot analysis with corresponding densitometry quantification of total and phosphor-c-Jun following NGF and NGF/Ang II addition with corresponding densitometry quantification. PC12 cells were cultured as described in Materials and Methods. Twenty-four hours after seeding, cells were kept in regular F-12 K media (C, control) or treated with either NGF-supplemented F-12K (50 ng/ml) (A) or NGF (50 ng/ml) and angiotensin II (10 uM) (NGF/AngII) (B) for 2 and 4 hours (+2h, +4h, respectively). Densitometry values are means of three independent experiments. Asterisk denotes statistical difference from the control. a-b: Values with different letters are significantly different. ($p < 0.05$). α-tubulin is a loading control.

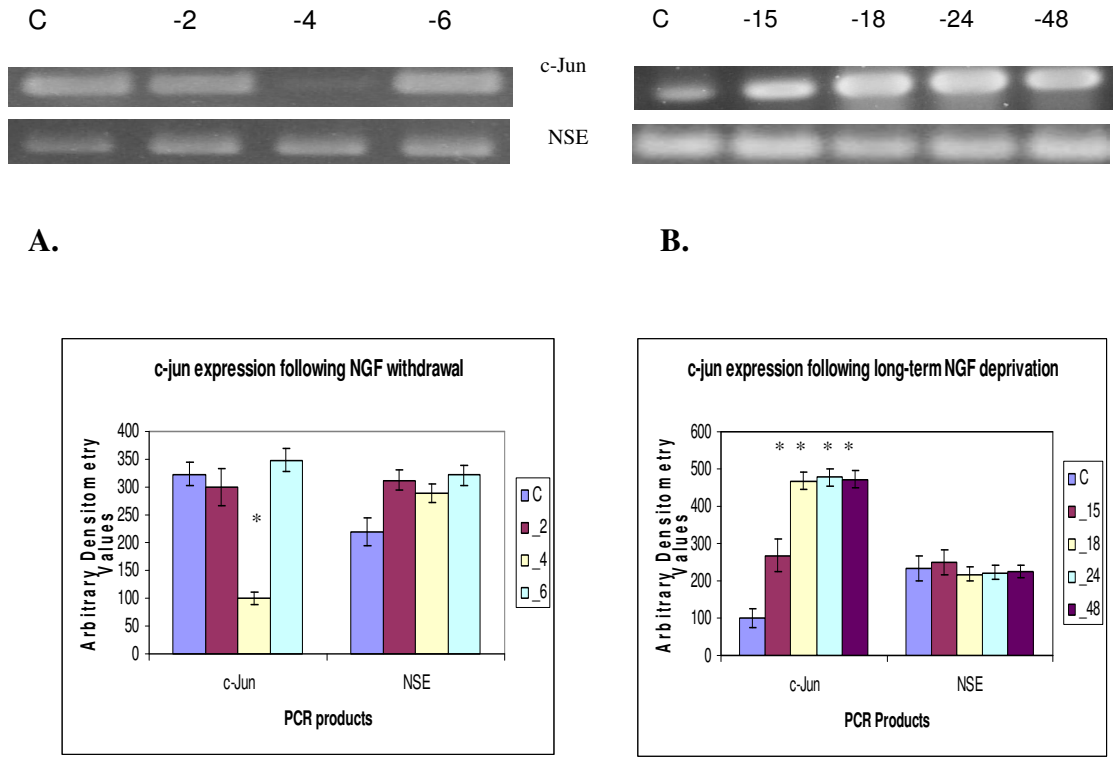


Figure 19. RT-PCR analysis of *c-jun* expression following NGF withdrawal from terminally differentiated PC12 cells with corresponding densitometry quantification. PC12 cells were terminally differentiated in NGF as previously described in Materials and Methods. Cells were kept in the presence of NGF (Control C), or in absence of NGF for 2, 4, 6 (A), 15, 18, 24 and 48 hours(B). NSE is Neuron-Specific Enolase is a loading control. Minus sign represents withdrawal of NGF. Each bar represents an average of three independent experiments. Error bars indicate S.E (* $p < 0.05$).

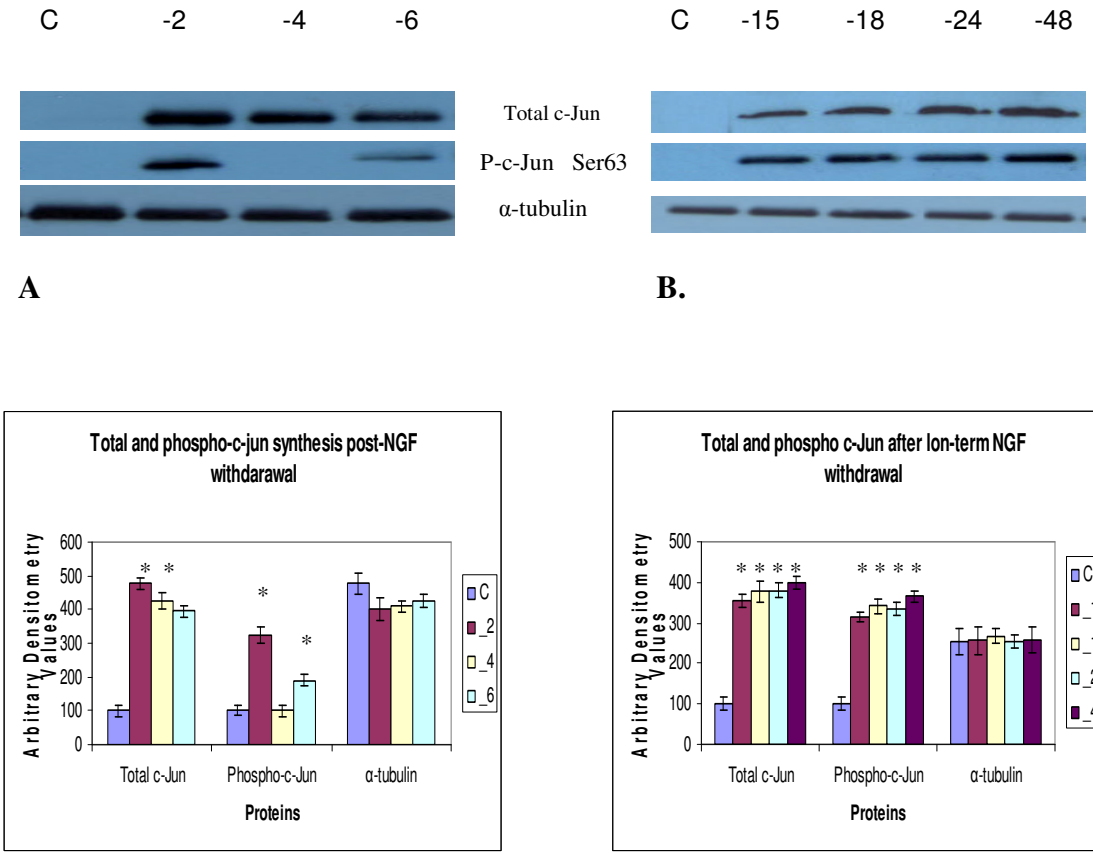


Figure 20. Western blot analysis of Total and Ser63 phosphorylated c-Jun following NGF withdrawal with corresponding densitometry quantification. PC12 cells were terminally differentiated in NGF as previously described in Materials and Methods. Cells were kept in the presence of NGF (Control, C), or in absence of NGF for 2, 4, 6 (A), 15, 18, 24 and 48 hours(B). α-tubulin is a loading control. Minus sign represents withdrawal of NGF. Each bar represents an average of three independent experiments. Error bars indicate S.E (* $p < 0.05$).

Differential AT2 expression following NGF and NGF/AngII treatment of PC12 cells, reversed by addition of DPI and PD123177

AT2 expression following NGF treatment produced a slight decrease in transcript levels initially but expression increased for about one to two fold with NGF addition (Fig 21A). On the other hand, NGF/AngII-treated cells produced an earlier upregulation of AT2 receptor mRNA increasing transcript levels by six-fold on average (Fig. 21B). When earlier time points were investigated, AT2 increase in expression was detected as soon as 1h following NGF/AngII treatment (data not shown). Western blot analysis revealed a similar fold increase to the transcript response for both NGF and NGF/Ang II addition (Fig. 22 A&B). The upregulation of AT2 receptor in response to NGF/AngII treatment could in part explain the accelerated rate of PC12 cell differentiation observed in the previous study. When DPI and PD123177 inibitors were co-administered added with NGF/AngII, AT2 expression and protein synthesis were completely downregulated (Fig. 23).

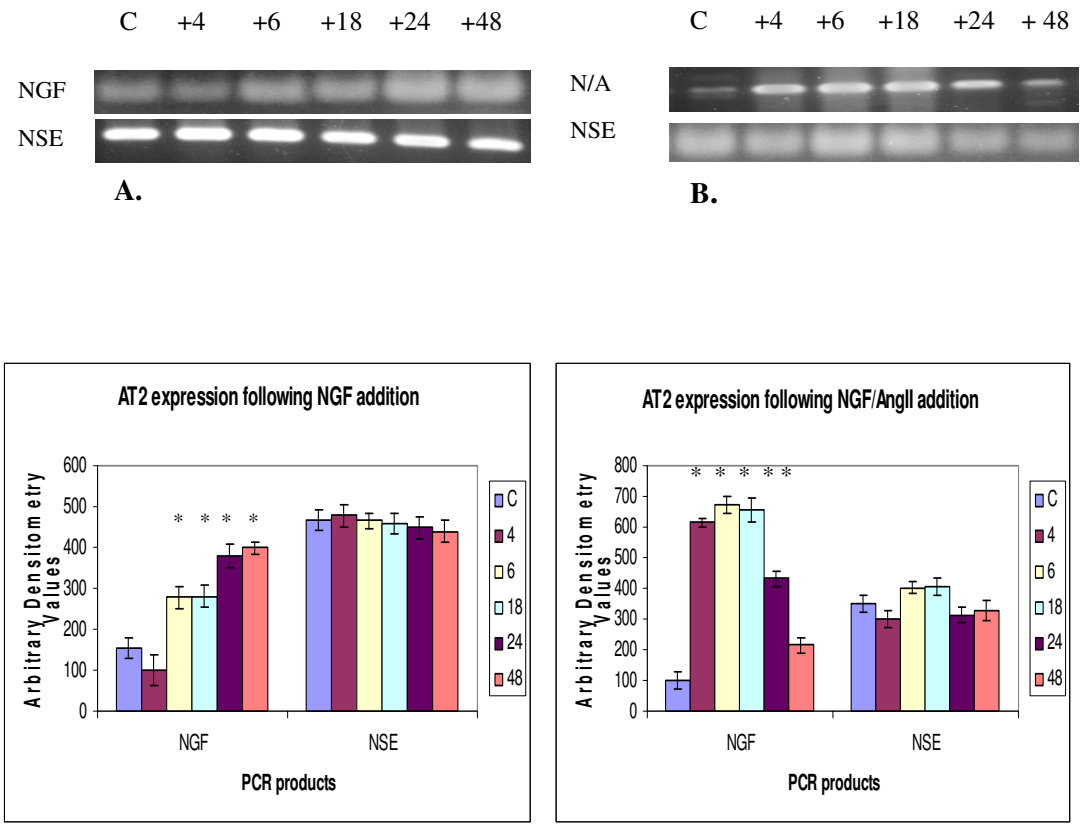


Figure 21. RT-PCR analysis of *AT2* expression in the presence of NGF or NGF and angiotensin II with corresponding densitometry quantification. PC12 cells were cultured as described in Materials and Methods. Twenty-four hours after seeding, cells were kept in regular F-12 K media (C, control) or treated with either NGF-supplemented F-12K (50 ng/ml) (A) or NGF (50 ng/ml) and angiotensin II (10 uM) (NGF/AngII) (B) for 4, 6, 18, 24, and 48 hours. Densitometry values are means of three independent experiments and represent values corrected for loading control (* $p < 0.05$). Equal loading control is Neuron Specific Enolase (NSE).

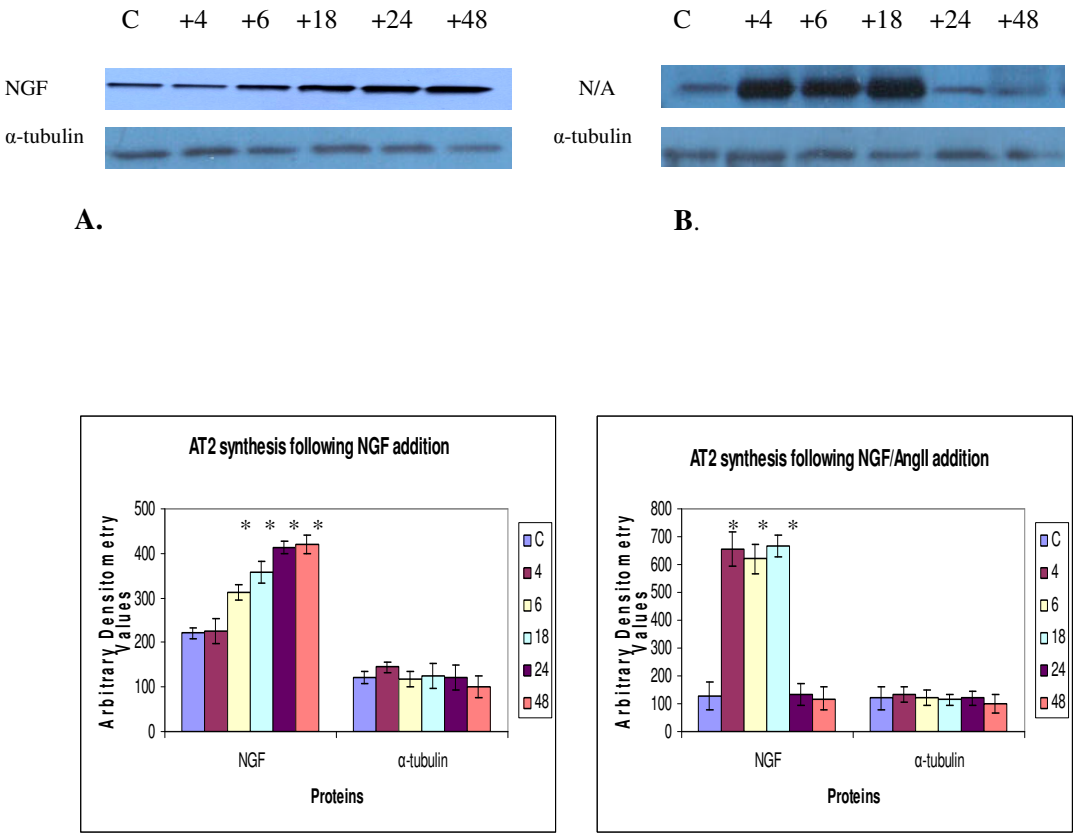


Figure 22. Western Blot analysis of AT2 receptor synthesis following NGF and NGF/AngII addition with corresponding densitometry analysis. PC12 cells were cultured as described in Materials and Methods. Twenty-four hours after seeding, cells were kept in regular F-12 K media (C, control) or treated with either NGF-supplemented F-12K (50 ng/ml) (A) or NGF (50 ng/ml) and angiotensin II (10 uM) (NGF/AngII) (B) for 4, 6, 18, 24, and 48 hours. Densitometry values are means of three independent experiments. Asterisk denotes statistical difference from the control. a-b: Values with different letters are significantly different. ($p < 0.05$). α-tubulin is a loading control.

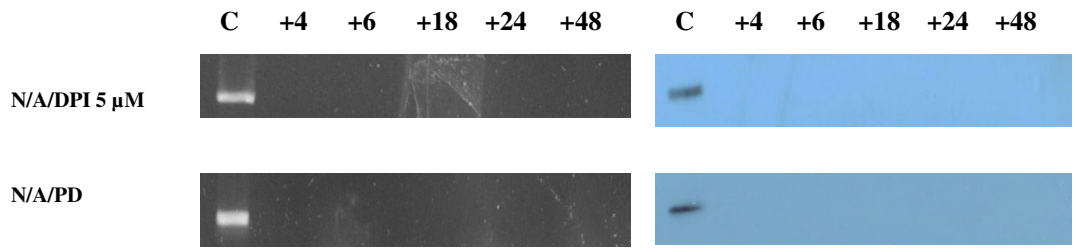


Figure 23. DPI and PD123177 downregulate AT2 expression and synthesis. PC12 cells were cultured as described in Materials and Methods. Twenty-four hours after seeding, cells were kept in regular F-12 K media (C, control) After 24 hours, the cells were either kept in regular F-12 K media (Cu, control) or treated with NGF/AngII /DPI 5 µM, or NGF/AngII /PD- supplemented media at different time-points. Two sets of plates per experimental group were prepared to collect RNA and proteins simultaneously. The PCR products were initially at 25 cycles, when repeated the cycles were increased to 30 cycles.

AT2 levels increase following NGF withdrawal from PC12 cells

Recent studies demonstrated that the AT2 receptor has an angiotensin II-dependent and independent roles in cellular apoptosis (Yamada et al., 2000; Miura and Karnik, 2000). The TUNEL assay and the DCF fluorescence results suggested a potential role for AT2 receptor activation following NGF withdrawal from terminally differentiated PC12 cells. To further elucidate possible angiotensin II-independent AT2 upregulation and protein synthesis following NGF removal, we performed RT-PCR and western blot analyses. AT2 transcripts were evident by 18h post-NGF withdrawal (Fig. 24). This suggests that AT2 receptors may play a role in the apoptotic effect following NGF withdrawal observed in the TUNEL assay.

Discussion

NGF/AngII -treated cells produce higher levels of c-jun transcript when compared to their NGF-treated counterparts

c-jun is an early response gene subject to regulation by external and internal stimuli (Angel and Karin, 1991). The results obtained so far have hinted toward a connection between c-Jun and AT2 receptors as two major regulators of apoptosis and differentiation. NGF addition significantly increased c-jun expression as well as protein levels. Previous reports linked the increase in c-jun levels to cellular differentiation (Leppa et al., 1998). In addition, NGF/AngII significantly upregulated c-jun transcript and produced a gradual increase in protein levels between the two experimental time

points of 2h and 4h post-treatment. Overexpression of c-jun in PC12 cells has been associated with enhanced induction of neurite growth, which suggests a link between c-jun overexpression and the accelerated rate of PC12 differentiation observed in the

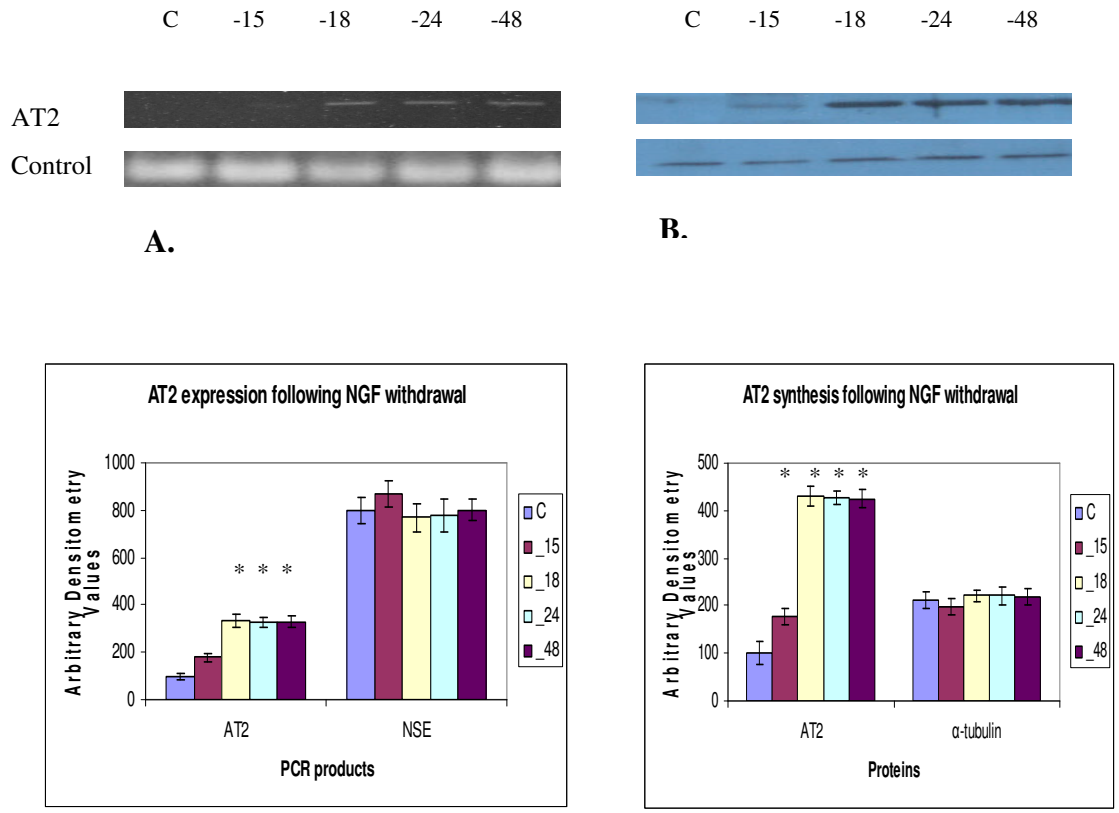


Figure 24. RT-PCR and western blot analyses of AT2 receptor following NGF removal from terminally differentiated PC12 cells. PC12 cells were terminally differentiated in NGF as previously described in Materials and Methods. Cells were kept in the presence of NGF (Control, C), or in absence of NGF for 15, 18, 24, and 48 hours (A) Neuron Specific Enolase is loading control for the RT-PCR. (B) α-tubulin is the loading control for the western blot. Error bars indicate S.E (* $p < 0.05$).

previous study (Dragunow et al., 1993). However, there are conflicting reports in the literature about the complete role of c-jun expression on stimulation of neuronal differentiation. One group argued that c-jun induction is necessary but not sufficient for a full activation of the neuronal differentiation cascade (Dragunow et al., 1993). In contrast, another report showed that NGF-dependent c-jun induction was enough to stimulate neurite sprouting in PC12 cells (Leppa et al., 1998). The discrepancy has been attributed to the type of PC12 cell clone used as one necessitates NGF and cAMP for differentiation, while the other requires only NGF (Dragunow et al., 1993).

c-Jun expression decreases shortly after NGF removal but remains high at later stages of apoptosis

Increased JNK activity has been correlated with neuronal death through phosphorylation of the N-terminal of c-Jun transcription factor (Carimalo et al., 2005). Withdrawal of NGF caused a decrease in c-jun expression at 4h but produced an upregulation of the gene at longer time points. This finding was puzzling as c-jun expression was expected to increase following NGF withdrawal while cells undergo apoptosis. Western blot analysis revealed similar enigmatic results. Phospho-Ser63 c-Jun paralleled the decrease depicted in the RT-PCR; however, total c-Jun levels showed a slow decrease. When shorter time points were performed, the western blot analysis depicted a decrease in phospho-Ser63 c-Jun starting after the first hour of NGF removal and lasting for about 4 hours. In fact, there are few reports in the literature that support these findings. c-jun expression in neurons undergoing apoptosis after hypoxia-ischemia and status epilepticus was delayed and prolonged. Indeed, those neurons produced a

substantial expression of c-Jun in their nuclei peaking at 24h after the insult, which did not totally agree with our results as total c-jun showed a steady increase up to the 48h time-course (Dragunow et al., 1993). The same group debated that the type of insult differentially upregulates certain immediate early-genes including c-jun. In addition, JNK activity increased twofold in sympathetic neurons following NGF removal and reached a peak at 4 and 8 hr after NGF withdrawal, the time at which it was reported that c-Jun protein levels and N-terminal phosphorylation started to increase (Rukenstein et al., 199; Ham et al., 1995). Our study looked at very early time points, and described an increase after the 4h time-point. However, the apoptosis signaling pathways of PC12 cells and sympathetic neurons differ. In differentiated PC12 cells, both JNK and p38 kinase activity increased after NGF withdrawal. However, in the case of sympathetic neurons there was no increase detected in p38 kinase activity (Eilers et al., 1998). Hence, the discrepancy is obviously tissue-related. The slow decrease after 60 min in band intensity detected (data not shown) reveals a possible degradation of the protein. The parallel response detected by RT-PCR might indicate that the c-jun system is switching from survival to apoptotic. When the survival signal is discontinued, c-jun expression is halted as well as the phosphorylation of the c-Jun protein. However, the transcription and c-Jun activation resumes when apoptotic signals are activated. The switch happens through ERK inactivation and JNK stimulation which was shown to peak at 4h followed by an increase in c-Jun proteins (Rukenstein et al., 1991).

NGF/AngII produces a premature AT2 expression and synthesis

AT2 receptors have been associated with cellular differentiation and apoptosis (Nakajima et al., 1995; Yamada et al., 1996). The addition of NGF produced an AT2 receptor upregulation over the course of the experiment which suggests that expression of AT2 receptors could be angiotensin II-independent and could be influenced by high availability of trophic factors, as is the case during embryogenesis (Nakajima et al., 1995, Yamada et al., 1996). However, administration of NGF/AngII produced an earlier upregulation of AT2 expression and synthesis. The precipitated upregulation of AT2 expression and synthesis might explain the accelerated rate of PC12 cell differentiation observed earlier. Angiotensin II increases cell surface receptor number by simply binding to its receptor (Csikos et al., 1998). AT2 ligands produce very slow dissociation which allows them to remain active for a period of time (Hein et al., 1997).

AT2 expression increases at a later stage of apoptosis at the mRNA and protein levels

Angiotensin II-dependent AT2 receptor implication in PC12 cell apoptosis has been well established and documented. Therefore, we systematically examined angiotensin II-independent AT2 receptor regulation (Yamada et al., 1996). In fact, a recent report pointed out toward possible angiotensin II-independent implication of these receptors in PC12 cell apoptosis (Miura and Karnik, 2000). Several lines of evidence suggests that induction of apoptosis is a constitutive function of AT2 receptor through p38 MAPK stimulation. Conversely, the effect of AT2 receptor on cellular

differentiation involves hindering of p38 MAPK signaling. In addition, it was reported that p38 MAPK activation is linked to the level of expression of AT2 receptor protein and not to activation of the AT2 receptor by the ligand (Miura and Karnik, 2000).

Chapter V

Conclusions and Future Directions

The broad significance of this research

Neurodegeneration is a big challenge for researchers and health care providers as many of the integrated underlying mechanisms are yet to be identified. There are a number of independent environmental and genetic risk factors proposed. None however were confirmed to be the definite perpetrators (Mayeux, 2003). Nevertheless, the oxidative stress theory has gained common ground in the etiology of neurodegeneration (Coyle and Puttfarcken, 1993). However, the machinery of oxidative stress has not been fully elucidated. On the other hand, adult neurogenesis is emerging as a promising therapy for brain degenerative diseases. In fact, neural stem cells were discovered in the adult brain and were found to be able to give rise to new neurons, astrocytes, and oligodendrocytes, in the same way as a developing brain (Reynolds and Weiss, 1992; Richards et al., 1992). Another significance for this type of research stems from the need to "reinforce synapses." The implication of this phenomenon ranges from using the brain model as a computer to neuronal adaptability and plasticity (Gage, 2002). Therefore, research pertaining to understanding neuronal differentiation as well as halting the onset of neurodegeneration is deemed valuable.

NADPH oxidase contributes to PC12 cells differentiation and apoptosis via oxidative stress

Here we present evidence that NADPH oxidase, through generation of reactive oxygen species (ROS), plays a dual role in neuronal-like PC12 cell differentiation and

apoptosis. The differentiation study has supplied many missing pieces to the puzzle concerning PC12 cell differentiation in relation to NADPH oxidase. First, it corroborated the emmerging reports pointing toward the implication of NADPH oxidase in PC12 cell differentiation. In fact, there are a number of recent published papers that link directly or indirectly NADPH oxidase-induced ROS to PC12 cell differentiation (Suzukawa et al., 2000, Li et al., 2006). Conversely, no one study has looked at the complete course of PC12 cell differentiation in relation to ROS production and gene expression of NADPH oxidase. The current study has identified the potential catalytic subunits involved in PC12 cell differentiation. Nox4 constitutive expression suggests that it is responsible for the basal-level of ROS production. Its activity however was reported to be inducible (Lambeth, 2004). The western blot analysis showed that p67-phox is present along with Nox4 during the short-term treatment with NGF. It is possible that NGF treatment leads to the production of p67-phox as a potential activator of the enzyme. When angiotensin II was added, Nox4 and p67-phox levels increased, however the major phenomenon occurring was the increase in Nox1 and p47-phox levels as a potential way to keep up with the increase demand for ROS production. The long-term treatment with NGF produced a parallel pattern of Nox4/p67-phox and Nox1/p47-phox increase. The long-term treatment with N/A expedited the synthesis of Nox1, Nox4 and p47-phox. The impact, however on p67-phox was less significant. Therefore, Nox4 and p67-phox may be involved in the initial process of PC12 cell differentiation and possibly in the production of basal ROS levels in unstimulated cells. Nox1 and p47-phox are more likely needed for the later and accelerated stage of differentiation. In fact, the combination of NGF and angiotensin II produced a need for an earlier employment of

Nox1 and p47 making these two subunits vital parts of the early stages of PC12 differentiation with NGF and angiotensin II co-treatment.

The apoptosis study has substantiated many data from experiments performed on sympathetic neurons in our laboratory, and provided novel findings about PC12 cell apoptosis that should be deemed useful for the understanding of many aspects of neurodegeneration. Indeed, NADPH oxidase is involved in PC12 cell apoptosis as it is implicated in sympathetic neuronal death (Tammariello et al., 2000, Hilburger et al., 2005). In addition, NADPH oxidase contributes to cell death by means of reactive oxygen species production. The cascade of events including the increase in NADPH oxidase synthesis with co-incubation of NGF and the enzyme activator angiotensin II, the enhancement of ROS production and the accelerated apoptosis are supportive of this finding. The decrease in ROS production concomitant with the decline in the number of apoptotic cells with the use of DPI furthers this strong relationship. In addition, the rapid and sustained increase in the protein levels of the enzyme following NGF-deprivation confirms that Nox1, p47 and p67 are the major participants in the apoptotic process.

PD123177 was able to diminish to basal levels DCF values when DPI at both concentrations failed to so. Although other investigators have found that AT2 receptors are engaged in the process of PC12 cell apoptosis (Horiuchi et al., 1997, Yamada et al., 1996), our findings, however furthered these data and implies that AT2 receptors in PC12 cell apoptosis may be independent of angiotensin II. In addition, the upregulation of AT2 receptors following NGF withdrawal and the PD123177-induced slower death rate even when angiotensin was added provide evidence that AT2 receptors are involved in both angiotensin II-dependent and -independent apoptosis of PC12 cells.

Future Directions

Although NADPH oxidase subunits involved in PC12 cell differentiation and apoptosis were potentially identified, we still need to gain further knowledge of the enzyme activation mechanisms and the kinetics of the catalytic subunits. The subunit kinetics might be responsible for the differential amount and rate of ROS produced in differentiation and apoptosis. According to our data, redox signaling is an integral part of both differentiation and apoptosis processes. The signaling pathways activated and possibly integrated still need to be identified. The differential ROS production whereby a second burst of ROS was detected in both studies points toward an upregulation of the ambient antioxidant system that presumably was insufficient to offset the high production of ROS in apoptosis and following angiotensin II addition in differentiation. Further analysis is needed to understand this phenomenon. The mechanisms of AT2 receptors are still not fully understood at the molecular levels, as they appear to be unusual G-Protein coupled receptors. The fact that NGF and angiotensin II exhibit a synergistic effect that was abolished by an AT2-antagonist demonstrates that there may be a signaling pathway cross talk between activated Trk and AT2 receptors. Further investigation is required in this area as some molecular signals could hold the key for successful neuronal differentiation or decelerated neurodegeneration. Finally, the effect of DPI or PD123177 on gene expression and protein synthesis of NADPH oxidase should be additionally studied. Preliminary data from RT-PCR analyses revealed Nox1 and Nox4 expression decreased following short-term DPI addition. Furthermore, c-jun expression decreased in a dose dependent manner. Analyzing the expression of NADPH

oxidase in response to those inhibitors will bring this research to a different level as many questions posed previously could be answered. In fact, controlling the enzyme's expression through redox control or use of inhibitors might prove extremely crucial for averting apoptosis.

References

Abo A., Pick E., Hall A., Totty N., Teahan C.G., Segal A.W. 1991. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature.* 353 (6345): 668-670.

Ago T., Kuribayashi F., Hiroaki H., Takeya R., Ito T., Kohda D., Sumimoto H. 2003. Phosphorylation of p47-phox directs phox homology domain from SH3 domain toward phosphoinositides, leading to phagocyte NADPH oxidase activation. *Proc Natl Acad Sci U S A.* 100 (8) : 4474-4479.

Ahsan H., Ali A., Ali R. 2003**.** Oxygen free radicals and systemic autoimmunity. *Clin Exp Immunol*. 31**:** 398-404.

Ames B.N., Shigenaga M.K., Hagen T.M. 1993**.** Oxidants, Antioxidants, and the Degenerative Diseases of Aging. *Proc. National Acad. Sc. U.S.A.* 90 (17): 7915-7922.

Angel P. and Karin M. 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta*. 1072 (2-3): 129–157.

Aoshima H., Satoh T., Sakai N., Yamada M., Enokido Y., Ikeuchi T., Hatanaka H. 1997. Generation of free radicals during lipid hydroperoxide-triggered apoptosis in PC12h cells. Biochim Biophys Acta. 1345 (1):35-42.

Arias J., Alberts A.S., Brindle P., Claret F.X., Smeal T., Karin M., Feramisco J., Montminy M. 1994**.** Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature.* 370 (6486): 226-229.

Arimura N. and Kaibuchi K. 2005. Key regulators in Neuronal Polarity. *Neuron*. 48 (6): 881-884.

Babior B.M. 1999. NADPH oxidase: an update. *Blood*. 93 (5) : 1464-1476.

Babior B.M., Lambeth J.D., Nauseef W. 2002. The Neutrophil NADPH Oxidase. *Archives of Biochemistry and Biophysics***.** 397 : 342-344.

Babior B.M. and Peters W.A. 1981. The O_2^--producing enzyme of human neutrophils: Further properties. *J. Biol Chem.* 256 : 2321- 2323.

Babcock G.T. 1999. How oxygen is activated and reduced in respiration. *Proc. Natl Acad. Sci. USA*. 96: 12971–12973.

Baehner R.L. and Nathan D.G. 1967. Leukocyte Oxidase: Defective Activity in Chronic Granulomatous Disease. *Science*. 17: 835-836.

Bainton D.F., **Ullyot J.L**., **Farquhar M.G**. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J. Exp Med.* 134 (4) : 907-934.

Bakhar A., Howell J.L., Paul C.E., Salehi A.H., Becker E.B., Said F. Bonni A., Barker P.A. 2003. Apoptosis induced by p75NTR overexpression requires Jun Kinase-dpendent phosphorylation of Bad. *J. Neurosc.* 23 (36):11373-11381.

Balcerczyk A., Soszynski M., Rybaczek D., Przygodzki T., Karowicz-Bilinska A., Maszewski J., Bartosz G. 2005. Induction of apoptosis and modulation of production of reactive oxygen species in human endothelial cells by diphenyleneiodonium. *Biochem Pharmacol*. 69 (8) : 1263-1273.

Bamji S.X., Majdan M., Pozniak C.D., Belliveau D.J., Aloyz R., Kohn J., Causing C.G., Miller F.D. 1998. The p75 Neurotrophin Receptor Mediates Neuronal Apoptosis and Is Essential for Naturally Occurring Sympathetic Neuron Death. *J. Cell Biol*. 140 (4): 911-923.

Banfi B., Clark R.A., Steger K., Krause K.H. 2003. Two Novel Proteins Activate Superoxide Generation by the NADPH Oxidase NOX1. *J. Biol. Chem*. 278 (6) : 3510-3513.

Banfi B., Maturana A., Jaconi S., Arnaudeau S., Laforge T., Sinha B., Ligeti E., Demaurex N., Krause K.H. 2000. A mammalian H^+ channel generated through alternative splicing of the NADPH oxidase homolog NOH-1. *Science.* 287 : 138-142.

Barbakadze T., Zhuravliova E., Sepashvili M., Zaalishvili E., Ramsden J.J., Bator J., Szeberenyi J., Mikeladze D. 2005. Production of homocysteine in serum-starved apoptotic PC12 cells depends on the activation and modification of Ras. *Neurosci Lett.* 391: 56-61.

Barde Y.A. 1989. Trophic factors and neuronal survival. *Neuron* 2: 1525– 1534.

Barker P.A. and Shooter E.M. 1994. Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. *Neuron.* 13**:** 203–215.

Barrett G.L and Bartlett P.F. 1994**.** The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proc Natl Acad Sci U S A*. 91 (14) : 6501-6505.

Batistatou A. and Greene L.A. 1993. Internucleosomal DNA cleavage and neuronal cell survival/death. *J. Cell Biol*. 722: 523-532.

Bazenet C.E., Mota M.A., Rubin L.L. 1998. The small GTP-binding protein Cdc42 is required for nerve growth factor withdrawal-induced neuronal death. *Proc Natl Acad Sci U S A*. 95 (7): 3984-3989.

Bedard K. and Krause K. 2007**.** The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology. *Physiol. Rev.* 87: 245-313.

Belliveau D.J., Krivko I., Kohn J., Lachance C., Pozniak C., Rusakov D., Kaplan D., Miller F.D. 1997. NGF and neurotrophin-3 both activate *trk*A on sympathetic neurons but differentially regulate survival and neuritogenesis. *J Cell Biol.* 136 : 375-388.

Berry C., Touyz R., Dominiczak A.F., Webb R.C., Johns D.G. 2001 Angiotensin receptors: signaling, vascular pathophysiology, and interactions with ceramide. *Am J Physiol Heart Circ Physiol*. 281 (6): H2337 – H2365.

Blume A., Herdegen T., Unger T. 1999. Angiotensin peptides and inducible transcription factors. *J Mol Med*. 77 (3) : 339-357.

Bobyn P.J., Franklin J.L., Wall C.M., Thornhill J.A., Juurlink B.H., Paterson P.G. 2002. The effects of dietary sulfur amino acid deficiency on rat brain glutathione concentration and neural damage in global hemispheric hypoxia-ischemia. *Nutr Neurosci*. 5 (6) : 407-416.

Boksay I., Boksay E., Reisberg B., Torossian C., Krishnamurthy M. 2005. Alzheimer's disease and medical disease conditions: a prospective cohort study**.** *J Am. Geriatr. Soc*. 53 (12): 2235-2236.

Bompard G., Sharp S.J., Freiss G., Machesky L.M. 2005. Involvement of Rac in actin cytoskeleton rearrangements induced by MIM-B. *J Cell Sci.* 118 (22) : 5393-5403.

Bottari S.P., De Gasparo M., Steckelings U.M., Levens N.R. 1993. Angiotensin II receptor subtypes: characterization, signaling mechanisms, and possible physiological implications. *Front Neuroendocrinol.* 14:123–171.

Braun-Menendez E., **Fasciolo J.C**., **Leloir L.F**., **Muñoz J.M**. 1940. The substance causing renal hypertension. *J Physiol*. 98 (3) : 283-298.

Bridges R.A., **Berendes H**., **Good R.A**. 1959. A fatal granulomatous disease of childhood; the clinical, pathological, and laboratory features of a new syndrome. *AMA J Dis Child*. 97 (4) : 387-408.

Bruckner S.R., Tammariello S.P., Kuan C.Y., Flavell R.A., Rakic P., Estrus S. 2001**.** JNK3 contributes to c-Jun activation and apoptosis but not oxidative stree in nerve growth factor-deprived sympathetic neurons. *J. Neurochem*. 78 : 298-303**.**

Burridge K. and Chrzanowska-Wodnicka M. 1996. Focal adhesions, contractility and signaling. *Annu Rev Cell Dev Biol.* 12 : 463–519.

Buytaert E., Callewaert G., Vandenheede J.R., Agostinis P. 2006. Deficiency in Apoptotic Effectors BAX and BAK Reveals an Autophagic Cell Death Pathway Initiated by Photodamage to the Endoplasmic Reticulum. *Autophagy.* 2 (3) : 238-240.

Carimalo J., Cronier S., Petit G., Peyrin J.M., Boukhtouche F., Arbez N., Lemaigre-Dubreuil Y., Brugg B., Miquel M.C. 2005. Activation of the JNK-c-Jun pathway during the early phase of neuronal apoptosis induced by PrP106-126 and prion infection. *Eur J Neurosci*. 21: 2311-2319.

Chabrashvili T., Kitiyakara C., Blau J., Karber A., Aslam S., Welch W.J., Wilcox C.S. 2003. Effects Of Ang II Type I And Type II Receptors On Oxidative Stress And Renal Nadph Oxidase And Sod Expression **.** *Am J Physiol Regul Integr Comp Physiol.* R117 – R124*.*

Chan J.H., Mellon E., C. N. Schanen, Twiss J.L. 2003**.** Persistent TrkA Activity Is Necessary to Maintain Transcription in Neuronally Differentiated PC12 Cells. *J. Biol. Chem*. 278 (44): 42877-42885.

Chang J.H., Mellon E., Schanen N.C., Twiss J.L. 2003. Persistent TrkA Activity Is Necessary to Maintain Transcription in Neuronally Differentiated PC12 Cells. *J. Biol. Chem.* 278: 42877-42885.

Chandra V., Pandav R., Dodge H.H., Johnston J.M., Belle S.H. 2001. Incidence of Alzheimer's disease in a rural community in India: the Indo-US study. *Neurology* 57 : 985–989.

Chanock S.J., El Benna J., Smith R.M., and Babior B.M. 1994. The respiratory burst oxidase *J. Biol. Chem*. 269 : 24519-24522.

Chao M.V. and Hempstead B.L. 1995. p75 and Trk: a two-receptor system. *Trends Neurosc.* 18 : 321–326.

Cheng G., Cao Z., Xu X., van Meir E.G., Lambeth J.D. 2001. Homologs of gp91phox cloning and tissue expression of Nox3, Nox4, and Nox5. *Gene.* 269 : 131–140.

Cheng G., Diebold B.A., Hughes Y., Lambeth J.D. 2006. Nox1-dependent reactive oxygen generation is regulated by Rac1. *J Biol Chem.* 281 (26) : 17718-17726.

Csikos T., Balmforth A.J., Grojec M., Gohlke P., Culman J., Unger T. 1998. Angiotensin AT_2Receptor Degradation Is Prevented by Ligand Occupation. *Biochem. Biophys. Res. Comm.* 243 (1) : 142-147.

Claret F.X., Hibi M., Dhut S., Toda T., Karin M. 1996. A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. *Nature.* 383 (6599) : 453-457.

Clement M.V., Ponton A., Pervaiz S. 1998. Apoptosis induced by hydrogen peroxide is mediated by decreased superoxide anion concentration and reduction of intracellular milieu. *FEBS Letters*. 440 (1-2) : 13-18.

Connolly J.L., Greene L.A., Viscarello R.R., Riley W.D. 1979. Rapid, Sequential Changes in Surface Morphology of PC12 Pheochromocytoma Cells in Response to Nerve Growth Factor. *J. Cell Bio*. 82 (3) : 820-827.

Cooper C., Patel R., Brookes P., Darley-Usmar V. 2002**.** Nanotransducers in cellular redox signaling: modification of thiols by reactive oxygen and nitrogen species. *Trends in Biochemical Sciences.* 27 (10) : 489-492.

Cory S. and Adams J.M. 2002. The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Reviews Cancer.* 2 : 647-656.

Coyle J.T. and Puttfarcken P. 1993. Oxidative stress, glutamate and neurodegenerative disorders. *Science.* 262 : 689–695.

Cross A.R. 2000. p40-phox participates in the activation of NADPH oxidase by increasing the affinity of p47-phox-phox for flavocytochrome b_{558}. *Biochem. J*. 349 : 113–117.

Cross A.R. and Curnutte J.T. 1995. The cytosolic activating factors p47-phoxphox and p67-phoxphox have distinct roles in the regulation of electron flow in NADPH oxidase. *J Biol Chem*. 270 (12) : 6543-6548.

Croteau D.L. and Bohr V.A. 1997. Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. *J. Biol. Chem.* 272 : 25409–25412.

Crowder R.J. and Freeman R.S. 1998. Phosphatidylinositol 3-Kinase and Akt Protein Kinase Are Necessary and Sufficient for the Survival of Nerve Growth Factor-Dependent Sympathetic Neurons**.** *J Neurosc*. 18 (8): 2933-2943.

Cutler R.G., Pedersen W.A., Camandola S., Rothstein J.D., Mattson M.P. 2002. Evidence that accumulation of ceramides and cholesterol esters mediates oxidative stress-induced death of motor neurons in ALS. *Ann. Neurol.* 52 : 448–457.

Dahl M.K. and Richardson T. 1978. Photogeneration of superoxide anion in serum of bovine milk and in model systems containing riboflavin and amino Acids. *J. Dairy Sc.* 61 **:** 400–407.

Datta S.R., Dudek H., Tao X., Masters S., Fu H., Gotoh Y., Greenberg M.E. 1997. Akt Phosphorylation of BAD Couples Survival Signals to the Cell-Intrinsic Death Machinery. *Cell*. 91 : 231-241.

De Coursey T.E., Morgan D., Cherny V.V. 2002. The gp91phox component of NADPH oxidase is not a voltage-gated proton channel. *J Gen Physiol* 120 : 773–779.

De Gasparo M., Catt K.J., Inagami T., Wright J.W., Unger T.H. 2000. The angiotensin II receptors. *Pharmacol Rev.* 52 : 415-72

De Leo F. and Quinn M. 1996**.** Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins**.** *J. Leukoc. Biol*. 60 : 677-691.

De Mendez I., Homayounpour N., Leto T. L. 1997. Specificity of p47-phoxphox SH3 domain interactions in NADPH oxidase assembly and activation. *Mol Cell Biol.* 17 (4): 2177–2185.

Del Tredici K., Rub U., De Vos R.A., Bohl J.R., Braak H. 2002. Where does Parkinson disease pathology begin in the brain? *J. Neuropathol. Exp. Neurol.* 61 : 413–426.

Delano F., Parks D., Ruedi J., Babior B., Schmid-Schönbein G. 2006. Microvascular Display of Xanthine Oxidase and NADPH Oxidase in the Spontaneously Hypertensive Rat. *Microcirculation.* 13 (7): 551-566.

Derijard B., Hibi M., Wu I.H., Barret T., Su B., Deng T., Karin M., Davis R.J. 1994. JNKI: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*. 76: 1025-1037.

Deshmukh M. and Johnson E.M. 1997. Programmed cell death in neurons: Focus on the pathway of Nerve Growth Factor deprivation-induced death of sympathetic neurons. *Mol. Pharmac.* 51 : 897-906.

Di Carlo A., Baldereschi M., Amaducci L., Lepore V., Bracco L. 2002. Incidence of dementia, Alzheimer's disease, and vascular dementia in Italy. The ILSA Study. *J. Am. Geriatr Soc*. 50 (1) : 41-48.

Dimmeler S., Rippmann V., Weiland U., Haendeler J., Zeiher A.M. 1997. Angiotensin II induces apoptosis of human endothelial cells. *Circ. Res.* 81: 970-976.

Dooneef G., Mirabello E., Bell K., Marder K., Stern Y., Mayeux R. 1992. An estimate of the incidence of depression in idiopathic Parkinson's disease. *Arch Neurol*. 49 : 305-307.

Dorseuil O., Vazquez A., Lang P., Bertoglio J., Gacon G., Leca G. 1992**.** Inhibition of superoxide production in B lymphocytes by rac antisense oligonucleotides. *J Biol Chem.* 267 (29) : 20540-20542.

Dragunow M., Young D., Hughes P., MacGibbon G., Lawlor P., Singleton K., Sirimanne E., Beilharz E., Gluckman P. 1993. Is c-Jun involved in nerve cell death following status epilepticus and hypoxic-ischaemic brain injury? *Mol. Brain Res.* 18 : 347–352.

Droge W. 2002. Free Radicals in the Physiological Control of Cell Function**.** *Physiological Reviews.* 82 (1) : 47-95.

Duke R.C. and Ojcius D.M. 1996. Cell Suicide In Health And Disease. *Scientific American.* 275 (6): 8733-8736.

Dusi S., Donini M., Rossi F. 1996. Mechanisms of NADPH oxidase activation: translocation of p40phox, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47-phoxphox or p67-phoxphox. *Biochem J*. 314 (Pt 2) : 409-412.

Dworakowski R., Anilkumar N., Zhang M., Shah A.M. 2006. Redox signaling involving NADPH oxidase-derived reactive oxygen species**.** *Biochem SocTrans*. 34: 960-964.

Dychtwald, K. *Age Wave: The challenges and opportunities of an aging North America*, St. Martins Press, New York, 1989.

Ebert B.L. and Bunn H.F. 1999. Regulation of the erythropoietin gene**.** *Blood*. 94 : 1864–1877.

Edwards S.N. and Tolkovsky A.M. 1994. Characterization of apoptosis in cultured rat sympathetic neurons after nerve growth factor withdrawal. *J. Cell Biol.* 124 : 537–546.

Eilers A., Whitfield J., Babij C., Rubin L. L., Ham J. 1998. Role of the Jun Kinase Pathway in the Regulation of c-Jun Expression and Apoptosis in Sympathetic Neurons. *J. Neurosc*. 18 (5) : 1713-1724.

El-Benna J., Dang P.M., Gougerot-Pocidalo M.A., Elbim C. 2005. Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses. *Arch Immunol Ther Exp.* 53 (3) : 199-206.

Elmore S. 2007. Apoptosis: A Review of Programmed Cell Death. *Toxic Pathol.* 35 (4) : 495–516.

Esther C.R., Marino E.M., Howard T.E. 1997. The critical role of tissue angiotensin-converting enzymes as revealed by gene targeting in mice. *J Clin Invest.* 99 : 2375-2385.

Fagan A.M., Zhang H., Landis S., Smeyne R.J., Silos-Santiago I., Barbacid M. 1996 TrkA, but not TrkC, receptors are essential for survival of sympathetic neurons *in vivo*. *J. Neurosc.* 16 : 6208-6218.

Fawthrop D.J., Boobis, A.R., Davies, D.S. 1991. Mechanisms of cell death. *Arch. Toxicol*. 65 : 437–444.

Finan P., **Koga H**., **Zvelebil M.J**., **Waterfield M.D**., **Kellie S**.1996. The C-terminal SH3 Domain of p67-phox^{phox} Binds its Natural Ligand in a Reverse Orientation. *J Mol Biol.* 261 (2): 173-180.

Fisher M., Meadows M.E., Do T., Weise J., Trubetskoy V., Charette M., Finklestein S.P. 1995**.** Delayed treatment with intravenous basic fibroblast growth factor reduces infarct size following permanent focal cerebral ischemia in rats. *J Cereb Blood Flow Metab*. 15 (6) : 953-959.

Forman H.J., Torres M., and Fukuto J. 2002. Redox signaling. *Mol Cell Biochem* 234–235 (1-2) : 49–62.

Franke T. F., Yang S. I., Chan T.O., Datta K., Kazlauskas A., Morrison D.K., Kaplan D.R., Tsichlis P.N. 1995**.** The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*. 81 (5) :727-736.

Fratiglioni L., Launer L.J., Andersen K., Breteler M.M., Copeland J.R., Dartigues J., Lobo A., Martinez-Lage J., Soininen H., Hofman A. 2000. Incidence of dementia and major subtypes in Europe: A collaborative study of population-based cohorts. *Neurology.* 54 (11 Suppl 5) : S10-15.

Freeman J.L., Abo A., Lambeth J.D. 1996. Rac "insert region" is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65. *J Biol Chem*. (33) : 19794-19801.

Freeman J.L. Kreck M.L., Uhlinger D.J., Lambeth J.D. 1994. Ras Effector-Homologue Region on Rac Regulates Protein Associations in the Neutrophil Respiratory Oxidase Burst Complex. *Biochemistry*. 33: 13431 – 13435.

Freeman J.L. and Lambeth J.D. 1996. NADPH oxidase activity is independent of p47-phox-phox *in vitro*. *J. Biol. Chem*. 271 : 22578-22585.

Freeman R.S., Burch R.L., Crowder R.J., Lomb D.J., Schoell M.C., Straub J.A., Xie L. 2004. NGF deprivation-induced gene expression: after ten years, where do we stand? *Prog Brain Res.* 146: 111-126. Review.

Fridovich I. 1989. Superoxide dismutases. An adaptation to a paramagnetic gas. *J. Biol. Chem*. 264: 7761–7764.

Fridovich I. 1999. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann. N. Y. Acad. Sci*. 893: 13–18.

Fujita, K., Lazarovici, P. and Guroff, G. 1989. Regulation of the differentiation of PC12 pheochromocytoma cells. *Environ. Health Perspect.* 80 : 127–142.

Gabig G. and Lefker B.A. 1985. Activation of the human neutrophil NADPH oxidase results in coupling of electron carrier function between ubiquinone-10 and cytochrome b559. *J. Biol. Chem*. 260 : 3991-3995.

Galbiati F., Volonte D., Gil O., Zanazzi G., Slazer J., Sargiacomo M., Scherer P., Engleman J., Sclegel A., Parenti., M., Okamoto T., Lisanti M. 1998. Expression of caveolin-1 and -2 in differentiating PC12 cells and dorsal root ganglion neurons: Caveolin-2 is up-regulated in response to cell injury. *Proc Natl Acad Sci USA*. 95: 10257-10262.

Galbusera C., **Orth P**., **Fedida D**., **Spector T**. 2006**.** Superoxide radical production by allopurinol and xanthine oxidase. *Biochem Pharmacol.* 71 (12) : 1747-1752.

Gallinat S., Yu M., Dorst A., Unger T., Herdegen T. 1998. Sciatic nerve transection evokes lasting up-regulation of angiotensin AT2 and AT1 receptor mRNA in adult rat dorsal root ganglia and sciatic nerves. *Brain Res Mol Brain Res.* 57 : 111–122.

Geiszt M., Dagher M., Molnár G., Havasi A., Faure J., Paclet M., Morel F., Ligeti E. 2001. Characterization of membrane-localized and cytosolic Rac-GTPase-activating proteins in human neutrophil granulocytes: contribution to the regulation of NADPH oxidase. *Biochem J*. 355 (3) : 851–858.

Geiszt M., Kopp J., Varnai P., Leto T. 2000**.** Identification of Renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U.S.A*. 97 (14) : 8010–8014.

Geiszt M., Lekstrom K., Brenner S., Hewitt S.M., Dana R., Malech H.L., Leto T.L. 2003. NAD(P)H oxidase 1, a product of differentiated colon epithelial cells, can partially replace glycoprotein 91phox in the regulated production of superoxide by phagocytes. *J Immunol*. 171 (1) : 299-306.

Geiszt M., Lekstrom K., Witta J., Leto T.L. 2003. Proteins homologous to p47-phoxphox and p67-phoxphox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. *J Biol Chem*. 278 (22) : 20006-20012.

Gendron L., Payet M.D., Gallo-Payet N. 2003. The angiotensin II type 2 receptor of angiotensin II and neuronal differentiation: from observations to mechanisms. *J. Mol. Endocrin*. 31 : 359-372.

Glebova N.O. and Ginty D.D. 2004. Heterogeneous requirement of NGF for sympathetic targets innervation *in vitro. J neurosc.* 24 (3) : 743-751.

Ghadge G.D., Lee J.P., Bindokas V.P., Jordan J., Ma L., Miller R.J., Roos R.P. 1997. Mutant superoxide dismutase-1-linked familial amyotrophic lateral sclerosis: molecular mechanisms of neuronal death and protection. *J Neurosci.* 17 (22): 8756-8766.

Goldblatt H., Lynch J., Hanzal R.F., Summerville W.W. 1934. Studies on experimental hypertension, I: the production of persistent elevation of systolic blood pressure by means of renal ischemia. *J Exp Med.* 59 : 347–379.

Gorin Y., Block K., Hernandez J., Bhandari B., Wagner B., Barnes J., Abboud E. 2005. Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. *J Biol Chem*. 280 (47) : 39616-39626.

Gotz R., Karch C., Digby M.R., Troppmair J., Rapp U.R., Sendtner M. 2000. The neuronal apoptosis inhibitory protein suppresses neuronal differentiation and apoptosis in PC12 cells. *Hum Mol Genet*. 9 (17): 2479-2489.

Gorelick P.B. 1995. Stroke prevention. *Arch. Neurol.* 52 : 347–355.

Gorman A.M., **McGowan A**., **O'Neill C**., **Cotter T**. 1996**.** Oxidative stress and apoptosis in neurodegeneration. *J Neurol Sci.* 139 : 45-52.

Gorzalczany Y., **Sigal N**., **Itan M**., **Lotan O**., **Pick E**. 2000. Targeting of Rac1 to the phagocyte membrane is sufficient for the induction of NADPH oxidase assembly. *J Biol Chem*. 275 (51) : 40073-40081.

Gottlieb D. I. 2002. Large-scale sources of neural stem cells. *Annu Rev Neurosc.* 25: 381-407.

Gotz R. 2000. Regulation of neuronal cell death and differentiation by NGF and IAP family members. *J Neural Transm Suppl.* (60): 247-59.

Grady E.F., Sechi L.A., Griffin C.A., Schambelan M., Kalinyak J.E. 1991. Expression of AT2 receptors in the developing rat fetus. *J Clin Invest.* 88 : 921–933.

Greene L.A. and Tischler A.S. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA*. 73 (7) : 2424-2428.

Greenlund L.J.S., Deckwerth T.L., Johnson E. M. Jr. 1995. Superoxide Dismutase delays neuronal apoptosis: Role of reactive oxygen species in programmed neuronal death. *Neuron.* 14: 303-315.

Griendling K. 2006. NADPH Oxidases: New Regulators of Old Functions *Antioxidants & Redox Signaling*. 8 (9-10) : 1443-1445.

Greindling K., Lassegue B., Alexander R. 1996. Angiotensin Receptors and Their Therapeutic Implications. *Ann. Rev. of Pharm. and Toxic*. 36 : 281-306.

Griendling K., Sorescu D., Lassègue B., Ushio-Fukai M. 2000. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arteriosc. Thromb. Vasc. Biol.* 20 (10) : 2175-2183.

Groemping Y., Lapouge K., Smerdon S.J., Rittinger K. 2003. Molecular Basis of Phosphorylation-Induced Activation of the NADPH Oxidase. *Cell*. 113 (3) : 343-355.

Guan J. L. 1997. Role of focal adhesion kinase in integrin signaling. *Int. J. Biochem Cell Biol.* 29: 1085-1096.

Gunther S. 1984**.** Characterization of angiotensin II receptor subtypes in rat liver. *J Biol Chem.* 259 (12) : 7622-7629.

G**uroff G**. 1985. PC12 cells as a model of neuronal differentiation. JE editor. New York.

Gusev E. **and Skvortsova V.I.** 2003. Brain Ischemia. Kluwer Academic/Plenum Publisher. New York.

Gutteridge J.M., and Halliwell B. 2000. Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann N Y Acad Sci*. 899 : 136-147.

Guzik T. J., **Sadowski J**., **Guzik B**., **Jopek A**., **Kapelak B**., **Przybylowski P**., **Wierzbicki K**., **Korbut R**., **Harrison D. G**., **Channon K. M**. 2006. Coronary artery superoxide production and nox isoform expression in human coronary artery disease. *Arterioscler Thromb Vasc Biol.* 26 (2) : 333-339.

Halliwell B. and Gutteridge J.M.C. 2006. Free Radicals in Biology and Medicine, 4th edn, Oxford University Press, Oxford.

Halliwell B. and Whiteman M. 2004. Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? *Br. J. Pharmacol.* 142: 231–255.

Ham J., Babij C., Whitfield J., Pfarr C.M., Lallemand D., Yaniv M., Rubin L.L. 1995. A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron*. 14 : 927-939.

Ham J., Eilers A., Whitfield J., Neame S.J., Shah B. 2000**.** c-Jun and the transcriptional control of apoptosis. *Biochem. Pharamac*. 60 : 1015-1021.

Harrington A.W., Kim J.Y., Yoon S.O. 2002. Activation of Rac GTPase by p75 in necessary for c-jun N-terminal kinase-mediated apoptosis. *J. Neurosci.* 22 : 156–166.

Han D., Antunes F., Canali R., Rettori D., Cadenas E. 2003. Voltage-dependent anion channels control the release of superoxide anion from mitochondria to cytosol. *J Biol Chem* 278 : 5557–5563.

Hebert L.E., **Scherr P.A**., **Bienias J.L**., **Bennett D.A**., **Evans D.A**. 2003. Alzheimer disease in the US population: prevalence estimates using the 2000 census**.** *Arch Neuro* . 60 (8) : 1119-1122.

Hein L., Barsh G.S., Pratt R.E., Dzau V.J., Kobilka B.K. 1995. Behavioral and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature.* 377 : 744–747.

Helin K., Stoll M., Meffert S., Stroth U., Unger T. 1997. The role of angiotensin receptors in cardiovascular diseases. *Ann Med*. 29:23–29.

Henderson L.M. and Chappell. J. B. 1996. NADPH oxidase of neutrophils. *Biochim. Biop/ro. Acta Bio-Energetics.* 1273: 87-107.

Hengartner M.O. 2000. The biochemistry of apoptosis. *Nature*.407:770-776.

Herdegen T., Claret F.X., Kallunki T., Martin-Villalba A., Winter C., Hunter T., Karin M. 1998. Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. *J. Neurosci*. 18 (14): 5124-5135.

Heyworth P.G., Curnutte J.T., Nauseef W.M., Volpp B.D., Pearson D.W., Rosen H., Clark R.A. 1991. Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox-phox and p67-phox-phox requires interaction between p47-phox-phox and cytochrome b558. *J Clin Invest.* 87 (1) : 352-356.

Hilburger E., Conte E., McGee D.W., Tammariello S.P. 2005. Localization of NADPH oxidase subunits in neonatal sympathetic neurons. *Neuroscience Letters*. 377 (1):16-19.

Hilburger E. 2004. NADPH oxidase and oxidative stress in sympathetic neuronal apoptosis. Thesis.

Hilenski L.L., Clempus R.E., Quinn M.T., Lambeth J.D., Griendling K.K. 2004. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 24 : 677–683.

Hollenberg N.K., Fisher N.D.L., Price D.A. 1998. Pathways for angiotensin II generation in intact human tissue. Evidence from comparative pharmacological interruption of the renin system. *Hypertension* 32: 387-392.

Horiuchi M., Hayashida W., Kambe T., Yamada T., Dzau V. 1997. Angiotensin II Type 2 receptor dephosphorylates Bcl-2 by activating Mitogen- activated Protein kinase Phosphatase-1 and Induces apoptosis. *J. Bioch Chem.* 272 : 19022-19026.

Hoyle G. 2003. Neurotrophins and lung disease. *Cytokine & Growth Factor Review.* 14 (6) : 551-558.

Huang J. and Kleinberg M.E. 1999. Activation of the Phagocyte NADPH Oxidase Protein p47-phox^{phox} :Phosphorylation Controls SH3 Domain-Dependent Binding to p22^{phox} . *J Biol Chem*. 274 (28) : 19731-1973.

Ibi M., **Katsuyama M**., **Fan C**., **Iwata K**., **Nishinaka T**., **Yokoyama T**., **Yabe-Nishimura C**. 2006. NOX1/NADPH oxidase negatively regulates nerve growth factor-induced neurite outgrowth. *Free Radic Biol Med.* 40 (10) : 1785-95.

Imanishi T., Hano T., Nishio I. 2005. Angiotensin II accelerates endothelial progenitor cell senescence through induction of oxidative stress. *J. Hypertens.* 23: 97–104.

Inoue I., Rohrwasser A., Helin C., Jeunemaitre X., Crain P., Bohlender J., Lifton R.P., Lalouel J.M. 1997. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription *in vitro*. *J Clin Invest.* 99 : 1786–1797.

Ito T., Nakamura R., Sumimoto H., Takeshige K., and Sakaki Y. 1996. An SH3 domain-mediated interaction between the phagocyte NADPH oxidase factors p40phox and p47-phox. *FEBS Lett.* 385 : 229-232.

Jenkins R. and Hunt S.P. 1991. Long-term increase in the levels of c-Jun mRNA and Jun protein-like immunoreactivity in motor and sensory neurons following axon damage. *Neurosci. Lett.* 129 : 107–110.

Jenkins R., Tetzlaff W., Hunt S.P. 1993. Differential Expression of Immediate Early Genes in Rubrospinal Neurons Following Axotomy in Rat. Europ. *J. Neurosc*. 5 (3) : 203-209.

Jerusalem F., Pohl C., Karitzky J., Ries F. 1996. ALS. *Neurology.* 47 (6 Suppl 4): S218 - S220.

Jiang H., Guo W., Liang X., Rao Y. 2005. Both the Establishment and the Maintenance of Neuronal Polarity require Active Mechanisms Critical Roles of GSK-3B and its Upstream Regulators. *Cell*. 120 (1) : 123-135.

Johnson D., Lanahan A., Buck R., Sehgal A., Morgan C., Mercer E., Bothwell M., Chao M. 1986. Expression and structure of the human NGF receptor. *Cell*. 47 (4) : 545-554.

Johnson E. and Yip H. 1985.Central nervous system and peripheral nerve growth factor provide trophic support critical to mature sensory neuronal survival. *Nature.* 314 : 751 – 752.

Joneson, **T. and D**. **Bar-Sagi**. 1998. A Rac1 effector site controlling mitogenesis through superoxide production. *J. Biol. Chem.* 273 (29): 17991-17994.

Joo J.H, Wang S., Chen J.G., Jones A.M., Fedoroff N.V. 2005. Different Signaling and Cell Death Roles of Heterotrimeric G Protein α and β Subunits in the Arabidopsis Oxidative Stress Response to Ozone. *Plant Cell*. 17 (3) : 957–970.

Jordan J., Galindo M.F., Calvo S., Gonzalez-Garcia C., Cena V. 2000. Veratridine induces apoptotic death in bovine chromaffin cells through superoxide production. *Br. J. Pharmacol.* 130 (7) : 1496-1504.

Joseph G., Gorzalczany Y, Koshkin V, Pick E. 1994**.** Inhibition of NADPH oxidase activation by synthetic peptides mapping within the carboxyl-terminal domain of small GTP-binding proteins. Lack of amino acid sequence specificity and importance of polybasic motif. *J Biol Chem*. 269 (46) : 29024-29031.

Kamata H., Tanaka C., Yagisawa H., Matsuda S., Gotoh Y., Nishida E., Hirata H. 1996. Suppression of Nerve Growth Factor-induced Neuronal Differentiation of PC12 Cells. *N*-Acetylcysteine Uncouples The Signal Transduction From Ras To The Mitogen-Activated Protein Kinase Cascade**.** *J. Biol. Chem.* 271 : 33018-33025.

Kambayashi Y., Bardhan S., Takahashi K., Tsuzuki S., Inui H., Hamakubo T., Inagami T. 1993. Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. *J Biol Chem*. 268: 24543–24546.

Kanazawa I. 2001. How do neurons die in neurodegenerative diseases**?** *Trends in Molecular Medicine*. 7 (8): 339-344.

Kaplan D., Martin-Zanca D., Parada L. 1991. Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature* 350 : 158–160.

Kaplan D.R. and Miller F.D. 1997. Signal transduction by the neurotrophin receptors**.** *Curr. Opin. Cell Biol.* 9**:** 213221.

Karathanassis D., Stahelin R.V., Bravo J., Perisic O., Pacold C.M., Cho W., Williams R.L. 2002. Binding of the PX domain of p47-(phox) to phosphatidylinositol 3,4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction. *EMBO J*. 21 (19) : 5057-5068.

Karin M. 1995.The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* 270:16483–16486.

Katsuyama M., Fan C., Arakawa N., Nishinaka T., Miyagishi M., Taira K., Yabe-Nishimura C. 2005. Essential role of ATF-1 in induction of NOX1, a catalytic subunit of NADPH oxidase: involvement of mitochondrial respiratory chain. *Biochem J.* 386 (2): 255-261.

Kellogg E.W. and Fridovich I. 1975. Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system. *J. Biol. Chem*. 250: 8812-8817.

Kerr J.F., Wyllie A.H., Currie A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer.* 26 (4): 239-257.

Kesavapany S., Li B. S., Pant H. C. 2003. Cyclin-Dependent Kinase 5 in Neurofilament Function and Regulation. *Neurosignals*. 12 : 252-264.

Kim B.C., Yi J.Y., Yi S.J., Shin I.C., Ha K.S., Jhun B.H., Hwang S.B., Kim J.H. 1998. Rac GTPase activity is essential for EGF-induced mitogenesis. *Mol Cells.* 8 (1) : 90-95.

Kim M.J., Kim E., Ryu S.H., Suh P.G. 2000**.** The mechanism of phospholipase C-g1 regulation. *Experimental and Molecular Medicine*. 32 (3) : 101-109.

Kim J.R., Yoon H.W., Kwon K.S., Lee S. R., Rhee S.G. 2000. Identification of proteins containing cysteine residues that are sensitive to oxidation by hydrogen peroxide at neutral pH. *Anal Biochem.* 283 : 214–221.

Kimball R.A. and Saier M.H. 2002.Voltage-gated H^+ channels associated with human phagocyte superoxide-generating NADPH oxidases: sequence comparisons, structural predictions, and phylogenetic analyses. *Mol Memb Biol.* 19 : 137–147.

Klesse L.J., Meyers K.A., Marshall C.J., Parada L.F. 1999. Nerve growth factor induces survival and differentiation through two distinct signaling cascades in PC12 cells. *Oncogene.* 18 (12) : 2055-2068**.**

Knight J.A. 2000. Review: Free radicals, antioxidants, and the immune system *Ann Clin Lab Sci.* 30 : 145-158.

Kobayashi S., Nominal Y., Shibuya M., Mara Y. 2004. Nox1 regulates apoptosis and potentially stimulates branching morphogenesis in sinusoidal endothelial cells. *Experimental Cell Research.* 300 (2) : 455-462.

Kohn J., Causing C. G., Miller F. D. 1998. The p75 Neurotrophin Receptor Mediates Neuronal Apoptosis and Is Essential for Naturally Occurring Sympathetic Neuron Death. *J. Cell Biol*. 140 (4) : 911-923.

Krause K.H. 2004. Tissue Distribution and Putative Physiological Function of NOX Family NADPH Oxidases. *J. J. Infect*. *Dis*. 57 : S28-S29.

Kreck M.L., Freeman J.L., Abo A., Lambeth J.D. 1996. Membrane association of Rac is required for high activity of the respiratory burst oxidase *Biochemistry.* 35: 15683-15692.

Kreck M.L., Uhlinger D.J., Tyagi S.R., Inge K.L. Lambeth J.D. 1993 Participation of the small molecular weight GTP binding protein Rac1 in cell-free activation and assembly of the respiratory burst oxidase: Inhibition by a C-terminal Rac peptide. *J. Biol. Chem*. 269 : 4161-4168.

Kruman I.I., Pedersen W.A., Springer J.E., Mattson M.P. 1999. ALS-linked Cu/Zn-SOD mutation increases vulnerability of motor neurons to excitotoxicity by a mechanism involving increased oxidative stress and perturbed calcium homeostasis. *Exp. Neurol.* 160 : 28–39.

Kuan C.Y. and Burke R.E. 2005. Targeting the JNK Signaling Pathway for Stroke and Parkinson's Diseases Therapy. *CNS & Neurological Disorders.* 4 (1): 63-67.

Kuan C.Y., Yang D.D., Samanta Roy D.R., Davis R.J., Rakic P. Flavell R.A. 1999. The Jnk1 and Jnk2 kinases are required for regional specific apoptosis during early brain development. *Neuron* 22: 667–676.

Kuribayashi F., Nunoi H., Wakamatsu K., Tsunawaki S., Sato K., Ito T., Sumimoto H. 2002. The adaptor protein p40(phox) as a positive regulator of the superoxide-producing phagocyte oxidase. *EMBO J*. 21 (23) : 6312-6320.

Kurth T., Gaziano J.M., Berger K., Kase C.S., Rexrode K.M., Cook N.R., Buring J., J.E. Manson. 2002. Body mass index and the risk of stroke in men. *Arch. Intern. Med.* 162 : 2557–2562.

Kyriakis J.M., **Banerjee P**., **Nikolakaki E**., **Dai T**., **Rubie E.A**., **Ahmad M.F**., **Avruch J**., **Woodgett JR**. 1994. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature.* 369 : 156–160.

Lafuse W. P., Alvarez G. R., Zwilling B. S. 2000. Regulation of Nramp1 mRNA stability by oxidants and protein kinase C in RAW264.7 macrophages expressing Nramp1 (Gly169). *Biochem J.* 351 (Pt 3) : 687– 696.

Laloi C., Apel K., Danon A. 2004. Reactive oxygen signaling: the latest news. *Curr. Opin. Plant Biol.* 7: 323–328.

Lambeng N., Michel P.P., Brugg B. Agid Y., Ruberg M. 1999. Mechanisms of apoptosis in PC12 cells irreversibly differentiated with nerve growth factor and cAMP. *Brain research*. 821 : 60-68.

Lambeth J.D. 2004. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol*. 4 (3) : 181-189.

Lambeth J.D., Cheng G., Arnold R.S., Edens W.E. 2000. Novel homologs of gp91phox. *Trends Biochem. Sci.* 25 : 459–461.

Lassegue B. and Clempus R.E. 2003.Vascular NAD(P)H oxidases: specific features, expression and regulation . *Am J Physiol Lung Regul Integr Comp Physiol*. 285 : R277-297.

Lassegue B., Sorescu D., Szocs K., Yin Q.Q., Akers M., Zhang Y., Grant S.L., Lambeth, J.D., Griendling K.K. 2001. Novel $gp91^{phox}$ homologues in vascular smooth muscle cells: Nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circulation Research.* 88 (9) : 888-894.

Laufs U., Wassmann S., Czech T., Munzel T., Eisenhauer M., Bohm M., Nickenig G. 2005. Physical inactivity increases oxidative stress, endothelial dysfunction, and atherosclerosis. *Arterioscler Thromb Vasc Biol* 25 : 809–814.

Lavigne M. C., Malech H. L., Holland S. M., Leto T. L. 2001. Genetic demonstration of p47phox-dependent superoxide anion production in murine vascular smooth muscle cells. *Circulation* 104 : 79 – 84.

Lebrun C. J., Blume A., Herdegen T., Seifert K., Bravo R., Unger T. 1995. Angiotensin II induces a complex activation of transcription factors in the rat brain: expression of Fos, Jun and Krox proteins. *Neuroscience.* 65 (1) : 93-99.

Lee J.M., Zipfel G.J., Choi D.W. 2001. The changing landscape of ischemic brain injury mechanisms. *Nature.* 399: A7–A14.

Lee S.B., Bae I.H., Bae Y.S., Um H.D. 2006. Link between Mitochondria and NADPH Oxidase 1 Isozyme for the Sustained Production of Reactive Oxygen Species and Cell Death. *J. Biol. Chem*. 281 (47): 36228-36235.

Lehninger A.L. and Kennedy E.P. 1948. Oxidation of fatty acids and tricarboxylic acid cycle intermediates by isolated rat liver mitochondria. *J. Biol. Chem*. 179: 957–963.

Lenkei Z., Palkovits M., Corvol P., Llorens-Cortes C. 1997. Expression of angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNAs in the adult rat brain: a functional neuroanatomical review. *Front Neuroendocrin.* 18 : 383–439.

Leppa S., Saffrich R., Ansorge W., Bohmann D. 1998. Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. *EMBO J.* 17 : 4404–4413.

Leto Thomas L.L., Adams A. G., De Mendez I. 1994. Assembly of the phagocyte NADPH oxidase: Binding of Src homology 3 domains to praline-rich targets. *Proc. Natl. Acad. Sci., USA*. 91 : 10650-10654.

Leusen J.H., Bolscher B.G., Hilarius P.M., Weening R.S., Kaulfersch W., Seger R.A., Roos D., and Verhoeven A.J. 1994. 156Pro-->Gln substitution in the light chain of cytochrome b558 of the human NADPH oxidase (p22-phox) leads to defective translocation of the cytosolic proteins p47-phox and p67-phox *J. Exp. Med.* 180 : 2329-2334.

Levi-Montalcini R. and Angeletti P.U. 1968. Nerve growth factor. *Physiol Rev*.48 : 534-569.

Levi-Montalcini R., Caramia F., Luse S.A., Angeletti P.U. 1968. In vitro effects of the nerve growth factor on the fine structure of the sensory nerve cell. *Brain Res.* 8 : 347-62.

Levi-Montalcini R. 1987. The nerve growth factor 35 years later. *Science*. 237 : 1154–1162.

Li J. M., Mullen A. M., Yun S., Wientjes F., Brouns G. Y., Thrasher A. J., Shah A. M. 2002. Essential role of the NADPH oxidase subunit p47-phox in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor-*Circ Res.* 90 : 143–150.

Li J., Stouffs M., Serrander L., Banfi B., Bettiol E., Charnay Y., Steger K., Krause K., Jaconi M. 2006**.** The NADPH Oxidase NOX4 Drives Cardiac Differentiation: Role in Regulating Cardiac Transcription Factors and MAP Kinase Activation *Mol. Biol. Cell.* 17 (9) : 3978-3988.

Li Q., Clark S., Lewis, Darrell V., Wilson, W.A. 2002**.** NMDA Receptor Antagonists Disinhibit Rat Posterior Cingulate and Retrosplenial Cortices: A Potential Mechanism of Neurotoxicity *J. Neurosci.* 22 : 3070-3080.

Lindwall C., Fothergill T., Richards L. J. 2007. Commissure formation in the mammalian forebrain. *Curr. Opinion Neurobio*. 17 (1) : 3-14.

Liu R.Y. and Snider W.D. 2001. Different signaling Pathways Mediate Regenerative versus developmental Sensory Axon Growth. *J. Neurosc*. 21 (RC164) : 1-5.

Lo Y., Johnson M. S., Cruz T. F. 1996. Reactive Oxygen Species Mediate Cytokine Activation of c-Jun NH_2-terminal Kinases. *J Biol Chem*. 271 : 15703-15707.

Lodish H., Berk A., Zipursky L., Matsudaira P., Baltimore D. and Darnell J. Molecular Cell Biology. 4th, 2000, W.H. Freeman.

Lucius R., Gallinat S., Rosentiel P., Herdegen T., Sievers J., Unger T. 1998. The angiotensin II type 2 (AT2) receptor promotes axonal regeneration in the optic nerve of adult rats. *J. Exp. Med.* 188 : 661-670.

Lynch R.E. and Fridovich I. 1978. Permeation of the erythrocyte stroma by superoxide radical. *J Biol Chem* 253 : 4697–4699.

Mahadev K., Motoshima H., Wu X., Ruddy J.M., Arnold R.S., Cheng G., Lambeth J.D., Goldstein B.J. 2004. The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H2O2 and plays an integral role in insulin signal transduction. *Mol Cell Biol*. 24 (5) : 1844-1854.

Makino I., Shibata K., Shibaguchi H., Niwa M., Katsuragi T., Furukawa T. 1998. The increase in angiotensin type-2 receptor mRNA level by glutamate stimulation in cultured rat cortical cells. *Brain Research*. 804 (2) : 296-305.

Margioris A., Markogiannakis E., Makrigiannakis A., Gravanis A. 1992**.** PC12 rat pheochromocytoma cells synthesize dynorphin. Its secretion is modulated by nicotine and nerve growth factor. *Endocrinology*. 131 : 703–709.

Matsui H., **Nishinaka K**., **Oda M**., **Komatsu K**., **Kubori T**., **Udaka F**. 2006. Minor depression and brain perfusion images in Parkinson's disease. *Mov Disord.* 21 (8) : 1169-1174.

Matsunaga Y, Kawai Y, Kohda Y, Gemba M. 2005**.** Involvement of activation of NADPH oxidase and extracellular signal-regulated kinase (ERK) in renal cell injury induced by zinc. *J Toxicol Sci*. 30 (2) : 135-144.

Matus A., Bernhardt R., Hugh-Jones T. 1981. High molecular weight microtubule-associated proteins are preferentially associated with dendritic microtubules in brain. *Proc Natl Acad Sci U S A*. 78 (5) : 3010–3014.

Matsusaka T. and Ichikawa I. 1997. Biological functions of angiotensin and its receptors. *Annu Rev Physiol.* 59 : 395-412.

Maurer K., Volk S., **Gerbaldo H.** 1997. Auguste D and Alzheimer's disease. *Lancet* 349 (9064) : 1546-1549**.**

Mayeux R. 2003**.** Epidemiology of Neurodegeneration. *Annual Rev. Neurosc* 26 : 81-104.

McCubrey J.A. and Franklin R.A. 2006. Reactive Oxygen Intermediates and Signaling Through Kinase Pathways. *Antioxidants & Redox Signaling*. 8 (9-10): 1745-1748.

Meffert S., Stoll M., Steckelings U.M., Bottari S.P., Unger T. 1996. The angiotensin II AT2 receptor inhibits proliferation and promotes differentiation in PC12W cells. *Mol Cell Endocrinol*. 122 (1) : 59-67.

Mehta P.K. and Griendling K.K. 2007. Angiotensin II Cell Signaling: Physiological and Pathological Effects in the Cardiovascular System. *Am J Physiol Cell Physiol.* 292: C82–C97.
Meinertzhagen I.A. 1994. The early influence of cell size upon synaptic number: the mutant gigas of Drosophila. *J. Neurogenet*. 9 (3) : 157-176.

Millan M.A., Jacobowitz D.M., Aguilera G., Catt K.J. 1991. Differential distribution of AT1 and AT2 angiotensin II receptor subtypes in the rat brain during development. *Proc Natl Acad Sci U S A*. 88 (24) : 11440-11444.

Mills E.M., Takeda K., Yu Z.X., Ferrans V., Katagiri Y, Jiang H., Lavigne M.C., Leto T.L., Guroff G. 1997. Nerve growth factor treatment prevents the increase in superoxide in epidermal growth factor in PC12 cells. *J Biol Chem*. 273 (28) : 22165-22168.

Mills J.C., Kim L.H., Pittman R.N. 1997. Differentiation to an NGF-Dependent state and apoptosis following NGF removal both occur asynchronously in cultures of PC-12 cells. *Experimental Cell research*. 231 : 337-345.

Mitsushita J., Lambeth D., Kamata T. 2004. The Superoxide-Generating Oxidase Nox1 Is Functionally Required for Ras Oncogene Transformation**.** *Cancer Res.* 64 : 3580-3585.

Mischel P.S., Smith S.G., Vining E.R., Valletta J.S., Mobley W.C., Reichardt L.F. 2001. The extracellular domain of p75NTR is necessary to inhibit neurotrophin-3 signaling through TrkA. *J. Biol. Chem.* 276 : 11294–11301.

Miura S. and Karnik S.S. 2000. Ligand-independent signals from angiotensin II type 2 receptor induce apoptosis. *EMBO J*. 19 (15) : 4026-4035.

Miyano K., Ueno N., Takeya R., and Sumimoto H. 2006**.** Direct Involvement of the Small GTPase Rac in Activation of the Superoxide-producing NADPH Oxidase Nox1. *J. Biol. Chem*. 281 : 21857-21868.

Morehouse L.A., **Tien M**., **Bucher J.R**., **Aust S.D**. 1983. Effect of hydrogen peroxide on the initiation of microsomal lipid peroxidation. *Biochem Pharmacol.* 32 (1) : 123-127.

Mukoyama M., Nakajima M., Horiuchi M., Sasamura H., Pratt R.E., and Dzau V.J. 1993. Expression cloning of type-2 angiotensin II receptor reveals a unique class of 7-transmembrane receptors. *J Biol Chem* 268 : 24539-24542.

Murphy H. S., Yu C., Quddus J. 2000. Functional expression of NAD(P)H oxidase p47phox subunit in lung microvascular endothelial cells. *Biochem Biophys Acta.* 278: 584–589.

Nakajima M., Hutchinson H.G., Fujinaga M., Hayashida W., Morishita R., Zhang L., Horiuchi M., Pratt R.E., Dzau V.J. 1995. The angiotensin II type 2 (AT2) receptor antagonizes the growth effects of the AT1 receptor: gain-of-function study using gene transfer. *Proc Natl Acad Sci U S A.* 92 (23) : 10663-10667.

N'Diaye E. N., Vaissiere C., Gonzalez-Christen J., Grégoire C., Le Cabec V. Maridonneau-Parini I. 1997. Expression of NADPH oxidase is induced by all-*trans* retinoic acid but not by phorbol myristate acetate and 1,25 dihydroxyvitamin D3 in the human promyelocytic cell line NB4. *Leukemia.* 11 (12) : 2131-2136.

Nio Y., Matsubara H., Murasawa S., Kanasaki M. and Inada M. 1995. Regulation of gene transcription of angiotensin II receptor subtypes in myocardial infarction. *J Clin Invest.* 95 : 46–54.

Nishio K., Arioka H., Ishida T., Fukumoto H., Kurokawa H., Sata M., Ohata M., Saijo N. 1995. Enhanced interaction between tublin and microtubule-associated protein 2 via inhibition of map kinase and cdc2 kinase by paclitaxel. *Int J Cancer*. 63 : 688-693.

Noh K.M. and Koh J.Y. 2000. Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes**.** *J Neurosci.* 20 (23) : RC111.

Nouet S. and Nahmias C. 2000. Signal transduction from the angiotensin II AT2 receptor. *Trends Endocrinol Metab.* 11 : 1–6.

Obata T. 2003. Phytic acid suppresses 1-methyl-4-phenylpyridinium ion-induced hydroxyl radical generation in rat striatum. *Brain Res.* 978 : 241–244.

Obermeier A., Bradshaw R.A., Seedorf K., Choidas A., Schlessinger J. Ullrich A. 1994. Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC gamma. *EMBO J.* 13 : 1585-1590.

Obermeier A., Lammers R., Wiesmuller K.H., Jung G., Schlessinger J. Ullrich A. 1993. Identification of Trk binding sites for SHC and phosphatidylinositol 3'-kinase and formation of a multimeric signaling complex. *J. Biol. Chem.* 268 : 22963-22966.

Oppenheim R.W. 1991. Cell death during development of the nervous system. *Annual Review of Neuroscience.* 14: 453-501.

Orient A., Donko A., Szabo A., Leto T.L., Geiszt M. 2007. Novel sources of reactive oxygen species in the human body. *Nephrol. Dial. Transplant*. 22 : 1281-1288.

Paclet M.H., Coleman A.W., Burritt J., Morel F. 2001. NADPH oxidase of Epstein-Barr-virus immortalized B lymphocytes. Effect of cytochrome b(558) glycosylation. *Eur J Biochem.* 268 : 5197–5208.

Pagano P. J., Clark J. K., Cifuentes-Pagano E. M., Clark S. M., Callis G. M., Quinn M. T. 1997. Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. Proc Natl Acad Sci USA. 94 : 14483-14488.

Page I.H. and Helmer O.M. 1940**.** Angiotonin-Activator, Renin- And Angiotonin-Inhibitor, And The Mechanism Of Angiotonin Tachyphylaxis In Normal, Hypertensive, And Nephrectomized Animals *J. Exp. Med*. 71: 495-519.

Pang L., Sawada T., Decker S. J., Saltiel A. R. 1996 Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by NGF. *J Biol Chem* 270:13585–13588.

Papadakis E., Finegan K., Wang X., Robinson A., Guo C., Kayahara M., Tournier C. 2006. The regulation of Bax by c-Jun N-terminal protein kinase (JNK) is a prerequisite to the mitochondrial-induced apoptotic pathway. *FEBS Letters*. 580 (5) : 1320-1326.

Papavassiliou A.G., Treier M., Bohmann D. 1995. Intramolecular signal transduction in c-Jun. *EMBO J*. 14 (9): 2014–2019.

Park D., Stefanis L., Yan C., Farinelli S., Greene L. 1996. Ordering the cell death pathway: differential effects of Bcl2, an interleukin-1-converting enzyme family protease inhibitor, and other survival agents on JNK activation in serum/nerve growth-deprived PC12 cells. *J Biol Chem.* 271 : 21898-21905.

Pascale A., Amadio M., Scapagnini G., Lanni C., Racchi M., Provenzani A., Govoni S., Alkon D.L., Quattrone A. 2005. Neuronal ELAV proteins enhance mRNA stability by a PKC-dependent pathway *Proc. Nat. Acad. Sc.* 102 : 12065-12070.

Pedruzzi E., Guichard C., Ollivier V., Driss F., Fay M., Prunet C., Marie J.C., Pouzet C., Samadi M., Elbim C., O'dowd Y., Bens M., Vandewalle A., Gougerot-Pocidalo M.A., Lizard G., Ogier-Denis E. 2004**.** NAD(P)H oxidase Nox-4 mediates 7-ketocholesterol-induced endoplasmic reticulum stress and apoptosis in human aortic smooth muscle cells. *Mol Cell Biol*. 24 (24) : 10703-10717.

Perry S.W., Norman J.P., Litzburg A., Gelbard H.A. 2004. Antioxidants are required during the early critical period, but not later, for neuronal survival. *J Neurosci Res***.** 78 (4) : 485-492.

Phillips M. and Schmidt-Ott K. 1999**.** The Discovery of Renin 100 Years Ago *News Physiol Sci* 14 : 271-274.

Pittman R.N., Wang S., DiBenedetto, Mills J.C. 1993. A system for characterizing cellular and molecular events in programmed neuronal cell death. *J. Neurosci*. 13 : 3669-3680.

Price M.O., Atkinson S.J., Knaus U.G., Dinauer M.C. 2002. Rac activation induces NADPH oxidase activity in transgenic COS phox cells, and the level of superoxide production is exchange factor-dependent. *J Biol Chem*. 277 (21) : 19220-19228.

Pullar J.M. and Hampton M.B. 2002. Diphenelyneiodonium triggers the efflux of glutathione from cultured cells. *J. Bio Chem.* 277 (22) : 19402-19407.

Pulverer B.J., Kyriakis J.M., Avruch J., Nikolakaki E., Woodgett J.R. 1991. Phosphorylation of c-jun mediated by MAP kinases. *Nature.* 353 (6345) : 670–674.

Putcha G.V., Harris C.A., Moulder K.L., Easton R.M., Thompson C.B., Johnson E.M., Jr. 2002. Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice**.** *J. Cell Biol*. 157 : 441-453.

Quinn M.T., Cloud M., Ammons B., DeLeo F.R. 2006. The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. *Clinical Science.* 111: 1–20.

Raffioni S. and Bradshaw R.A. 1992. Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor, and nerve growth factor in PC12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA.* 89 : 9121-9125.

Rao G.N. 1996. Hydrogen peroxide induces complex formation of SHC-Grb2-SOS with receptor tyrosine kinase and activates Ras and extracellular signal-regulated protein kinases group of mitogen-activated protein kinases. *Oncogene.* 13 (4) : 713-719.

Rasouly D., Lazarovici P., Stephens R. M., Kaplan D. R. 1995. Signal Transduction of Neurotrophin Receptors: Basic Concepts and Available Pharmacological Tools. *Promega Notes Magazine.* 50 : 24-26.

Reinecke K., Lucius R., Reinecke A., Rickert U., Herdegen T., Unger T. 2003. Angiotensin II accelerates functional recovery in the rat sciatic nerve in vivo: role of the AT2 receptor and the transcription factor NF-κB. *The FASEB J.* 17: 2094-2096.

Repine J.E., **Fox R.B**., **Berger E.M**. 1981. Hydrogen peroxide kills Staphylococcus aureus by reacting with staphylococcal iron to form hydroxyl radical. *J Biol Chem.* 256 (14) : 7094-7096.

Rhee S.G. 1999. Redox signaling: hydrogen peroxide as intracellular messenger. *Exp Mol Med.* 31 (2) : 53-59.

Rhee S.G., Bae Y.S., Lee S.R., Kwon J. 2000. Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. *Sci. STKE* 2000 (53) : pE1.

Rhee S.G., Chang, Tong-Shin B., Yun Soo L., Seung-Rock K., Sang W. 2003. Cellular Regulation by Hydrogen Peroxide. *J Am Soc Nephrol*. 14 : S211-S215.

Riccio A., Pierchala B. A., Ciarallo C. L., Ginty D .D. 1997. An NGF-TrkA-Mediated Retrograde Signal to Transcription Factor CREB in Sympathetic Neurons. *Science*. 277 : 1097-1100.

Risso. A. 2000. Leukocyte antimicrobial peptides: multifunctional effector molecules of innate immunity *J. Leukoc. Biol*. 68: 785-792.

Rodriguez M.H., Arnaud S., Blanchet J.P. 1995. IL-11 directly stimulates murine and human erythroid burst formation in semi-solid cultures. *Exp Hematol.* 23: 545–550.

Rothfork J.M., Timmins G.S., Harris M.N., Chen X., Lusis A.J., Otto M., Cheung A.L., Gresham H.D. 2004. Inactivation of a bacterial virulence pheromone by phagocyte-derived oxidants: new role for the NADPH oxidase in host defense. *Proc Natl Acad Sci U S A*. 101 (38) : 13867-13872.

Rukenstein A., Rydel R.E., Greene L.A. 1991. Multiple agents rescue PC12 cells from serum-free cell death by translation and transcription-independent mechanisms. *J. Neurosci.* 11: 2552-2563.

Sadok A., **Bourgarel-Rey V**., **Gattacceca F**., **Penel C**., **Lehmann M**., **Kovacic H**. 2008. Nox1-dependent superoxide production controls colon adenocarcinoma cell migration *Bioch. Biophys. Acta.* 1783 (1) : 23-33.

Sadoshima J. 1998. Versatility of the Angiotensin II Type 1 Receptor. *Circulation Research.* 82 : 1352-1355.

Sathyamoorthy, M., de Mendez, **I., Adams**, **AG and Leto**, **T. L**. 1997. p40 (phox) Down-regulates NADPH Oxidase Activity through Interactions with its SH3 Domain *J. Biol. Chem*. 272 : 9141-9146.

Sato T., Overduin M., Emr S. 2001. Location, location, location: membrane targeting directed by PX domains. *Science*. 294 (5548) : 1881-1885.

Satoh T., Enokido Y., Aoshima H., Uchiyama Y., Hatanaka H. 1997. Changes in mitochondrial membrane potential during oxidative stress-induced apoptosis in PC12 cells. *J Neurosci Res*. 50 (3) : 413-420.

Saunders, J.W. 1966. Death in Embryonic Systems. *Science* 154: 604-612.

Sawyer D.T. and Valentine J.S. 1981. How super is superoxide. *Acc. Chem. Res*. 14 : 393-400.

Scandalios J.G., Guan L., Polidoros A.N. 1997. Catalases in plants: Gene structure, properties, regulation, and expression. In: Scandalios JG (Editor), *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*. Cold Spring Harbor Laboratory Press, Plainview, NY, USA, 343-406.

Scandalios J.G. 1997. Molecular genetics of superoxide dismutases. In: Scandalios JG (Editor), *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*. Cold Spring Harbor Laboratory Press, Plainview, NY, USA, 527-568.

Scandalios J.G. 2005. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. *Braz. J. Med. Biol. Res.* 38 : 995–1014.

Schenkel J. 2004**.** Activation of the c-Jun transcription factor following neurodegeneration in vivo. *Neuroscience letters*. 361(1-3) : 36-39.

Shanmugam S., Llorens-Cortes C., Clauser E., Corvol P., Gasc J.M. 1995. Expression of angiotensin II AT2 receptor mRNA during development of rat kidney and adrenal gland. *Am J Physiol* 268: F922–F930.

Shibata K., Makino I., Shibaguchi H., Niwa M., Katsuragi T., Furukawa T. 1997. Up-regulation of Angiotensin Type 2 Receptor mRNA by Angiotensin II in Rat Cortical Cells. *Biochem Biophys Res Com.* 239 (2) : 633-637.

Sbarra A.J., Karnovsky M.l. 1960. The biochemical basis of phagocytosis. 2. Incorporation of C14-labeled building blocks into lipid, protein, and glycogen of leukocytes during phagocytosis. *J Biol Chem.* 235: 2224–2229.

Sebok A., Nusser N., Debreceni B., Guo Z., Santos M. F., Szeberenyi J., Tigyi G. 1999. Different roles for RhoA during neurite initiation, elongation, and regeneration in PC12 cells. *J. Neurochem*. 73 (3) : 949-960.

Seeley P.J. and Greene L.A. 1983. Short-latency local actions of nerve growth factor at the growth cone. *Proc Natl Acad Sci U.S.A.* 80 (9) : 2789–2793.

Segal A.W. and Jones O.T.G. 1978. Novel cytochrome b system in phagocytic vacuoles of human granulocytes. *Nature.* 276 : 515-517.

Serrano F., **Kolluri N.S**., **Wientjes F.B**., **Card J.P**., **Klann E**. 2003. NADPH oxidase immunoreactivity in the mouse brain. *Brain Res.* 988 : 193–198.

Seta K., Nanamori M., Modrall J., Neubig R., Sadoshima J. 2002. AT1 receptor mutant lacking heterotrimeric G protein coupling activates the Src-Ras-ERK pathway without nuclear translocation of ERKs. *J Biol Chem*. 277 (11) : 9268-79277.

Shigeno T., Mima T., Takakura K., Graham D.I., Kato G., Hashimoto Y., and Furukawa S. 1991**.** Amelioration of delayed neuronal death in the hippocampus by nerve growth factor. *J. Neurosci*. 11 : 2914-2919.

Shiose A., Kuroda J., Tsuruya K., Hirai M., Hirakata H., Naito S., Hattori M., Sakaki Y., Sumimoto H. 2001. A novel superoxide-producing NAD(P)H oxidase in kidney. *J Biol Chem*. 276 (2) : 1417-1423.

Shiose A. and Sumimoto H. 2000. Arachidonic acid and phosphorylation synergistically induce a conformational change of p47-phoxphox to activate the phagocyte NADPH oxidase. *J Biol Chem*. 275 (18) : 13793-13801.

Siderowf A. and Stern M. 2003. Update on Parkinson Disease**.** *Ann Intern Med*. 651-658.

Simonian N.A. and Coyle J.T. 1996. Oxidative stress in neurodegenerative diseases**.** *Annu Rev Pharmacol Toxicol*. 36: 83-106.

Skalnik D.G., Strauss E.C., Orkin, S.H. 1991. CCAAT displacement protein as a repressor of the myelomonocytic- specific gp91-phox gene promoter *J. Biol. Chem.* 266: 16736-16744.

Skeggs L. Jr., **Kahn Jr**., **Shumway N., Woods K.** 1956. The amino acid sequence of hypertensin. II. *J Exp Med.* 104 (2) : 193-197.

Saunders J.W. 1966. Death in Embryonic Systems. *Science* 154: 604-612.

Siderowf A. and Stern M. 2003. Update on Parkinson Disease**.** *Ann Intern Med*. 651-658.

Slee E. A., Harte M. T., Kluck R. M., Wolf B. B., Casiano C. A., Newmeyer D. D., Wang H. G., Reed J. C., Nicholson D. W., Alnemri E. S., Green D. R., Martin S. J. 1999. Ordering the Cytochrome c-initiated Caspase Cascade: Hierarchical Activation of Caspases-2, -3, -6, -7, -8, and -10 in a Caspase-9-dependent Manner. *J. Cell Biol.* 144 : 281-292.

Smart E., Graf G., McNiven M., Sessa W., Engelman J., Scherer P., Okamoto T., Lisanti M. 1999. Caveolin, liquid-ordered domains, and signal transduction. *Mol Cell Biol.* 19 : 7289–7304.

Smeal T., Binetruy B., Mercola D.A., Birrer M., Karin M. 1991. Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serine-63 and serine-73. *Nature* 354 : 494-496.

Stahelin R.V., Burian A., Bruzik K.S., Murray D., Cho W. 2003. Membrane binding mechanisms of the PX domains of NADPH oxidase p40phox and p47-phoxphox. *J Biol Chem*. 278 (16) : 14469-14479.

Steckelings U.M., Henz B.M., Wiehstutz S., Unger T., Artuc M. 2005. Differential expression of angiotensin receptors in human cutaneous wound healing. *Br J Dermatol*. 153 (5) : 887-893.

Stocker H. and Hafen E. 2000. Genetic Control of cell size. *Curr. Opin. Genet. Dev*. 10 : 529-535.

Stoll M., Steckelings U. M., Paul M., Bottari S. P., Metzger R., Unger T. 1995**.** The angiotensin AT_2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J. Clin. Invest.* 95: 651–657.

Stroth U., Meffert S., Gallinat S., Unger T. 1998. Angiotensin II and NGF differentially influence microtubule proteins in PC12W cells: role of the AT_2 receptor. *Molecular Brain Research.* 53 (1-2) : 187-195.

Stroth U., Blume A., Mielke K., Unger T. 2000. Angiotensin AT(2) receptor stimulates ERK1 and ERK2 in quiescent but inhibits ERK in NGF-stimulated PC12W cells. *Brain Res Mol Brain Res*. 78 (1-2) : 175-180.

Su B., Mitra S., Gregg H., Flavahan S., Chotani M.A., Clark K.R., Goldschmidt-Clermont P.J., Flavahan N.A. 2001. Redox regulation of vascular smooth muscle cell differentiation. *Circ Res*. 89 (1) : 39-46.

Suh Y.A., Arnold R.S., Lassegue B., Shi J., Xu X., Sorescu D., Chung A.B., Griendling K.K., Lambeth J.D. 1999. Cell transformation by the superoxide-generating oxidase Mox1. *Nature.* 401 (6748) : 79-82.

Sumners C., Tang W., Zelezna B., Raizada M.K. 1991 Angiotensin II receptor subtypes are coupled with distinct signal-transduction mechanisms in neurons and astrocytes from rat brain. *Proc Natl Acad Sci USA*. 88 : 7567–7571.

Suzukawa K., Miura K., Mitsushita J., Resau J., Hirose K., Crystal R., Kamata T. 2000. Nerve growth factor-induced neuronal differentiation requires generation of Rac1-regulated reactive oxygen species. *J. Biol. Chem.* 275 : 13175–13178.

Takeya R, Ueno N, Kami K, Taura M, Kohjima M, Izaki T, Nunoi H, Sumimoto H. 2003. Novel human homologues of p47-phox and p67-phox participate in activation of superoxide-producing NADPH oxidases. *J Biol Chem*. 278 (27) : 25234-25246.
Takeya R., **Ueno N**., **Sumimoto H**. 2006. Regulation of superoxide-producing NADPH oxidases in nonphagocytic cells. *Methods Enzymol.* 406 : 456-468.

Tammariello S.P., Quinn M.T., Estus S. 2000. NADPH Oxidase Contributes Directly to Oxidative Stress and Apoptosis in Nerve Growth Factor-Deprived Sympathetic Neurons. *J. Neuroscience.* 20 (RC53) : 1-5.

Tapon N., Nagata K-I., Lamarche N., Hall A. 1998. A new rac target POSH is an SH3 containing scaffold protein involved in the JNK and NF-kappa B signaling pathways. *EMBO J*. 17*:* 1395-1404.

Tejada-Simon M., Serrano F., Villasana L., Kanterewicz B., Wu G., Quinn M., Klann E. 2005. Synaptic localization of a functional NADPH oxidase in the mouse hippocampus. *Molecular and Cellular Neuroscience*. 29 (1) : 97-106.

Thannickal V.J. and Fanburg B.L. 2000. Reactive Oxygen Species in cell signaling. *Am J Physiol Lung Cell Physiol.*. 279 : L1005-L2000.

Thomas W.G. 1999. Regulation of angiotensin II type 1 (AT_1) receptor function. *Regulatory Peptides.* 7 (1): 9-23.

Thommessen B., Aarsland D., Braekhus A., Oksengaard A.R., Engedal K., Laake K. 2002. The psychosocial burden on spouses of the elderly with stroke, dementia and Parkinson's disease**.** *Int J Geriatr Psychiatry*. 17 (1) : 78-84.

Thornberry N.A. and Lazebnik Y. 1998. Caspases: enemies within. *Science.* 281:1312-1316.

Torres M. and Forman H. J. 2003. Redox signaling and the MAP kinase pathways. *Biofactors.* 17*:* 287–296.

Tsai C.T., Lai L.P., Lin J.L., Chiang F.T., Hwang J.J., Ritchie M.D., Moore J.H., Hsu K.L., Tseng C.D., Liau C.S., Tseng Y.Z. 2004. Renin-angiotensin system gene polymorphisms and atrial fibrillation. *Circulation.* 109 : 1640 –1646.

Tsatmali M., Walcott E.C., Makarenkova H., Crossin K. L. 2006. Reactive Oxygen Species Modulate the Differentiation of Neurons in Clonal Cortical Culture *Mol. Cell. Neurosc.* 33 (4) : 345-357.

Tsay H., Wang P., Wang S., Ku H. 2000.Age-Associated Changes of Superoxide Dismutase and Catalase Activities in the Brain. *J Biomed Sci*. 6 : 466-474.

Tsui-Pierchala B. A., Putcha G. V., Johnson E. M. 2000. Phosphatidylinositol 3-kinase is required for the trophic, but not the survival-promoting, actions of NGF on sympathetic neurons. *J. Neuroscience*. 20 (19) : 7228-7237.

Tsutsumi K. and Saavedra J.M. 1991. Characterization and development of angiotensin II receptor subtypes (AT1 and AT2) in rat brain. *Am J Physiol*. 261 (1 Pt 2) : R209-216.

Tsuzuki S., Eguchi S., Inagami T. 1996 Inhibition of cell proliferation and activation of protein tyrosine phosphatase mediated by angiotensin II type 2 (AT_2) receptor in R3T3 cells. *Biochem Biophys Res Commun*. 228 : 825–830.

Tucker R.P. 1990. The roles of microtubule-associated proteins in brain morphogenesis: a review. *Brain Res Brain Res Rev*. 15 (2) : 101-20.

Unger T., Chung O., Csikos T., Culman J., Gallinat S., Gohlke P., Hohle S., Meffert S., Stoll M., Stroth U., Zhu Y.Z. 1996. Angiotensin receptors. *J Hypertens.* Suppl 14: S95–103.

Vaghefi H., Hughes A.L., Neet K.E. 2004. Nerve growth factor withdrawal-mediated apoptosis in naive and differentiated PC12 cells through p53/caspase-3-dependent and -independent pathways. *J Biol Chem*. 279: 15604–15614.

Vallet P., Charnay Y., Steger K., Ogier-Denis E., Kovari E., Herrmann F., Michel J.P., Szanto I. 2005. Neuronal expression of the NADPH oxidase NOX4, and its regulation in mouse experimental brain ischemia. *Neuroscience*.132 (2) : 233-238.

Vaudry D., Stork P.J., Lazarovici P., Eiden L.E. 2002**.** Signaling Pathways for PC12 Cell Differentiation: Making the Right Connections. *Science*. 296 (5573) : 1648 – 1649.

Vergnaud S., Paclet M.H., El Benna J., Pocidalo M.A., Morel F. 2000. Complementation of NADPH oxidase in p67-phox-deficient CGD patients p67-phox/p40-phox interaction. *Eur J Biochem*. 267 (4) : 1059-1067.

Voet D., Voet J.G., Pratt C.W. 2006. *Fundamentals of Biochemistry, 2nd Edition*. John Wiley and Sons, Inc., 547. ISBN 0471214957.

Wang R., Zagariya A., Ibarra-sunga O., Giidea C., Ang E., Deshmukh S., Chaudhary G., Baraboutis J., Filippatos G., Uhal B. 1999. Angiotensin II induces apoptosis in human and rat alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 276 : L885 - L889.

Wang Y., Huang S., Sah V.P., Ross J. Jr., Brown J.H., Han J., Chien K.R. 1998. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38-mitogen activated protein kinase family. *J. Biol Chem*. 273 : 2161-2168.

Warne P.H., Viciana P.R., Downward J. 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. *Nature*. 364 (6435) : 352-355.

Watson A., Eilers A., Lallemand D., Kyriakis J., Rubin L.L., Ham J. 1998. Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J. Neurosci.* 18 : 751–762.

Willis S., Day C. L., Hinds M. G. Huang D.C. 2003. The Bcl-2-regulated apoptotic pathway. *J. Cell Sci.* 116: 4053 - 4056.

Wolf G. 2002. Angiotensin II and tubular development. *Nephrol Dial Transplant*. 17 (9) : 48–51.

Wolin M.S. 2004. Subcellular localization of Nox-containing oxidases provides unique insight into their role in vascular oxidant signaling. *Arterioscler Thromb Vasc Biol.* 24 (4) : 625-627.

Wiggin G. F., Fawcett J. P., Pawson T. 2005. Polarity proteins in axon specification and synaptogenesis. *Dev. Cell*. 8: 803-816.

Wyatt S. and Davies A.M. 1995. Regulation of nerve growth factor receptor gene expression in sympathetic neurons during development. *J. Cell Biol.* 130:1435-1446.

Xia Z., Dickens M., Raingeaud J., Davis R.J., Greenberg M.E. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis induced by neurotrophic factor withdrawal. *Science* 270: 1326-1331.

Xu Z., Kukekov N.V., Greene, L.A. 2003. POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis. *EMBO J.* 22 : 252–261.

Xu, Z., Kukekov, N.V., Greene, L.A. 2003. POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis. *EMBO J.* 22 : 252–261.

Yamada T., Horiuchi M., Dzau V.J. 1996.Angiotensin II type 2 receptor mediates programmed cell death. *Proc Natl Acad Sci U.S.A.* 93:156–160.

Yang S., Madyastha P., Bingel S., Ries W., Key L. 2001. A new superoxide-generating oxidase in murine osteoclasts, *J Biol Chem.* 276 : 5452–5458.

Yang D., Kuan C-Y., Whitmarsh A.J., Rincon M., Zheng T.S., Davis R.J., Rakic P., Flavell R.A. 1997. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the JNK3 gene. *Nature*. 289: 865-870.

Yankner B.A. and Shooter E.M. 1979. Nerve growth factor in the nucleus: Interaction with receptors on the nuclear membrane. *Proc. Natl. Acad. Sci. U.S A*.76 : 1269-1273.

Yu L., DeLeo F.R., Biberstine-Kinkade K.J., Renee J., Nauseef W.M., Dinauer M.C. 1999. Biosynthesis of flavocytochrome b558 . gp91(phox) is synthesized as a 65-kDa precursor (p65) in the endoplasmic reticulum. *J Biol Chem*. 274 (7) : 4364-4369.

Yu Z., Nikolova-Karakashian M., Zhou D., Cheng G., Schuchman E.H., Mattson M.P. 2000. Pivotal role for acidic sphingomyelinase in cerebral ischemia-induced ceramide and cytokine production, and neuronal apoptosis. *J. Mol. Neurosci.* 15: 85–98.

Zapf-Colby A. and Olefsky J.M. 1998. Nerve Growth Factor processing and trafficking events following TrkA-mediated endocytosis. *Endocrinology*. 139 (7): 3232- 3240.

Zhang N., Gao G., Bu X., Han S., Fang L., Li J. 2007. Neuron-specific phosphorylation of c-Jun N-terminal kinase increased in the brain of hypoxic preconditioned mice. *Neuroscience Letters.* 423 (3): 219-224.

Zhao Y., Biermann T., Luther C., Unger T., Culman J., Gohlke P. 2003. Contribution of bradykinin and nitric oxide to AT_2 receptor-mediated differentiation in PC12W cells. *J. Neurochem*.85 : 759–767.

Zhou L.J., Zhu X.Z. 2000. Reactive oxygen species-induced apoptosis in PC12 cells and protective effect of bilobalide. *J Pharmacol Exp Ther*. 293(3):982-988.

Zohn I. E., Yu H., Li X., Cox A. D., Earp H. S. 1995.Angiotensin II stimulates calcium-dependent activation of c-Jun N- terminal kinase. *Mol. Cell. Biol.* 15: 6160-6168.