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I am submitting herewith a dissertation written by Brian A. Jull entitled "The Effects of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone on the MAP Kinase Cascade in Neoplastic and Non-neoplastic Pulmonary Neuroendocrine Cells". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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We have read this dissertation
and recommend its acceptance:

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THE EFFECTS OF 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE ON THE MAP KINASE CASCADE IN NEOPLASTIC AND NON-NEOPLASTIC PULMONARY NEUROENDOCRINE CELLS.

A Dissertation
presented for the
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Brian Ashley Jull, DVM
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Dedication

This thesis is dedicated to my parents Mr. Kelly Jull and Mrs. Della Jull as well as to my wife Krista who have lovingly provided me with the support and encouragement needed to achieve all of lifes dreams. To my daughter Abigail, whose young existence has brought immense joy to my life.

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Abstract

Small cell lung carcinoma (SCLC) is a highly malignant pulmonary neoplasm derived from a normally sparse population of pulmonary neuroendocrine cells (PNEC) that proliferate when exposed to tobacco associated carcinogens and hypoxic environments. Although the precise mechanism by which these mitogens result in cellular proliferation and subsequent transformation is not known, there has been abundant attention directed toward the mutagenic mechanisms of pulmonary carcinogenesis. However, a lack of specific mutations in many cases of SCLC fails to explain the inciting cause and the progression of this neoplasm. Therefore, a receptor mediated mechanism linking the specific tobacco derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), to cellular proliferation via the MAP kinase signaling pathway has been proposed. Understanding the involvement of this pathway in the development of SCLC provides a unique target for potential chemopreventive and chemotherapeutic measures.

NNK and hypoxia have a profound effect on the MAP kinase signal transduction pathway and nuclear phospho-protein activation. This intracellular signaling cascade has been shown to play a critical role in the regulation of cell cycle events and cell proliferation. Exposure of both neoplastic and non-neoplastic PNECs to NNK results in the activation of the protein kinases, Raf-1 and MAPK 1\2, within the MAP kinase

signaling pathway, as demonstrated by immunoblotting techniques and in vitro kinase assays. Additionally, NNK stimulates the phosphorylation of specific nuclear proteins involved in subsequent cell cycle regulation. These induced levels of activation can be significantly attenuated by various inhibitors that act on the receptor or on specific protein kinase domains. Based on these findings, the tobacco specific carcinogen, NNK, reliably induces an activating signal on the MAP kinase cascade and its downstream early gene nuclear transcripts. This, in turn, is believed to result in the proliferation of the neoplastic and non-neoplastic pulmonary neuroendocrine cells. Future investigations will be done to link these NNK induced intracellular signaling events with specific cell cycle responses.

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LIST OF ABBREVIATIONS

BSA: bovine serum albumin

Btx: α-bungarotoxin

Ca^{2+}: calcium ion

cAMP: adenosine 3' 5'-cyclic phosphate

cdk2: cyclin dependent kinase 2

CO_2: carbon dioxide

COPD: chronic obstructive pulmonary disease

CPM: counts per minute

CR(1,2,3): conserved amino acid regions 1,2, or 3 of Raf-1 protein

DAG: diacylglycerol

DMEM: Delbeccos modified eagles media

DNA: deoxyribonucleic acid

ERK: extracellular regulated kinase (a.k.a. MAPK)

FBS: fetal bovine serum

GPCR: GTP-protein coupled receptor

Grb2: adaptor protein

5-HT: 5-hydroxytryptamine (a.k.a. serotonin)

IP_3: inositol 1,4,5-triphosphate

MAD: negative regulating nuclear protein

MAX: positive regulating nuclear protein

MAPK: mitogen activated protein kinase (a.k.a. ERK)

MBP: myelin basic protein

MEK: MAPK/Erk kinase

MEKK: mitogen/extracellular-signal regulated kinase kinase kinase

MOPS: 3-[N-marpholino] propanesulfonic acid

Myc: nuclear phosphoprotein

c-myc: nuclear phosphoprotein gene

p-Myc: phosphorylated Myc

nAChR: nicotinic acetylcholine receptor

NCI-H69: small cell lung carcinoma cell line

NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

NSCLC: non-small cell lung carcinoma

PAGE: polyacrylamide gel

PBS: phosphate buffered saline

PD98059: [2-(2'-amino-3'-mehtylphenyl)-oxanaphthalen-4-one]; MEK inhibitor

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PMSF: phenylmethylsulfonyl flouride

PNEC: pulmonary neuroendocrine cell

Raf-1: cytoplasmic serine/threonine protein kinase

Ras: membrane associated GTP protein kinase

RIPA: cell lysing buffer

RTK: receptor tyrosine kinase

SCLC: small cell lung carcinoma

SDS: sodium dodecyl sulfate

Ser: amino acid; serine

SH2: Src homology 2 adaptor protein

TBS: Tris buffered saline

Thr: amino acid; threonine

Tyr: amino acid; tyrosine

PART I: GENERAL INTRODUCTION AND OVERVIEW

Chapter 1: Introduction

Lung cancer is the most frequently lethal form of cancer accounting for 28% of all cancer deaths in the United States, surpassed in incidence but not mortality only by breast cancer and colorectal cancer (American Cancer Society, 1998). Prior to 1930, mortality due to lung cancer was lower than any other cancer type in both males and females. However, by 1960 lung cancer deaths had risen 86% to become the leading cause of cancer related death in men (American Cancer Society, 1998). The same trend may be found in women after 1970. The major factor believed to account for this dramatic shift in lung cancer death rate is the increased incidence of smoking among men and later social acceptance of smoking among women (Centers of Disease Control and Prevention, 1997). Although amount and duration of smoking are considered the most significant influence on lung cancer rate, factors such as race, gender, environment, genotype, and concurrent pulmonary disease are also believed to play a major role in pulmonary cancer risk (Alberg, 1998).

Lung cancer is subcategorized into four major histologic types: adenocarcinoma, small cell carcinoma, large cell carcinoma, and squamous cell carcinoma (Yesner, 1993). Of the four types, small cell lung carcinoma (SCLC) accounts for about 25% of all lung cancer cases and up to 88% of pulmonary cancer deaths. Additionally, >90% of the

diagnosed cases of SCLC have been epidemiologically linked to smoking (Alberg, 1998). This cancer type is highly malignant with rapid growth and high metastatic potential (Polack, 1993; Bunn, 1997). Although SCLC is sensitive to combination radiation and chemotherapy, most tumors recur and 5 year survival rate after therapy is only 2-5% (Bunn, 1996). Currently there is no effective long term treatment for SCLC patients.

SCLC cells are characterized as having small (80-140 nm diameter) dense-core neurosecretory granules containing a wide variety of bio-active compounds including 5-hydroxytryptamine (5-HT, serotonin), bombesin-like peptides, insulin-like growth factor I, transferrin, calcitonin, cholecystokinin, neurotensin, vasopressin, and the multifunctional natriuretic peptide (Moody, 1998). The putative cell of origin for SCLC is the pulmonary neuroendocrine cell (PNEC) which shares many phenotypic and functional features with SCLC (Sheppard, 1993). PNECs are present in large numbers lining the bronchioles of late gestational fetal lungs. In the low oxygen tension environment of the fetal lungs, these cells release the bio-active peptides which have potent actions on airway smooth muscle, vasomotor tone, airway secretions, and fetal lung development (Becker, 1985). However, by 2 weeks following birth, the number of PNECs are greatly reduced within the oxygenated lung (Johnson and Georgieff, 1989). Although these cells are inherently sensitive to the degree of oxygenation/pulmonary hypoxia and have been shown to express oxygen sensing receptors (Youngson, 1993), the mechanisms of action of these receptors remain largely unknown. Hypoxic lungs of individuals with chronic obstructive pulmonary disease (COPD) frequently contain small hyperplastic foci of PNECs with elevated levels

of 5-HT, calcitonin, and bombesin-like peptide (Tabassian, 1989). Individuals suffering from COPD that also smoke are far more likely to develop SCLC than any other type of lung cancer or persons without COPD (Weiss, 1991). Accordingly, CO_2, which is greatly increased in the COPD lung, acts as a potent selective mitogen in SCLC and PNEC (Merryman, 1997; Schuller 1994); thus identifying elevated CO_2 levels as a promoting factor for the development of SCLC.

In an effort to link specific compounds in tobacco smoke to cellular proliferation and subsequent cellular transformation of PNEC, attention was directed toward nicotine and its classic agonistic effect on nicotinic acetylcholine receptors. There are multiple subtypes of neuronal nicotinic receptors that are based on the expression of various α and β subunit combinations. Nicotinic receptors are ligand-gated ion channels with a centrally located pore through which ions flow (Linstrom, 1995). Many of the neuronal nicotinic ion-channel receptors do not demonstrate preferential passage of any specific ion into the cell. However, nicotinic receptors composed of α_7 subunits operate exclusively as Ca^{2+} channel receptors (Goplakrishnan, 1995), and high affinity binding of nicotine to the α_7 Ca^{2+} channel receptor results in marked Ca^{2+} influx into both PNECs and SCLC cells (Sheppard, 2000), where it may play a role in the initiation of growth stimulating intracellular signaling processes. Schuller and Orloff (1998) demonstrated the presence of α-bungarotoxin (Btx) sensitive, α_7 neuronal nicotinic acetylcholine receptors (nAChR) in SCLC and PNECs. These studies also showed that nicotine and the tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), stimulate PNEC and

SCLC proliferation via high affinity binding to the α_7 nAChR which acts in concert with the yet uncharacterized oxygen sensitive receptors to stimulate 5-HT release (Youngson 1993; Codignola 1994).

Nicotine has long been established as a potent selective agonist of specific acetylcholine receptors (nAChR), and the physiologic neurotransmitter agonist for these receptors, acetylcholine, has been shown to stimulate the synthesis and secretion of a wide variety of biogenic compounds in PNECs (Nylen, 1988). It has been subsequently shown that nicotine stimulates PNEC proliferation in hamsters (Tabassian, 1989) via the nAChR (Schuller, 1989), as well as the release of 5-HT (Youngson, 1993), mammalian bombesin (Nylen, 1988), and other secretagogues (i.e. calcitonin) (Becker, 1984). The nicotine derived tobacco-specific n-nitrosamine, NNK, that is formed during tobacco processing and within the mammalian organism, shares structural similarities with nicotine, but has been found to bind to the α_7 nAChR with greater affinity (1300x greater) than nicotine (Schuller and Orloff, 1998). Therefore, it is logical to expect that NNK may also initiate a signaling event mediated by this nAChR in normal and neoplastic PNECs.

Previous studies have shown that dose dependent exposure to NNK results in increased cell proliferation and increased ^{3}H-thymidine incorporation in both neoplastic and normal PNECs (Schuller, 1994). It was also observed (Schuller, unpublished) that Syrian hamsters exposed to NNK and treated with bronchodilators had 40-70% reduced incidence in SCLC development as well as reduction in size of existing SCLC compared to groups exposed to NNK but not administered the bronchodilator. These bronchodilators

inhibit phosphodiesterase resulting in elevated levels of cAMP and protein kinase A (PKA). Increased PKA competitively inhibits the activation of Raf-1 by phosphorylation of the kinase domain; thus down regulating the kinase activity of Raf-1 (Hafner, 1994). Therefore, it was hypothesized that a signaling pathway used by NNK to induce cellular proliferation would involve Raf-1. The MAP kinase cascade is one such pathway. We subsequently began to investigate this intracellular pathway as a link between the NNK receptor-mediated signal and the resultant proliferative response by examining the activation and protein kinase levels of the critical components within the cascade, specifically Raf-1 and MAPK 1\2. As a result, we identified the MAP kinase pathway as a receptor-mediated signaling target for the orchestrated ligand binding of NNK, CO_2, and 5-HT. Furthermore, since cytosolic signaling pathways are intermediate steps leading to activation of early gene transcripts, we demonstrate concurrent phosphorylation of nuclear phospho-proteins. Future studies need to be conducted to determine the effect of these signaling changes on the cell cycle and its protein components.

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PART II: NNK INDUCED ACTIVATION OF THE MAP KINASE PATHWAY

Chapter 1: Abstract

Pulmonary neuroendocrine cells (PNEC) contain numerous intracellular signaling pathways that transmit receptor mediated mitogenic signals from the cell surface to the nucleus where that information is used to regulate cell growth, quiescence, or death. Chronic stimulation or alteration of these pathways may result in aberrant cell proliferation and transformation. By stimulating both normal and neoplastic PNECs with low concentrations of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), we were able to demonstrate activation of key protein kinases within the mitogen activated protein (MAP) kinase cascade, specifically Raf-1 and MAPK. A significant increase in Raf-1 and MAPK activation over unstimulated cells was detected by using an in vitro kinase assay and phospho-specific antibodies directed against the two protein kinases. This NNK inducible activation was blocked by intracellular inhibitors directed against protein kinase C (PKC) and MAPK/Erk kinase (MEK). As a result, these data demonstrate a PKC dependent activating effect of NNK on the MAP kinase pathway.

Chapter 2: Introduction

The MAP kinase cascade is a growth regulatory pathway found in most cell types that plays a role in cell proliferation, differentiation, or programed cell death (apoptosis). This cytosolic signal transduction is composed of specific protein kinases which are sequentially regulated by a series of phosphorylation and/or dephosphorylation reactions of highly conserved amino acid sequences (Heidecker, 1992; Mgnusun, 1994, Mischak, 1996). The MAP kinase cascade may be initiated by ligand-receptor binding of various tyrosine kinase receptors or via heterotrimeric guanine nucleotide binding proteins, commonly referred to as G protein coupled receptors (GPCR). GPCR have been shown to activate the MAP kinase pathway via downstream activation of protein kinase C (PKC) and subsequently Raf-1 (Ueffing, 1997). Raf-1 phosphorylates the MAPK/Erk kinase (MEK) which in turn activates MAPK or otherwise refered to as the extracellular signal-regulated protein kinase, Erk. Therefore, the serine-threonine protein kinase Raf-1 and the threonine-tyrosine protein kinase MAPK, in part convey ligand-receptor signals from the cell membrane to the nucleus where gene transcription and the cell cycle can be regulated. It has been suggested that excessive stimulation or aberrant regulation of this signaling process is linked to various non-neoplastic and neoplastic conditions, including SCLC (Graziano, 1991; Naumann, 1997).

Furthermore, signal transduction and subsequent cellular proliferation can result from autocrine, paracrine, or endocrine receptor-mediated processes that are generally cell type specific, and responsive to low concentrations of a stimulus. Accordingly, exposure to mitogenic agents such as the tobacco-specific nitrosamine, NNK, operating via the α_7 nAChR in PNEC may result in hyperactivation of downstream intracellular pathways that regulate cell growth or initiate the release of other biogenic compounds such as 5-HT. The released biogenic amine may have an autocrine or paracrine effect that also stimulates intracellular signaling. Therefore, 5-HT binding via a seven transmembrane GPCR may result in activation of the MAP kinase cascade via a PKC dependent pathway. In this way, a large proliferative response could result from a single small initiating stimulus. Therefore, by exposing neoplastic and non-neoplastic PNECs to NNK and subsequently examining the activation of two key components of the MAP kinase signaling pathway, a link between NNK exposure and specific kinase activation was demonstrated. This is of vital importance in elucidating the intracellular pathway of NNK induced mitogensis and its role in the development of SCLC.

Chapter 3: Materials and Methods

Cell Culture: Normal pulmonary neuroendocrine cells were obtained from the lungs of 15 day gestational Syrian golden hamster fetuses. Prior to obtaining fetal lung tissue, all hamsters were maintained in a standardized housing environment with *ad libitum* commercial feed and water. The lungs were harvested under aseptic conditions, followed by cellular dissociation using 2% trypsin for 1 hour in 10% CO_2 at 37° C. The cells were subsequently transferred and maintained in RPMI 1640 medium (Biofluids) supplemented with 10% fetal bovine serum (FBS) in a 10% CO_2 atmosphere. This environment inhibits the growth of fibroblasts and non-neuroendocrine lung cells while selectively promoting the growth of PNECs via activation of their hypoxia-sensitive receptor pathway (Schuller, 1994). The resulting primary cultures are >80% PNECs. All cells were grown to 60-80% confluence in 25 or 75 cm^2 vented culture flasks prior to experimental procedures. The NCI-H69 (small cell carcinoma) cell line was obtained from the American Type Culture Collection. NCI-H69 cells grow as floating cell aggregates and were maintained in RPMI 1640 medium supplemented with 10% FBS in a 5% CO_2 atmosphere prior to their use in experiments.

Cell treatment and lysate production: NCI-H69 (SCLC) cells and fetal PNECs were placed into low serum Dulbecco's modified eagles media (DMEM) (Biofluids) containing 0.1% bovine serum albumin (BSA), 210 mg L-glutamine, 500 units penicillin, and 500 mcg streptomycin for 12-24 hours prior to the experiment while maintained in 5% CO_2. Cells were washed with 1x Ca^{2+} free phosphate buffered saline (PBS) and reintroduced to low serum media 30 minutes to 1 hour prior to the experiment. Competitive receptor inhibitors were added to the designated treatment group 10 minutes before the addition of NNK. Protein kinase inhibitors were placed on the designated cells 1 hour preceding the addition of NNK. NNK and chemical inhibitors were made fresh within 3 hours of experimental use.

Collection of the fetal PNECs involved removal of the low serum DMEM media containing the designated treatment, washing the cells with ice-cold 1x Ca^{2+} PBS, and removal of adherent cells with 0.25% trypsin. The cells were washed twice with PBS and centrifuged at 500 rpms (30 g's) forming a cell pellet that was either lysed or stored intact at -80° C. Cells were lysed by 600 µl RIPA solution (1% NP40, 0.5% sodium deoxycholate, 0.1%SDS) containing protease inhibitors (10µl/ml phenylmethylsulfonyl flouride (PMSF) [10 mg/ml], 30 ul/ml aprotinin [1.9mg/ml], 10 ul/ml sodium orthovanadate (Na_3VO_4) [100 mM]) and phosphatase competitors (p-nitrophenyl phosphate (pNPP) [2-4 mM]). The cell lysate was immediately passed three times through a 21 gauge needle with subsequent addition of 10 µl PMSF [10 mg/ml]. Following 30 minutes incubation on ice, the cell lysates were centrifuged at 14000 rpms

(16000 g's) for 20 minutes at 4° C. The protein containing supernate was separated from the pellet and collected for subsequent assays.

Following the treatment of NCI-H69 cells, the floating cells and low serum media were placed in conical tubes, centrifuged at 500 rpms (30 g's) for 5 minutes, and washed with ice-cold PBS. The cells were subsequently processed in a manner similar to the fetal PNEC.

Raf-1 and MAPK immunoprecipitation: Cell lysates were obtained in a similar technique as for the western blot analysis by using a non-SDS containing lysis buffer (sodium chloride [150 mM], sodium phosphate [50 mM], 1% NP-40, 0.25% sodium deoxychloride, pH7.5). Lysates were incubated for 2 hours at 4° C in immunoprecipitation buffer (Tris-hydroxymethyl-aminomethane [50 mM], EDTA [1 mM], Na_3VO_4 [0.5 mM], 0.1% β-2-mercaptoethanol, 1% Triton X-100, sodium flouride [50mM], sodium pyrophosphate [5 mM], sodium glycerophosphate [10 mM], PMSF [0.1 mM], 1μg/ml aprotinin, 1 μg/ml leupeptin) containing 50% protein G agarose beads (Gibco) coated with anti-Raf-1 sheep polyclonal IgG (2 μg) (Upstate). The Raf-1 antibody recognizes a 12 residue peptide corresponding to amino-acids 627-638 of the human Raf-1 kinase.

Immunoprecipitation of MAPK from cell lysates was performed using an anti-MAPK 1\2 rabbit polyclonal IgG antibody that recognizes a 38 residue peptide that corresponds to residues 333-367 of the rat MAPK domain (Upstate). The amino-acid sequences are totally conserved in hamsters and highly conserved in human MAPK.

Raf-1 and MAPK 1\2 in vitro kinase assay: Cell lysates were assayed in vitro for Raf-1 and MAPK 1\2 activity using a specific kinase assay (Upstate Biotech.) that measures the phophotransferase activity of a kinase cascade reaction initiated by the specific activated protein kinase immunoprecipitated from cell lysates. The immunoprecipitated Raf-1 kinase incites downstream activation of non-active MEK and MAPK which results in phosphorylation of the final substrate myelin basic protein (MBP). Immunoprecipitated MAPK is used to directly transfer γ-^{32}P ATP to the MBP substrate. The Raf-1 immunocomplex was washed in ice-cold immunoprecipitation buffer containing 80 µl ice-cold assay dilution buffer (MOPS (3-[N-marpholino] propanesulfonic acid) [20 mM] pH 7.2, β-glycerol phosphate [25 mM], EGTA [5 mM], Na_3VO_4 [1 mM], dithiothreitol [1 mM]). Following the first wash, the immunocomplex mixture was centrifuged at 14000 rpms (16000 g's) for 15 seconds with subsequent removal of the supernate. The agarose beads with attached immunocomplexes were resuspended with 20 µl assay dilution buffer, 10 µl magnesium/ATP cocktail (ATP [500 µM], magnesium chloride [75 mM] in assay dilution buffer), 0.4 µg recombinant mouse non-active MEK 1 (MEK 1 activates p42 MAPK to a specific activity of 1300U/mg), and 1.0 µg recombinant mouse non-active p42 MAPK. Recombinant mouse MEK 1 and p42 MAPK were fused with a GST vector at the N terminus (71 kDa (MEK 1) and 62 kDa (p42) fusion protein) and expressed in *E. coli*. MEK 1 and p42 MAPK were purified via glutathione-sepharose chromatography to >75 and 90% respectively (Upstate Bio.). The immunocomplex-kinase reaction was incubated on a shaker for 15 minutes at 30° C followed by centrifugation for 15 seconds at

14000 rpms and subsequent separation of the immunocomplex-kinase reaction supernate from the agarose beads. The immunocomplex-kinase reaction supernate (4 µl) was added to 10 µl assay dilution buffer, 0.02 mg MBP, and 100 µCi γ-^{32}P ATP (Dupont-New England Nuclear) that has been diluted 1:10 with magnesium/ATP cocktail. The reaction mixture was incubated on a shaker for 10 minutes at 30° C. P81 phosphocellulose paper was slowly spotted with 25 µl of the reaction mixture followed by three, 5 minute washes in 0.75% phosphoric acid (Fisher) and a 5 minute wash in acetone (Fisher). The washed paper was placed in 5 ml Bio-Safe II™ scintillation cocktail (Research Products International) and read for 1 minute on a Tri-Carb 2300 TR scintillation counter (Packerd) at 98% counting efficiency. Results using the coupled phosphorylation assay demonstrate a lack of MBP phophorylation when activation kinases or target kinases are absent and when non-specific rabbit-IgG antibodies (Santa Cruz) were coated on agarose beads.

The coupled phosphotransferase reaction for MAPK 1\2 was similar to that of Raf-1. Immunoprecipitated MAPK 1\2 was combined with 10 µl 1x assay dilution buffer, 10 µl inhibitor cocktail (PKC inhibitor peptide [20 µM], PKA inhibitor peptide [2 µm], calmidazolium [20 µM]), and 100 µCi γ-^{32}P ATP on a shaker for 10 minutes at 30° C. Subsequent quantification of γ-^{32}P MBP substrate was similar to that performed for Raf-1 activity.

Western blot analysis of Raf-1 and MAPK 1\2 phosphorylation: The cell lysates and protein concentration were prepared and determined in the same manner as for the western blot analysis of Raf-1 and MAPK expression (Part IV). 20 µg of protein from

each sample was boiled in 2x SDS loading buffer, placed onto a 10% PAGE gel, and electrophoretically transferred to nitrocellulose as described for the analysis of Raf-1 and MAPK expression. The membrane was blocked in 5% nonfat dry milk for 1 hour at room temperature. Following three separate 5 minute washes in TBS/0.1% tween-20, the membranes were probed with primary polyclonal rabbit antibodies specific for phosphorylated Raf-1 or phosphorylated MAPK (New England Biolabs) at a dilution of 1:1000 in primary antibody dilution buffer (Raf-1: 5% bovine serum albumin in TBS/0.1% tween-20, MAPK: 5% non-fat dry milk in TBS/0.1% tween-20), overnight at 4° C with gentle agitation. The Raf-1 phospho-specific antibody is directed toward the phosphorylated serine residue at position 259, whereas the phospho-MAPK 1\2 antibody is directed toward the phosphorylated threonine and tyrosine at positions 202 and 204 respectively. The membrane was subsequently incubated with horse radish peroxidase conjugated anti-rabbit secondary antibody (New England Biolabs) at a 1:2000 dilution in blocking buffer for 1 hour at room temperature. Band signals were detected by using LumiGLO™ chemiluminescent detection reagent (New England Biolabs).

Densitometric analysis: Raf-1 and MAPK protein phosphorylation was quantified using SigmaGel™ gel analysis software V1.0 (SPSS Inc.). Threshold points were based on the degree of exposure for each western blot.

Statistical analysis: The results obtained from control groups and cells exposed to NNK were assessed via the unpaired t-test.

Chapter 4: Results

Raf-1 activation: In all experiments examining kinase activation, fetal PNECs were placed in low serum media (0.1% BSA) for 24 hours and washed 30 minutes prior NNK treatment in order to minimize growth factor associated stimulation of signal transduction pathways. Serum starved fetal PNECs were exposed to NNK (100 pM) for 15 minutes while maintained in a 5% CO_2 environment. Control cells were also exposed to low serum media, washed, and maintained in the same CO_2 environment for the same length of time as the treated cells. The PKC inhibitor, sphingosine (10 μM), was placed on cells in low serum media 1 hour prior to NNK exposure. Following, the 15 minute NNK treatment, cell lysates were examined for Raf-1 activation using an in vitro kinase assay and western blots probed with phospho-specific antibodies. Raf-1 activation in NNK treated cells was higher than unstimulated control cells ($p \leq 0.01$) when measuring the phosphotransferase activity within normal PNEC cell lysates (Figure 2-1). This assay quantifies the amount of phosphorylated Raf-1 from cell lysates through the subsequent activation of downstream protein kinases and radiolabeling of the final substrate, MBP. Controls for endogenous phosphorylation of proteins in the sample were performed by substituting assay dilution buffer for MBP. The counts per minute (CPM) were 1.6 to 1.9

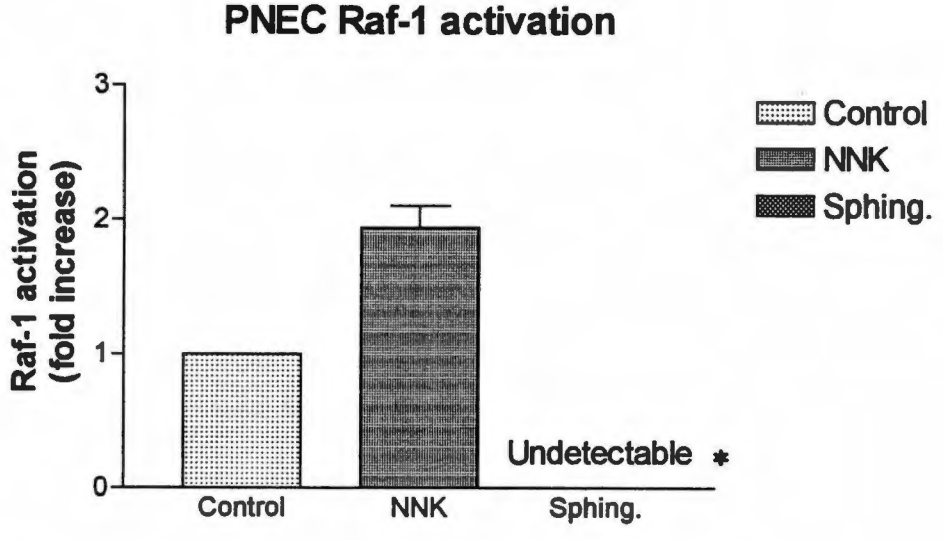

FIGURE 2-1. Activation of Raf-1 in non-neoplastic PNECs. The activation is measured as fold increase over control, and is the compilation of data from phosphotransferase assays and western blot analyses. Control values are normalized at 1. Cells were exposed to NNK 1 hour following sphingosine treatment. The levels of Raf-1 activation in cells pre-treated with sphingosine [10 μM] prior to NNK treatment were not detectable. NNK [100 pM]. (n=6) *(n=2).

fold higher in cells exposed to NNK as compared to untreated control cells. Similar results were obtained using a phospho-specific antibody directed against the phosphorylated serine residue within the Raf-1 CR2 (conserved region 2) domain at position 259. In Figure 2-2, there is a 1.9 fold increase in band (74 kDa) intensity from cell lysates of NNK treated cells (lane 2) over control cells (lane 1). The results of this western blot indicate increased phosphorylation of the Raf-1 CR2 domain in cells exposed to NNK compared to non-treated cells. Furthermore, NNK induced Raf-1 phosphorylation was inhibited by pretreatment of cells with sphingosine (lane 3), indicating PKC dependent activation of Raf-1 following stimulation by NNK (Figure 2-1; Figure 2-2). By examining Figure 2-2, the protein level of Raf-1 in sphingosine treated cells is similar to those cells exposed to NNK but not pretreated with sphingosine. This finding is consistent with the effect of (trans-D-erythro) sphingosine as a competitive inhibitor of the second messenger diacylglycerol (DAG), and blocks the subsequent activation of PKC substrates with little or no effect on protein levels (Bell, 1988). This further substantiates the effect of this PKC inhibitor on NNK induced activation of Raf-1. For all samples, protein was quantified and actin was probed to insure equal protein loading per well. Cells were also treated with sphingosine without the addition of NNK (0.1 fold fractional value of control).

The NNK induced Raf-1 activation in normal PNECs was also blocked by the α_7 nicotinic receptor antagonist, α-bungarotoxin (10 μM), and the 5-HT uptake inhibitor, imipramine (1 μM) (Figure 2-3). Data expressed as a percentage of NNK induced

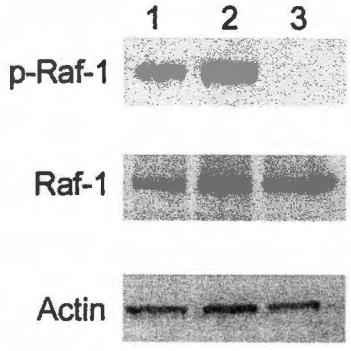

FIGURE 2-2. Western blot of Raf-1 phosphorylation in non-neoplastic PNECs. p-Raf-1 (74 kDa) represent phosphorylated Raf-1 in untreated control cells (lane 1), NNK [100 pM] treated cells (lane 2), and NNK exposed cells pre-treated with sphingosine [10 μM] (lane 3). Raf-1 bands are composed of both phosphorylated and non-phosphorylated Raf-1 protein, and the actin bands denote an internal control for equal loading of protein samples.

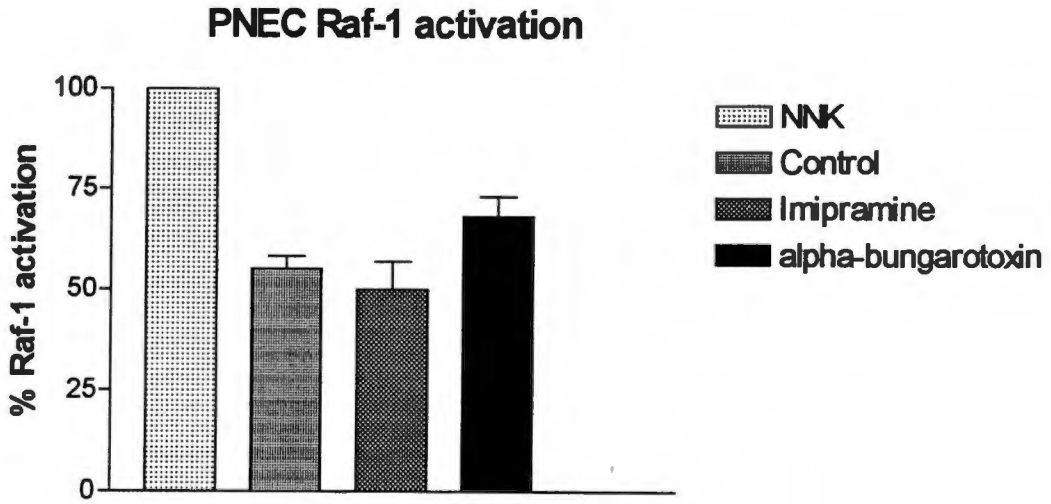

FIGURE 2-3. Receptor-mediated inhibition of Raf-1 activation in non-neoplastic PNECs. Using CPM from phosphotransferase assays, Raf-1 activation in cells exposed to NNK [100 pM] are normalized at 100%. Untreated control cells and cells pre-treated with the 5-HT uptake inhibitor imipramine [1 μM] or the nicotinic receptor antagonist α-bungarotoxin [10 μM] are expressed as a percentage of total Raf-1 activation. Cells were pre-treated with receptor antagonists and uptake inhibitor 10 minutes prior to NNK treatment (n=3).

activation demonstrates a 50% reduction in Raf-1 activation in NNK stimulated cells that were pretreated with imipramine ($p<0.01$) as compared to control cells which represent 55% of total Raf-1 activation. Blocking the α_7 nAChR with α-bungarotoxin resulted in a 32% reduction in total Raf-1 activation and 71% of the NNK induced Raf-1 activation ($p<0.05$). These data suggest that NNK binding to the α_7 nAChR results in 5-HT uptake via the GPCR which subsequently results in the activation of Raf-1.

NNK induced similar kinase activation in the neoplastic PNEC (NCI-H69). Measuring phosphotransferase activity and Raf-1 phosphorylation, there was a 1.9 fold increase ($p<0.01$) in Raf-1 activation compared to control cells (Figure 2-4). The band intensity of the 74 kDa protein was 1.5 to 2.2 fold higher in cells exposed to NNK than in unstimulated control cells (Figure 2-5). Furthermore, pretreatment of cells with the PKC inhibitor, sphingosine, resulted in decreased phosphorylation of Raf-1 in stimulated cells (Figure 2-5). Treatment of NCI-H69 cells with sphingosine resulted in a 0.6 fold change. Similar to the results demonstrated in the normal PNEC, sphingosine had little effect on protein levels of Raf-1. Cells pretreated with the receptor antagonist α-bungarotoxin and the 5-HT uptake inhibitor imipramine, also contained reduced levels of activated Raf-1 compared to cells exposed to NNK without pretreatment with a receptor antagonist. With data expressed as a percentage of NNK induced activation, the inhibition of Raf-1 activation via imipramine and α-bungarotoxin (figure 2-6) indicates the effect of NNK is mediated through the α_7 nAChR and the 5-HT receptor. Blocking the α_7 receptor with α-bungarotoxin resulted in an 80% reduction of the induced NNK activation of Raf-1 and

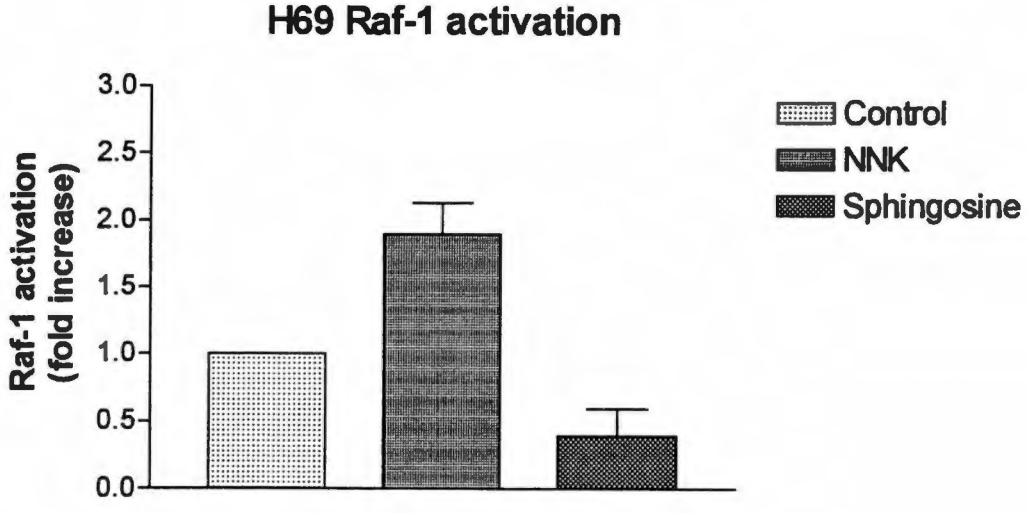

FIGURE 2-4. Activation of Raf-1 in neoplastic PNECs (NCI-H69 cells). Data represent a compilation of results from phosphotransferase assays (CPM) and western blot analyses (pixel units). Control values are normalized at 1. Cells were treated with the PKC inhibitor 1 hour prior to NNK exposure. (n=4). NNK [100 pM]; sphingosine [10 μM]

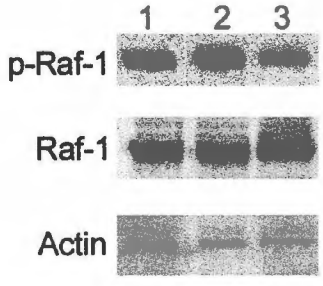

FIGURE 2-5. Western blot of Raf-1 phosphorylation in neoplastic PNECs (NCI-H69). p-Raf-1 (74 kDa) represents phosphorylated Raf-1 in untreated control cells (lane 1), NNK [100 pM] treated cells (lane 2), and NNK exposed cells pre-treated with sphingosine [10 μM] (lane 3). Raf-1 bands are composed of both phosphorylated and non-phosphorylated Raf-1 protein, and the actin bands denote an internal control for equal loading of protein samples. Blot is representative of at least three separate experiments.

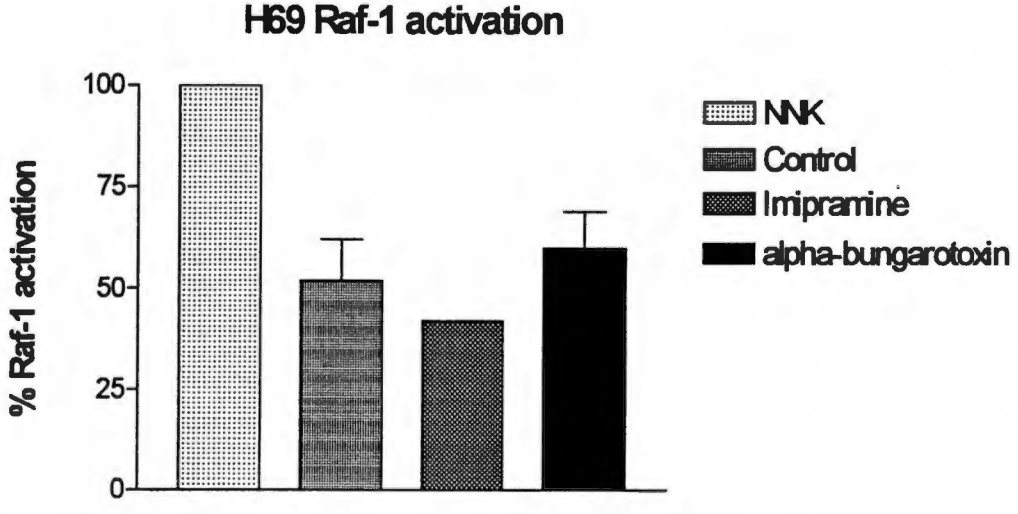

FIGURE 2-6. Receptor-mediated inhibition of Raf-1 activation in neoplastic PNECs (NCI-H69 cells). Using CPM from phosphotransferase assays, Raf-1 activation in cells exposed to NNK [100 pM] are normalized at 100%. Untreated control cells and cells pre-treated with the 5-HT uptake inhibitor imipramine [1 μM] or the nicotinic receptor antagonist α-bungarotoxin [10 μM] are expressed as a percentage of total Raf-1 activation. The receptor antagonist and uptake inhibitor were applied to cells 10 minutes prior to NNK treatment. (n=3).

a 40% reduction in total Raf-1 activation in stimulated cells($p<0.01$). Additionally, inhibition of serotonin uptake by imipramine completely prevented the activation of Raf-1 kinase by NNK. Therefore, in neoplastic and normal PNECs, NNK appears to stimulate the activation of Raf-1 protein kinase via α_7 nAChR ligand binding, the uptake of 5-HT, and a PKC dependent pathway.

MAPK (Erk) activation: In order to examine the effect of NNK on downstream components of the MAP kinase cascade, serum starved fetal PNEC were treated with 100 pM NNK for 15 minutes while maintained in a 5% CO_2 environment. Cell lysates were subsequently examined for MAPK p44/42 activity and phosphorylation by way of an in vitro kinase assay and western blot analysis using a phospho-specific antibody. Inhibition of MAPK activity was achieved through the use of the selective MEK inhibitor PD98059 (50 μM) which was placed onto the cells 1 hour prior to NNK exposure. MEK is a direct upstream effector of MAPK p44/42, and the inhibitor binds to inactive MEK 1/2 which blocks phosphorylation of the kinase domain by upstream effectors such as Raf-1 and MEKK (Alessi, 1995; Dudley, 1995). By using the in vitro kinase assay, a 3.0 to 3.4 fold increase in MAPK activity, as measured in CPM, was observed in normal PNECs exposed to NNK compared to untreated control cells ($p<0.01$). A similar 2.0 to 3.0 fold increase in MAPK phosphorylation was demonstrated when analyzed by western blots probed with a phospho-MAPK antibody (Figure 2-7). Examination of figure 2-7 reveals greater phosphorylation of the 42 kDa MAPK protein versus the 44 kDa protein, although the antibody is specific for either phosphorylated isoform. This difference may be the result of

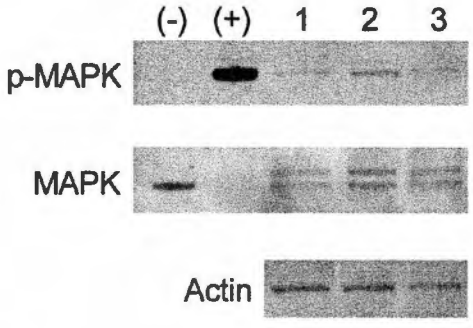

FIGURE 2-7. Western blot of MAPK phosphorylation in non-neoplastic PNECs. p-MAPK (44 and 42 kDa) represents phosphorylated MAPK in untreated control cells (lane 1), NNK [100 pM] treated cells (lane 2), and NNK exposed cells pre-treated with the MEK inhibitor PD98059 [50 uM] (lane 3). MAPK bands are composed of both phosphorylated and non-phosphorylated MAPK protein. +/- controls consist of bacterially expressed active and inactive MAPK 2 (42 kDa). Actin bands denote an internal control for equal loading of protein samples. Blot is representative of at least three separate experiments.

preferential phosphorylation of MAPK 2 (Erk 2) by the MEK 2 isoform. Therefore, as shown in figure 2-8, MAPK in NNK treated PNECs was activated and phosphorylated 3.0 fold over untreated cells ($p<0.01$). Furthermore, the NNK induced activation and phosphorylation of MAPK can be completely inhibited by pretreating cells with PD98059 (Figure2-7; Figure 2-8) ($p<0.01$). As controls, cells were treated with PD98059 without subsequent NNK exposure (0.9 fold fractional value of control). Although MAPK p44/42 phosphorylation is directly affected by the MEK inhibitor, protein levels were not significantly different between NNK exposed cells treated with PD98059 and NNK exposed cells not treated with the inhibitor (Figure 2-7). Therefore, as demonstrated in figure 2-7, the reduced signal observed by using a phospho-specific antibody on normal PNECs treated with NNK and PD98059 (lane3) is the result of inhibition of phosphorylation/activation of MAPK by MEK, and not specific protein degredation.

MAPK p44/42 is also activated in neoplastic H69 cells that have been exposed to NNK (100 pM) for 15 minutes in a 5% CO_2 environment ($p<0.05$). Similar to the effect observed in normal PNECs, NNK stimulated activation/phosphorylation of MAPK 3.0-6.5 fold over untreated control cells (Figure 2-9). Additionally, treatment with PD98059 completely blocked the effect of NNK on MAPK without altering protein levels (Figure 2-10) ($p<0.05$). Cells treated with PD98059 without NNK exposure resulted in a 0.14 fold response compared to controls cells. These data demonstrate a direct effect on MAPK p44/42 activation via a PKC/Raf-1/MEK dependent pathway by low concentrations of NNK in both normal and neoplastic PNEC. This NNK induced activation was inhibited by blocking the path of signal transduction at the level of MEK 1/2.

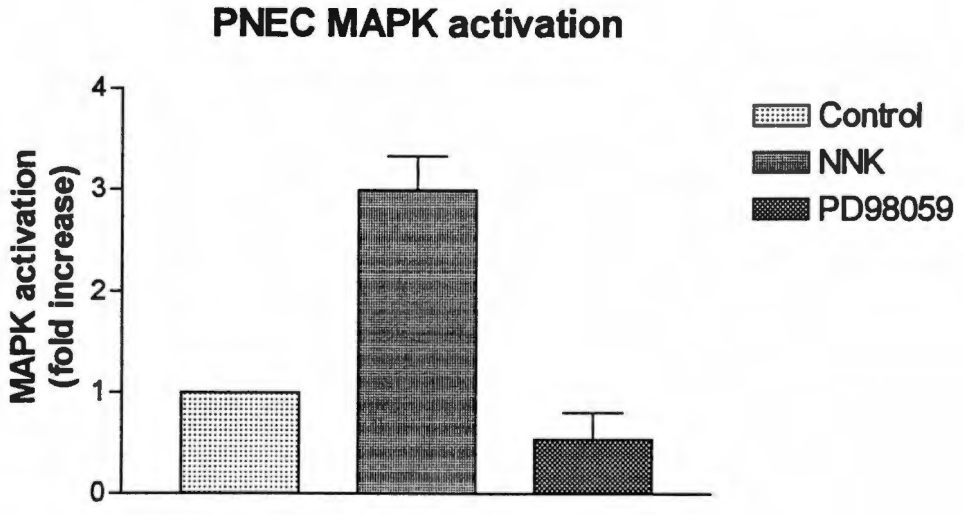

FIGURE 2-8. Activation of MAPK in non-neoplastic PNECs. Data represent a compilation of results from phosphotransferase assays (CPM) and western blot analyses (pixel units). Control values are normalized at 1. Cells were pre-treated with the MEK inhibitor 1 hour prior to NNK treatment. (n=4). NNK [100 pM]; PD98059 [50 μM].

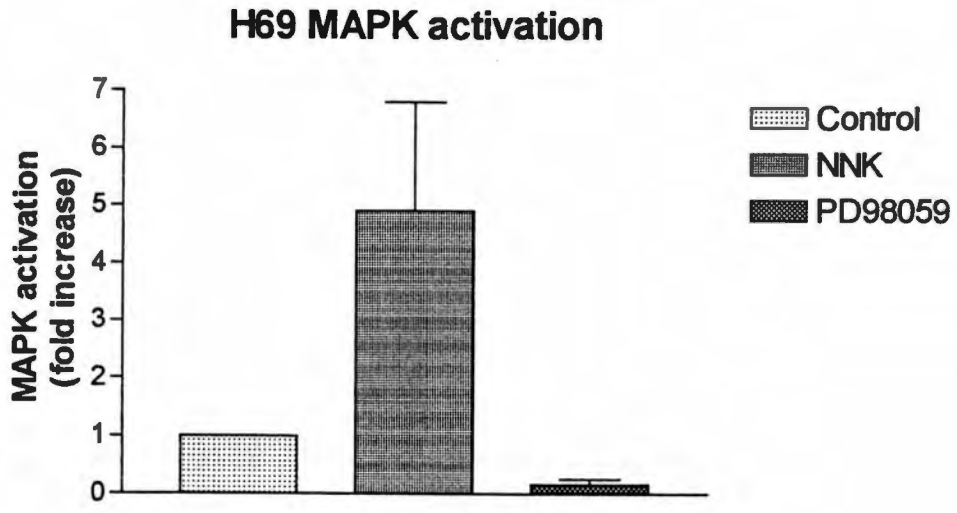

FIGURE 2-9. Activation of MAPK in neoplastic PNECs (NCI-H69). The data represent a compilation of results obtained from phosphotransferase assays (CPM) and western blot analyses (pixel units) where the control groups are normalized at 1. Cells were treated with the MEK inhibitor 1 hour prior to NNK treatment. (n=3). NNK [100 pM]; PD98059 [50 μM].

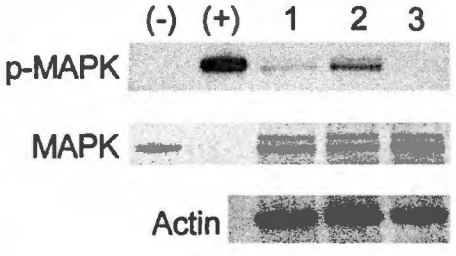

FIGURE 2-10. Western blot of MAPK phosphorylation in neoplastic PNECs (NCI-H69 cells). p-MAPK (44 and 42 kDa) represents phosphorylated MAPK in untreated control cells (lane 1), NNK [100 pM] treated cells (lane 2), and NNK exposed cells pre-treated with PD98059 [50 uM] (lane 3). MAPK bands are composed of both phosphorylated and non-phosphorylated MAPK protein.
+/- controls consist of bacterially expressed active and inactive MAPK 2 (42 kDa). Actin bands denote an internal control for equal loading of protein samples. Blot is representative of at least three separate experiments.

Chapter 5: Discussion

Intracellular signaling plays a vital, yet poorly understood role in transmitting an extracellular stimulus into a cellular response such as proliferation or apoptosis. The initiating signal and the interaction with its complementary receptor in a particular cell type determines the signaling pathway to be used in eliciting a specific response. Experiments were performed to evaluate the effect of the tobacco-derived nitrosamine, NNK, on the activation of the MAP kinase cascade and its major constituents, Raf-1 and MAPK (Erk 1/2). Figure 2-11 illustrates a proposed pathway by which NNK in conjunction with elevated CO_2 results in the concerted release/re-uptake of 5-HT and the subsequent activation of Raf-1 and MAPK. It is proposed that NNK acting through the α_7 nAChR Ca^{2+} channel receptor stimulates the activation of Raf-1 and MAPK. To test this hypothesis, normal and neoplastic PNECs were exposed to low concentrations of NNK for 15 minutes in a low CO_2 environment. The changes observed indicate that NNK stimulates a greater than 1.5 fold increase in Raf-1 and MAPK activation. In the case of MAPK, the level of activation was as high as 3 and 6 fold in normal and neoplastic PNECs respectively. In these experiments, serum starved control cells and treated cells were thoroughly washed and immediately replaced in low serum media to insure that the

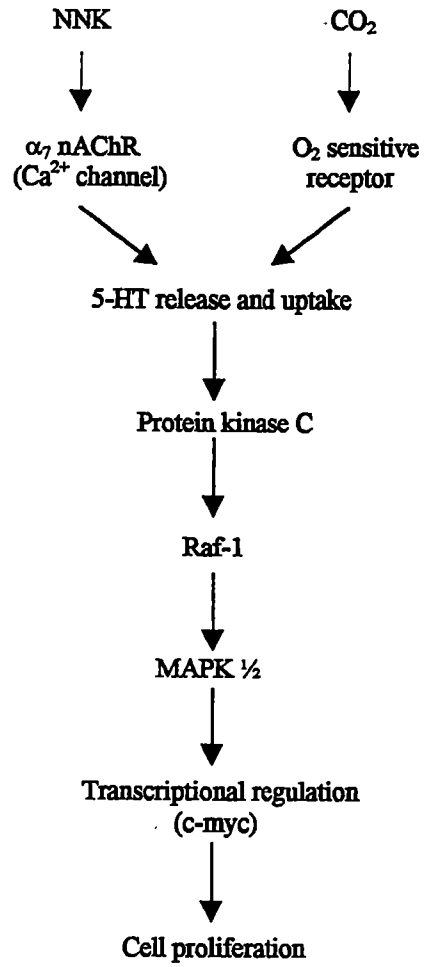

FIGURE 2-11. Proposed mechanism for the activation of the MAP kinase cascade by NNK.

observed activation was the result of NNK treatment and not due to serum induced growth factors or secretagogues. Also all cells were maintained before and during the experiment in 5% CO_2 to reduce the mitogenic effect of hypoxia on PNECs which has been demonstrated in previous studies (Merryman, 1997; Schuller, 1994). Our results were repeatable and consistent using an assay for phosphotransferase activity and western blots analyzing phosphorylation of specific kinase amino acid residues. Therefore, there appears to be a correlation between the activation of these kinases and the phophorylation of specific amino acids. In fact, previous studies have shown that phosphorylated serine 259 of Raf-1 (Williams, 1994) and phosphorylated Thr202/Tyr204 of MAPK (Marshall, 1995) result in the activation of these kinases. Kolch (1993), Morrison (1993), and Sozeri (1992) have demonstrated PKC mediated activation of Raf-1 through the phosphorylation of serine 259. The antibodies used in our experiments are highly specific for the phosphorylation of these amino acid residues.

It is interesting to note that MAPK in both normal and neoplastic cells was activated to a greater extent than the upstream Raf-1 kinase. This is likely due to a promoting effect in which a smaller stimulatory signal leads to sequentially increased amounts of activated substrate. This effect has been readily associated with a variety of receptor-mediated signaling events, including GPCRs and the MAP kinase pathway (Lamb, 1992). Furthermore, this amplification of a receptor-mediated signal is consistent with our findings of sequentially higher levels of activated protein kinases within the MAP kinase pathway that were induced by a low concentration of NNK (100 pM). Preliminary

experiments using higher concentrations of NNK induced similar activation of Raf-1 and MAPK as did low dose concentrations. Since NNK is normally present in tobacco smoke at parts-per-million range and within pulmonary airspaces in picomolar concentrations (Schuller, 1991), we decided to use a low (100 pM) concentration in all experiments.

The level of MAPK activation in the neoplastic PNECs was shown to be higher than in normal cells. This difference in the degree of activation may be attributed to higher levels of kinase protein expression or an upregulation of α_7 nicotinic receptors in the tumor cells compared to normal PNECs. Tumor cells expressing higher levels of Raf-1 protein may demonstrate a similar degree increase (fold increase) in activation compared to normal cells, but have a much higher level of total activation. This would result in a greater amplification of the signal and increased activation of downstream effectors such as MAPK. Additionally, high levels of MAPK protein may further contribute to the amplification of the signal by increasing the amount of protein available for activation. A second possibility pertains to the upregulation of the α_7 nicotinic receptor and increased receptor numbers in neoplastic PNECs. NNK saturation of larger α_7 receptor numbers would be expected to initiate a greater receptor-mediated response, and subsequent signal amplification. However, in considering the latter, one might expect also to see a higher fold increase of Raf-1 in neoplastic cells compared to normal PNECs. Although constitutively high levels of MAPK protein have been demonstrated in SCLC (Jull, Part IV), investigation into α_7 receptor upregulation is in progress.

In order to further characterize the pathway by which NNK stimulates the MAP

kinase pathway, various inhibitors were used to block the NNK induced activation of Raf-1 and MAPK. As demonstrated in figure 2-2 and figure 2-5, the PKC inhibitor, sphingosine, completely inhibited the NNK induced Raf-1 activation in normal and neoplastic PNECs. Sphingosine acts as a reversible competitive inhibitor of diacylglycerol dependent activation of PKC. Side by side comparison of Raf-1 activation and expression from each type of PNEC shows that although there is reduction in activation, protein levels remain uneffected by the inhibitor. Therefore, Raf-1 activation, but not expression, is a PKC dependent process in NNK stimulated cells. Our data are consistent with findings reported by Schuller (1994) when using the PKC inhibitor (dexniguldipine) on neoplastic PNECs to reduce NNK induced cell proliferation. Further examination of these western blots (Figure 2-2, Figure2-5) also showed that the signal from NNK exposed cells treated with sphingosine (lane3) was lower compared to untreated control cells (lane 1). This indicates a degree of dependency these cells have on PKC in spite of NNK stimulation. Although Ras involvement cannot be ruled out by our data, stimulation of a Ras mediated pathway would be expected to have resulted in MAP kinase pathway stimulation despite PKC inhibition. Previous studies examining non-small cell lung carcinomas (NSCLC) have reported point mutations in the Ras gene at codons 12, 13, or 61 as a result of nitrosamine induced DNA adduct formation with subsequent constitutive activation of this G protein (Richardson, 1993). This constitutive Ras activation in turn results in the recruitment and activation of Raf-1. However, Ras mutations have been shown to be absent in SCLC (Sekido, 1998), and our data suggest that NNK induced

Raf-1 activation via a PKC dependent receptor.

To further characterize the NNK induced stimulation of the MAP kinase pathway, an inhibitor targeting a kinase downstream of Raf-1 was used. The MEK inhibitor, PD98059, completely abolished the NNK induced MAPK activation in both normal and neoplastic PNECs (Figure 2-7, Figure 2-10). PD98059 ([2-(2'-amino-3'-methylphenyl)-oxanaphthalen-4-one] is a flavinoid compound that blocks the phosphorylation of the dual specificity kinase MEK 1\2 (Alessi, 1995; Dudley, 1995). This effectively and specifically severs the MAP kinase signaling cascade, preventing a signal via Raf-1 from activating MAPK. As demonstrated in Figures 2-7 and 2-10, the reduced band intensity of lane 3 (cells exposed to NNK and treated with PD98059) indicates complete blockage of NNK induced, MEK mediated MAPK 1\2 activation. Additionally, the degree of inhibition rules out NNK stimulated MAPK activation via an alternate pathway since this would be expected to result in no change or at least less of a reduction in the band intensity within lane 3 compared to lane 2. In fact, the intensity of the band in lane 3 is slightly less than the band in lane 1 (untreated control cells), suggesting some degree of suppression of basal MAPK activation. This is not surprising since the MAP kinase cascade serves as a universal pathway for a large variety of initiating stimuli. Furthermore, the reduction in MAPK activation resulting from PD98059 treatment had no effect on protein expression. This may be indicative of an MEK independent process responsible for MAPK protein expression which may include an NNK stimulated transcriptional pathway or NNK induced relocalization of the kinase within the cell.

Neither of these intracellular events would be affected by treatment with the MEK inhibitor. Protein levels were examined on the same membrane and from the same sample used to identify kinase activation, and protein loading was uniform as demonstrated by the actin internal control (Figure 2-7, Figure 2-10).

Therefore, inhibition of NNK induced activation at two non-consecutive points within the signaling cascade demonstrates the strong predilection for use of this pathway by the NNK initiated signal. It is interesting to note that in previous studies, NNK in a 5% CO_2 environment did not significantly increase neoplastic or normal PNEC proliferation within a 24 hour time period compared to cells exposed to NNK in 10% CO_2 (Schuller, 1994). This points to the potentiating effect of CO_2 in PNECs exposed to NNK. Although NNK stimulates the activation of the MAP kinase pathway in a short period of time, as demonstrated with our data, an increase in cell number may require a dramatic upregulation of the α_7 receptor via elevated CO_2. This may serve to magnify the effect of the receptor mediated NNK stimulus on Raf-1 and MAPK activation resulting in quantifiable cell proliferation. Our experiments were conducted in the absence of high CO_2 so as to diminish its potential mitogenic effect and examine the response to NNK alone. In light of our data, it would be reasonable to expect that upregulation of the α_7 nAChR would result in an amplification of the induced effect.

In order to verify the role of the α_7 receptor in the observed signaling response, normal and neoplastic cells were treated with the site-selective α_7 receptor antagonist, α-bungarotoxin (Gopalakrishnan, 1995; Bertrand, 1997), exposed to NNK, and

subsequently analyzed for Raf-1 activation. α-Bungarotoxin is a component of venom extracted from the Braided Krate snake that irreversibly binds to the α subunit of the nAChR, stabilizing the closed resting state of the Ca^{2+} channel (Atassi, 1988). As shown in figures 2-3 and 2-6, both normal and neoplastic cells treated with the receptor antagonist contained reduced amounts of activated Raf-1 kinase as measured by phosphotransferase activity. This demonstrates the selective receptor-ligand binding of NNK and its downstream effect on the activation of the MAP kinase pathway. Although the majority of the NNK induced activation was inhibited by α-bungarotoxin (71% and 83% for normal and neoplastic PNECs respectively), it did not completely inhibit the stimulated Raf-1 activation. This may be due to NNK binding to other nicotinic receptors not sensitive to α-bungarotoxin. Although other nicotinic receptors may exist, their concerted effect on 5-HT release and uptake and MAP kinase pathway activation, as outlined in figure 2-11, may be similar. To investigate this possibility and the downstream role of 5-HT, experiments were conducted using the 5-HT uptake inhibitor, imipramine. According to our proposed pathway in which NNK stimulates the MAP kinase cascade via the release and uptake of 5-HT (Figure 2-11), a 5-HT uptake inhibitor would be expected to result in broader receptor-mediated inhibition of NNK. This explanation is supported by data presented in figures 2-3 and 2-6, where NNK treated cells exposed to imipramine contained reduced levels of activated Raf-1 compared to NNK treated cells

with and without α-bungarotoxin. In many instances Raf-1 activation was reduced below basal levels in imipramine treated cells, supporting the documented role of 5-HT as an autocrine growth factor in these neuroendocrine cells (Schuller, 1998). Therefore, 5-HT not only plays a role in intracellular basal processes, but also plays a central role in NNK induced activation of the MAP kinase pathway. Additionally, the direct involvement of 5-HT and PKC in the activation of Raf-1 points to the role of the GPCR; since 5-HT receptors are characteristically transmembrane coupled GPCRs (Hoyer, 1994), and PKC may act as a downstream substrate of a GPCR mediated signal (Gutkind, 1998). Although there are as many as 15 various subtypes of 5-HT receptors, studies have linked the 5-HT_2 class of receptors to the activation of PLCβ (phospholipase C) via the α subunit of the G_q class ($G_{\alpha q}$) of heterotrimeric GTP binding proteins (Hoyer, 1994). PLC activation results in phosphatidylinositol hydrolysis which generates 1,4,5-trisphophate (IP_3) and DAG for the mobilization of intracellular Ca^{2+} and the activation of PKC. $G_{\alpha q}$ has also been shown to effect the MAP kinase pathway through a proline rich tyrosine kinase, Ras dependent mechanism mediated through activated PLC and IP_3 (Lev, 1995; Bourne, 1995). However, this is a PKC independent mechanism not expected to be blocked by the PKC inhibitor sphingosine. Utilization of this PKC independent pathway by NNK to induce Raf-1 activation is not supported by our data. Therefore, it is likely that NNK acting via the α_7 nicotinic receptor stimulates activation of the MAP kinase cascade to a significant extent through a 5-HT/PKC dependent pathway.

In addition to the $G_{\alpha q}$ GPCR, the $G_{\alpha o}$ subfamily of the G_i class of GPCR has also been shown to regulate activation of the MAP kinase pathway in a PKC dependent, Ras independent manner (van Biesen, 1996). Although the signaling mechanism is not entirely clear, this GPCR is known to mediate a signal leading to the hydrolysis of phosphoinositol and subsequently effect the MAP kinase cascade (Moriarty, 1990; van Biesen, 1996). As demonstrated in figure 2-12, $G_{\alpha o}$ GPCR mediated activation of PKC may be an alternative means by which Raf-1 and MAPK of the MAP kinase pathway could be activated by NNK mediated release and uptake of 5-HT. However, to date there is no evidence of 5-HT ligand binding to the G_o subfamily of GPCRs.

Of equal importance to the stimulatory effects mediated by the $G_{\alpha q}$ and $G_{\alpha o}$ on the MAP kinase cascade are the inhibitory effects of other GPCRs on this mitogenic pathway. As reported by Cook (1993) and Sevetson (1993), $G_{\alpha s}$ may have a growth inhibitory effect via negative regulation of Raf-1 signaling through protein kinase A (PKA). Upon stimulation of this GPCR, there is adenylyl cyclase (AC) mediated formation of cylic adenosine monophosphate (cAMP) which subsequently activates PKA. Mischak et al. (1996) has shown that PKA negatively regulates Raf-1 activation by directly phosphorylating ser 621 within the catalytic domain. This, in turn, would block MAP kinase signal transduction induced by mitogenic stimuli. However, our data indirectly suggests inapparent PKA inhibition of Raf-1 in both the neoplastic and non-neoplastic PNECs exposed to NNK. In both cell lines, PKA would be expected to have attenuated

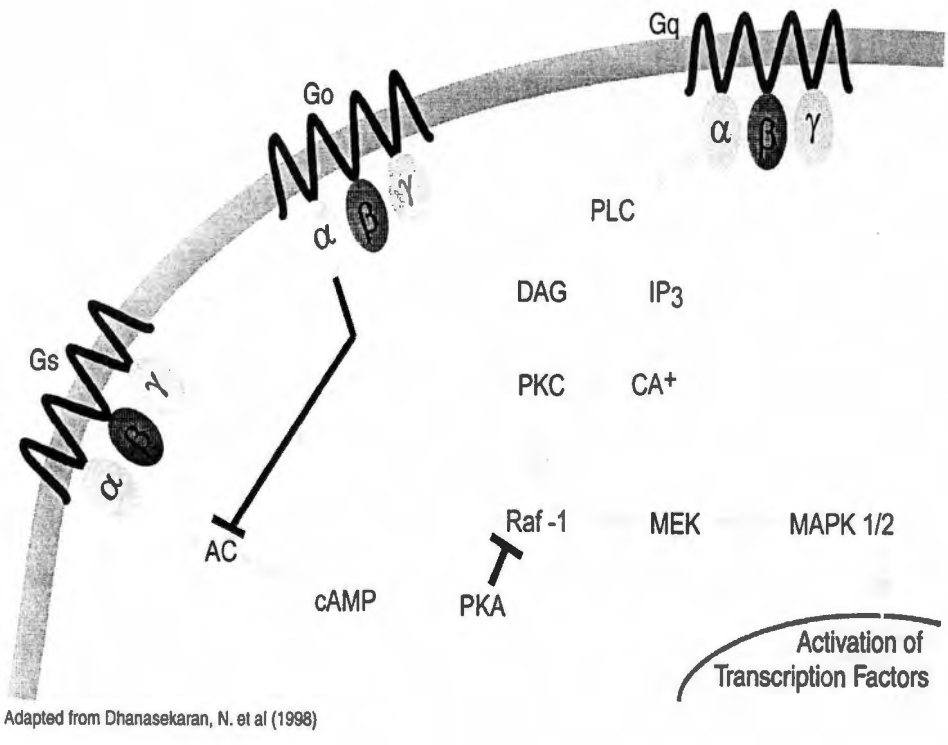

FIGURE 2-12. G-protein coupled receptor-mediated activation and inhibition of the MAP kinase pathway via 5-HT binding and uptake by GPCR subfamily receptors (Gq, Go, Gs). Activation of intracellular signaling pathways occurs through the phosphorylation of the GPCR subunits (α, β, γ).

or blocked NNK induced, PKC dependent activation of Raf-1 and subsequent activation of MAPK when compared to untreated control cells. This may be due to a lack of $G_{\alpha s}$ receptor mediated binding to 5-HT, inhibition of AC by G_o GPCR involvment, or NNK induced preferential signaling via PKC as opposed to PKA (Figure 2-12). Additionally, most evidence supporting the inhibitory role of PKA is in relation to a Ras-dependent pathway (Cook, 1993; Sevetson, 1993) which was found not to exist in our experiments. Characterization and subcatagorization of the 5-HT receptor involved in the NNK signaling pathway is the subject of future investigation, as is the mechanism of 5-HT release and uptake within NNK and CO_2 stimulated PNECs.

By examining the effect of NNK on the MAP kinase pathway and the activation of its major constituents Raf-1 and MAPK, we have shown in conjunction with previous work that NNK ligand binding to the α_7 nicotinic receptor results in the activation of the MAP kinase cascade which is mediated by 5-HT release and uptake via GPCRs. This is believed to be a highly relevant process in the development of PNEC hyperplasia and subsequent cellular transformation in the lung.

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PART III: NNK INDUCED PHOSPHORYLATION OF MYC

Chapter 1: Abstract

Numerous signaling pathways within the pulmonary neuroendocrine cell (PNEC) converge upon specific nuclear proteins that are directly and indirectly responsible for the regulation of cell growth, quiescence, and death. Myc is one such protein, that may regulate transcription and result in either growth or apoptosis depending on its interactions with other key protein kinases and transcription factors. We conducted experiments on normal and neoplastic PNECs to determine the effect of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on Myc expression and phosphorylation. Our data demonstrate NNK induced phosphorylation of Myc in both neoplastic and non-neoplastic cells with no apparent changes in levels of expression. These changes corresponded to changes in the activation of MAPK 1\2 following NNK exposure, and were subsequently inhibited at the level of MEK within the MAP kinase pathway. These results demonstrate a link between NNK receptor mediated stimulation, the MAP kinase pathway, and c-myc. It further links the NNK induced intracellular signaling events to an intranuclear transcriptional regulatory event.

Chapter 2: Introduction

In most cell types the c-myc nuclear phosphoprotein is a short-lived transcriptional regulator that has been implicated in growth, differentiation, and apoptosis (Ramsey, 1986; Gross-Mesilaty, 1998; Hershko, 1998). The c-myc protein effects its control on gene transcription through the formation of a heterodimer complex with another nuclear protein, MAX (Gupta, 1993; Sekido, 1998). This heterodimer in turn recognizes a specific consensus sequence within the DNA strand which results in downstream gene transcription. The action of the Myc/MAX complex is partially regulated and antagonized by the high affinity competitive binding of MAX by a seperate nuclear protein (MAD) (Gupta, 1993; Sekido, 1998). Although the mechanisms surrounding these interactions are not well understood, the phosphorylation of specific amino acids within the N-terminal domain of the c-myc protein has been suggested to play a role in its transactivating capabilities (Gupta, 1993). It has further been demonstrated that these specific amino acid residues are substrates for mitogenic signaling pathways and their constituent kinases (Lutterbach, 1994). Specifically, MAPK 2 has been shown to phosphorylate serine 62 *in vitro* and *in vivo* with resultant *in vitro* modulation of c-myc transactivation potential (Alverez, 1991; Gupta, 1993; Chuang, 1994).

Having demonstrated the effect of NNK on the MAP kinase cascade, our intention was to examine the effect of this tobacco-specific nitrosamine on the phosphorylation status of the c-myc protein. The elicited increase in specific Myc phosphorylation links the receptor mediated stimulation of the MAP kinase pathway by NNK to a nuclear transcription factor that has been implicated in the control of cell growth and differentiation. These findings are not only important in substantiating the effect of MAPK on this nuclear phosphoprotein, but they are the first to demonstrate a direct effect of NNK on Myc, the amplification and overexpression of which has been widely associated with SCLC (Krystal, 1988; Johnson, 1996; Sekido, 1998). This provides additional support and a potential mechanism by which NNK stimulates cell proliferation in normal and neoplastic PNECs.

Chapter 3: Materials and Methods

Cell Culture: Syrian golden hamsters were used as the source for normal pulmonary neuroendocrine cells, and were obtained from the 15 day gestational fetal lungs. General maintenance of hamsters prior to harvesting lung tissue included a standardized housing environment with *ad libitum* commercial feed and water. The lungs were aseptically harvested, followed by cellular dissociation using 2% trypsin for 1 hour in 10% CO_2 at 37° C. Subsequently cells were transferred and maintained in RPMI 1640 medium (Biofluids) supplemented with 10% fetal bovine serum (FBS) in a 10% CO_2 atmosphere. All cells were grown to 60-80% confluence in 25 or 75 cm^2 vented culture flasks prior to experimental procedures. The NCI-H69 (small cell carcinoma) cell line was obtained from the American Type Culture Collection. The H69 cells were cultured in RPMI 1640 medium supplemented with 10% FBS in a 5% CO_2 atmosphere prior to their use in experiments.

Cell treatment and lysate production: Twelve to 24 hours prior to the experiment, NCI-H69 cells and fetal PNEC were placed into low serum Dulbecco's modified eagles media (DMEM) (Biofluids) containing 0.1% bovine serum albumin (BSA), 210 mg L-glutamine,

500 units penicillin, and 500 mcg streptomycin and maintained in 5% CO_2. Cells were washed with 1x Ca^{2+} free phosphate buffered saline (PBS) and reintroduced to low serum media 30 minutes to 1 hour before the onset of the experiment. Protein kinase inhibitors were placed on the designated cells at least 1 hour preceding NNK exposure. NNK and chemical inhibitors were made fresh within 3 hours of experimental use.

Collection of the fetal PNEC involved removal of the low serum DMEM media containing the designated treatment, washing the cells with ice-cold 1x Ca^{2+} PBS, and removal of adherent cells with 0.25% trypsin. The cells were washed twice with PBS and centrifuged at 500 rpms (30 g's) forming a cell pellet that was either lysed or stored at -80° C. Cells were lysed by 600 µl RIPA solution (1x PBS, 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate) containing protease inhibitors (10µl/ml phenylmethylsulfonyl flouride (PMSF) [10 mg/ml], 30 µl/ml aprotinin [1.9mg/ml], 10 µl/ml sodium orthovanadate (Na_3VO_4) [100 mM]). A 21 guage needle was used to further break up cellular constituents by immediately passing the lysate three times through the needle with subsequent addition of 10 µl PMSF [10 mg/ml]. The lysate was incubated 30 minutes on ice, then centrifuged at 14000 rpms for 20 minutes at 4° C. The supernate containing the protein was separated from the pellet and collected for subsequent assays.

Following the treatment of NCI-H69 cells, the cells and low serum media were placed in conical tubes, centrifuged at 500 rpms (30 g's) for 5 minutes, and washed twice with ice-cold PBS. The cells were subsequently processed in a manner similar to the fetal PNEC.

Western blot analysis of c-myc protein phosphorylation: Protein concentration was determined in the same manner as for the western blot analysis of Raf-1 and MAPK expression. 20 μg of protein from each sample was boiled in 2x SDS loading buffer, placed onto a 10% PAGE gel, and electrophoretically transferred to nitrocellulose as described for the analysis of Raf-1 and MAPK expression. For previously probed membranes, attached antibodies were removed by incubating the blot in striping buffer (350 μl 2-2-mercaptoethanol [100 mM], 8 ml SDS [2%], 3.125 ml Tris-Cl pH 6.7 [62.5 mM], 38.5 ml deionized water) for 30 minutes to 1 hour in a 50° C water bath with gentle agitation. Following antibody removal, membranes were washed twice in TBS/0.1% tween-20 for 20 minutes prior to incubation with phospho-c-myc antibody. The membrane was blocked in 5% nonfat dry milk for 1 hour at room temperature. Following three separate 5 minute washes in TBS/0.1% tween-20, the membrane was probed with primary polyclonal rabbit antibodies specific for phosphorylated c-myc (New England Biolabs) at a dilution of 1:1000 in TBS/0.1% tween-20 containing 5% BSA overnight at 4° C with gentle agitation. The c-myc phospho-specific antibody is directed toward the phosphorylated threonine and serine residues at positions 58 and 62 respectively. The membrane was subsequently incubated with horse radish peroxidase conjugated anti-rabbit secondary antibody (New England Biolabs) at a 1:2000 dilution in blocking buffer for 1 hour at room temperature. The signal was detected by using LumiGLO™ chemiluminescent detection reagent (New England Biolabs). To insure equal loading

between protein samples, membranes were probed with polyclonal goat IgG antibody for actin (Santa Cruz Biolabs) at a dilution of 1:200 using the same protocol as for Raf-1 and MAPK (Chapter 3 of Parts 2 and 3). The secondary antibody against the actin IgG consisted of anti-goat IgG labeled with horse radish peroxidase (Santa Cruz Biolabs) at a dilution of 1:20,000.

Western blot analysis of c-myc protein expression: Antibodies attached to previously probed membranes were removed by incubating the membrane in striping buffer for 30 minutes to 1 hour in a 50° C water bath with gentle agitation. Following antibody removal, membranes were washed twice in TBS/0.1% tween-20 for 20 minutes prior to incubation with a c-myc antibody. The membrane was blocked in 5% nonfat dry milk for 1 hour at room temperature. Following three separate 5 minute washes in TBS/0.05% tween-20, the membrane was probed with primary polyclonal rabbit antibodies specific for c-myc (New England Biolabs) at a dilution of 1:300 in TBS/0.05% tween-20 containing 5% nonfat dry milk for 1 hour. The c-myc antibody was raised against a recombinant protein that corresponds to amino acid residues 1-262 of the N-terminal c-myc protein of human origin. The wash steps were repeated, and the membrane was incubated in TBS/0.05% tween-20 containing anti-rabbit IgG-horse radish peroxidase labeled antibody (400 µg/ml) (Santa Cruz Biolabs) and streptavidin-horse radish peroxidase conjugate (Amersham) at a dilution of 1:1000 for 1 hour at room temperature. Signals were detected through the use of a chemiluminescent detection system (Amersham).

Chapter 4: Results

c-myc protein (Myc) phosphorylation: Serum starved fetal non-neoplastic PNECs were exposed to a low concentration of NNK (100 pM) for 15 minutes in a 5% CO_2 environment. Western blots were probed with a phospho-specific antibody that only detects c-myc protein containing phosphorylated Thr-58 and/or Ser-62 residues within the N-terminal domain. The western blots revealed increased levels (>1.5 fold increase) of phosphorylated c-myc (p-Myc) protein in cells exposed to NNK as compared to untreated control cells (Figure 3-1). Furthermore, the level of Myc phosphorylation in NNK stimulated cells was reduced by pre-treating cells with the MEK inhibitor, PD98059, prior to NNK exposure. This is apparent in figure 3-2, where there is slightly increased band intensity in lane 2 (NNK treated cells) compared to lane 1 (control cells), while the lane representing cells pre-treated with PD98059 (lane 3) contains a band with a markedly attenuated signal. The similar intensity among the actin protein bands represents equal sample loading in each lane. Therefore, PD98059 blocks the NNK induced phosphorylation of this nuclear transcription factor resulting in levels that are equal to or below those in untreated cells.

Similar changes were observed in neoplastic H69 cells exposed to NNK (100 pM)

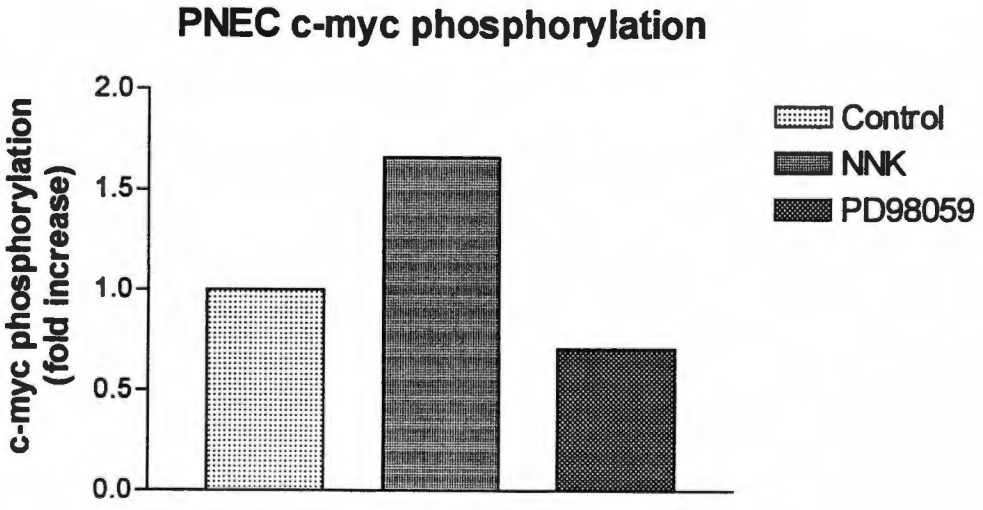

FIGURE 3-1. Phosphorylation of c-myc protein in non-neoplastic PNECs. Results indicate the level of Myc phosphorylation at threonine 58 and serine 62. Cells were treated with the MEK inhibitor (PD98059) 1 hour prior to NNK exposure. The data are representative of two independent experiments giving similar results where the controls have been normalized at 1.

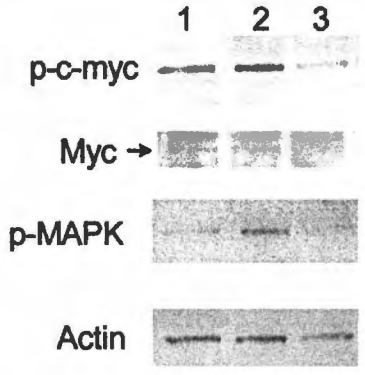

FIGURE 3-2. Western blot of Myc phosphorylation in non-neoplastic PNECs. p-Myc bands represent phosphorylated c-myc protein in untreated control cells (land 1), NNK [100 pM] treated cells (lane 2), and NNK exposed cells pre-treated with PD98059 [50 μ M] (lane 3). p-MAPK demonstrates phosphorylated MAPK protein from the same blot. Actin control bands reveal equal loading of protein sample into each well.

for 15 minutes in 5% CO_2; however, the increase in Myc phosphorylation was higher (>2.0 fold) than those levels previously found in non-neoplastic PNECs (Figure 3-3). As shown in figure 3-4, the signal intensity of the band in lane 2 (NNK exposed cells) is approximately 2 fold greater than the band in lane 1 (untreated cells), indicating elevated Myc phosphorylation at Thr-58 and/or Ser-62 in cells exposed to the nitrosamine. The band in lane 3, representing c-myc protein from cells treated with PD98059 prior to NNK exposure, has a signal intensity equal to or slightly less than that of the untreated control cells. Observing this reduction in p-Myc, following the inhibition of MEK phosphorylation, is suggestive of a MAPK mediated influence on the c-myc protein. These data may also signify that NNK not only has an influence on the MAP kinase cascade, but also on downstream Myc phosphorylation via MAPK.

c-myc protein (Myc) expression: In order to correlate the elevated levels of p-Myc to the level of Myc expression, the phospho-specific antibody was removed from previously probed membranes and incubated with an antibody specific for c-myc protein. The levels of Myc in non-neoplastic PNECs exposed to NNK did not vary significantly from those levels in untreated cells. This is demonstrated in figure 3-2, were the band intensity in lane 1 (untreated cells) and lane 2 (NNK treated cells) is similar. Interestingly, the signal strength of the band in lane 3, representing c-myc protein from cells pre-treated with PD98059, was lower than in lane 1 and lane 2. Therefore, although NNK alone does not

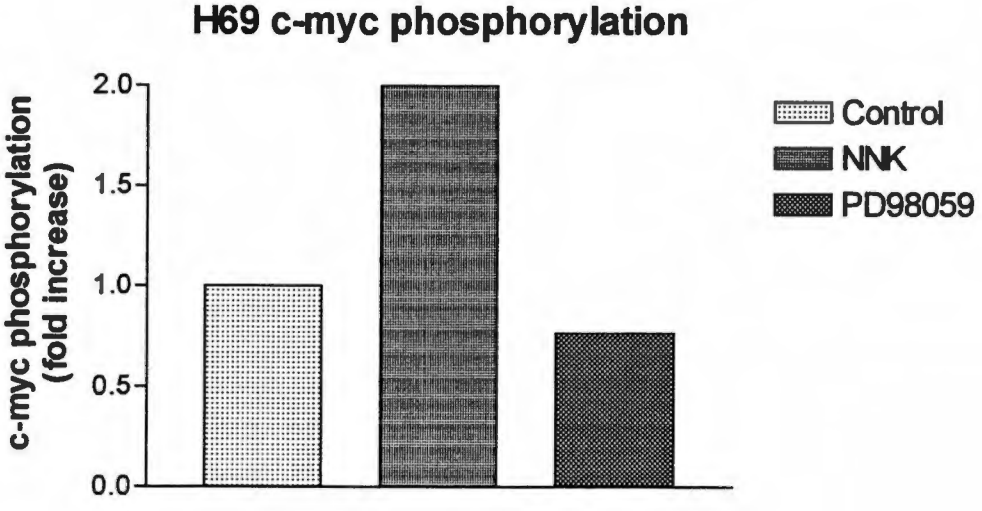

FIGURE 3-3. Phosphorylation of c-myc protein in neoplastic PNECs (NCI-H69). Results indicate the level of Myc phosphorylation at threonine 58 and serine 62. Cells were pre-treated with PD98059 one hour prior to treatment with NNK. The data are representative of two independent experiments giving similar results where the controls have been normalized at 1. NNK [100 pM]; PD98059 [50 μM].

appear to alter Myc levels, pre-treatment of these cells with a MEK inhibitor followed by NNK exposure does result in reduced protein levels.

Levels of c-myc protein expression were also examined in the H69 cells, and not unlike its non-neoplastic counterpart, NNK exposure did not alter the expression levels of this nuclear transcription factor. Figure 3-4 shows equal signal intensity of the band in lane 2 (NNK treated cells) when compared to lane 1 (untreated cells). Furthermore, there is little variation in the band intensity of lane 3 (PD98059 treated cells) as compared to lanes 1 and 2. Therefore, unlike in the normal PNECs, pre-treatment with the MEK inhibitor was ineffective at altering the c-myc protein levels in H69 cells. These data suggest that the MAP kinase pathway may contribute to the regulation of c-myc protein expression in normal PNECs, but not in neoplastic PNECs.

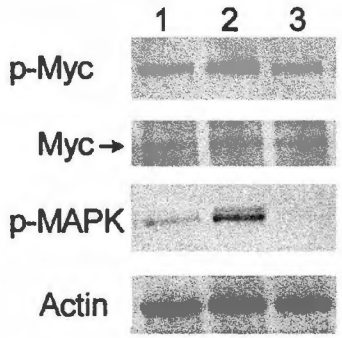

FIGURE 3-4. Western blot of Myc phosphorylation in neoplastic PNECs (NCI-H69 cells). p-Myc bands represent phosphorylated c-myc protein in untreated control cells (land 1), NNK [100 pM] treated cells (lane 2), and NNK exposed cells pre-treated with PD98059 [50 μM] (lane 3). c-myc protein levels (lanes labeled Myc) and phosphorylated MAPK protein (lanes labeled p-MAPK) are shown for the same blot/experiment. Actin control bands reveal equal loading of protein sample into each well.

Chapter 5: Discussion

The regulation of cell proliferation and differentiation is a tightly controlled process that is initiated by a receptor-mediated mitogenic stimulus and sustained by a sequential series of phosphorylation reactions. Ultimately, the initiating signal is transmitted to the nucleus were it activates various nuclear transcription factors and proteins involved in cell cycle progression. The c-myc protein (Myc) has long been recognized as a vital component in the regulation of cell growth and death (Cole, 1986; Luscher, 1990; Evan, 1992), and is present in the cytoplasm and in the nucleus were it controls the transcription of other key regulatory proteins (Lutterbach, 1994). Myc functions via the heterodimerization with the protein MAX which mediates specific complex binding to the DNA E-box sequence, CACGTG, and results in subsequent transactivation (Blackwell, 1990; Blackwood, 1991). MAX also dimerizes with the protein MAD, forming a similar complex interaction with the E-box sequence that blocks DNA transactivation (Ayer, 1993; Hurlin, 1995). Therefore, the ratio of Myc to MAD dictates which MAX heterodimer complex will predominate and whether transcription will be activated or repressed (Ayer, 1993). The dynamics of this ratio is based on the fact that MAX expression remains constant while Myc and MAD, with their short half-lives,

are influenced by the growth of the cell. Previous studies have shown that Myc levels decline while MAD levels inversely increase as cell proliferation ceases (Chin, 1995). These findings suggest that the role of Myc is heavily dependent on the levels of c-myc protein available, and that regulation of these levels may ultimately effect DNA transcription and cell growth.

It has long been established that the c-myc protein is phosphorylated at multiple sites within the C- and N-terminal domains (Ramsay, 1982; Hann, 1984; Lutterbach, 1997), but its significance on Myc function and regulation remains unclear. Although it has been demonstrated that the C-terminal domain of Myc is adjacent to the DNA E-box binding domain, mutational alteration of these phosphorylation sites within the C-terminus had no effect on transcriptional activation via Myc (Blackwell, 1990; Street, 1990). However, alteration of phosphorylation via mutation within the N-terminal transactivation domain did alter Myc mediated gene transcription (Chen, 1989; Symonds, 1989). Specifically, mutation of the serine amino acid residue at position 62 within the transactivation domain has been shown to reduce the transcriptional activity of Myc in a variety of cells (Alverez, 1991; Gupta, 1993; Lutterbach, 1994; Pulverer, 1994). Furthermore, these investigators linked the phosphorylation of Ser-62 to the in vitro and in vivo mitogen stimulation of the MAP kinase cascade and downstream activation of MAPK 2. Therefore, an important link was established between a specific receptor-mediated signaling pathway and the potential activation of the c-myc transcription factor. It was our intention to examine the effect of NNK exposure on the phosphorylation of Myc as a

downstream effect of previously demonstrated MAP kinase pathway activation in normal and neoplastic PNECs.

We observed that exposing both normal and neoplastic PNECs to a low concentration of NNK resulted in the increased binding of antibody that specifically recognizes phosphorylated Ser-62 of the c-myc protein. This phosphorylation took place under the same conditions (15 minute exposure in 5% CO_2) as those that resulted in the activation of the MAP kinase pathway. Although the phospho-specific antibody detects phosphorylation at both Thr-58 and Ser-62 residues, it should be noted that phosphorylation of Thr-58 is believed to require prior phosphorylation of Ser-62 (Lutterbach, 1994). A proposed mechanism for the observed increase in p-Myc likely involves the NNK induced activation of the MAP kinase pathway. Our previous data has demonstrated the activation of both Raf-1 and MAPK 1\2 in H69 and fetal PNECs following NNK exposure; and furthermore, as illustrated in figure 3-2 and 3-4, MAPK 2 (p42) appeared to be the isoform preferentially activated. This suggests that Myc is either directly phosphorylated by MAPK 2 or may receive the signal from a yet unknown MAPK 2 substrate. Nevertheless, these findings correlate MAPK 2 activation with Myc phosphorylation. Our data are substantiated by other studies showing the in vitro and in vivo phosphorylation of c-myc protein at Ser-62 by activated MAPK 2 (Gupta, 1993; Lutterbach, 1994; Pulverer, 1994). In addition to these investigations, Chuang (1994) demonstrated selective phosphorylation and activation of Myc by the MAPK 2 (p42), but not the MAPK 1 (p44) isoform in NIH/3T3 fibroblasts. Subsequent mutation of the Myc

phosphorylation site, Ser-62, prevented MAPK 2 activation of the c-myc protein. Therefore, the observed increase in p-Myc following NNK exposure is believed to be the result of intracellular signaling via the MAP kinase pathway and specifically MAPK 2 activation.

To further investigate the role of NNK induced MAPK activation on Myc, experiments using normal and neoplastic cells pre-treated with the MEK inhibitor , PD98059, were conducted. By specifically inhibiting the NNK induced phosphorylation of MEK and the activation of MAPK 1\2, we were also able to demonstrate concurrent inhibition of Myc phosphorylation. In H69 cells, the levels of p-Myc in PD98059 treated cells are similar to those in untreated control cells which likely indicates phosphorylation of Myc by one or more MAPK independent pathways under basal conditions. However, the degree of inhibition in normal fetal PNECs treated with PD98059 achieved levels lower than in untreated control cells. Therefore, it appears that normal PNECs rely heavily on the MAP kinase cascade for downstream phosphorylation of Myc and subsequent Myc associated transactivation. These data strongly support the hypothesis that the MAP kinase pathway, under the influence of NNK, exerts its effect on Myc at Ser-62.

Levels of c-myc protein expression were examined from the same experiments as those conducted for p-Myc by re-probing western blots with a non-phospho-specific Myc antibody. Although NNK directly or indirectly induced the phosphorylation of Myc in both normal and neoplastic cells, it did not effect expression of this protein. This indicates

that NNK does not influence the expression of this protein during the same time interval that it effects the Myc phosphorylation state. Furthermore, in the H69 cells, pre-treatment with PD98059 had no influence on Myc levels. In neoplastic PNECs, these data are likely to reflect constitutive overexpression of this protein, as has been reported in a significant proportion of SCLC (Sekido, 1998). The overexpression of this protein in SCLC is believed to be the result of marked c-myc gene amplification or transcriptional dysregulation (Krystal, 1988; Spencer, 1991), and occurs most frequently in cell lines derived from metastatic lesions rather than primary tumors (Johnson, 1996). Therefore, the H69 cells would contain higher levels of Myc not further augmented by NNK stimulation nor inhibited by blocking MAP kinase pathway activation. However, this explanation does not account for the lack of response in the non-neoplastic cells, since these cells have not been shown to overexpress this protein. Therefore, another explanation may be that NNK does not further increase levels of c-myc protein above the basally stimulated levels observed in untreated cells, but rather stabilizes protein levels. Interestingly, unlike in the H69 cells, Myc levels are reduced in non-neoplastic PNECs treated with PD98059 prior to NNK exposure as compared to untreated cells or cells treated with just NNK. Since no elevation in Myc was observed in NNK treated cells, this reduction in Myc likely reflects the degradation and reduced stability of existing protein. The c-myc protein has a very short half-life that is regulated by ubiquitin-mediated targeting and 26S proteasome destruction (Ramsey, 1986; Gross-Mesilaty, 1998; Hershko, 1998). As previously stated, it is believed that the level of c-myc protein

strongly influences its effect on DNA transcription as it relates to heterocomplex formation with MAX. Thus, it has been proposed that Myc levels and subsequent transcriptional activation are controlled at least in part by post-translational turnover and protein stability (Sears, 1999). Although there was no change in Myc levels in response to NNK, could NNK exposure and subsequent MAP kinase cascade activation affect Myc protein stability? If this is the case, NNK as well as other endogenous growth factors could increase the half-life of Myc which results in elevated protein levels and enhanced transcriptional activation. Our data demonstrating the reduced levels of Myc in PD98059 pre-treated normal PNECs supports this proposed hypothesis. Reduced levels of Myc following the inhibition of MEK phosphorylation strongly suggests a MAPK dependent pathway responsible for maintaining c-myc protein stability and increasing its half-life in these cells. Sears (1999) has shown that activation of the MAP kinase pathway resulted in a 5 fold increase in Myc half-life. In fact, these findings more specifically demonstrated that Raf-1 and MAPK activation increased the stability and half-life of Myc which in turn resulted in Myc accumulation within the cell. The increased stability and elevated protein levels were subsequently inhibited by PD98059. These investigations are consistent with our data, and support the idea that NNK may contribute to the stability of Myc and increase or maintain its levels within the PNEC. Additionally, it is reasonable to suspect that NNK exposure, resulting in Myc accumulation, could increase transcriptional activation and ultimately lead to cell cycle progression. In support of this, the stabilization of Myc by the activated MAP kinase pathway has been reported to correlate with E2F

activation and progression through the S phase of the cell cycle (Sears, 1999). Furthermore, it has been shown that accumulation of Myc results in the activation of cyclin E/cdk 2 (Leone, 1997) and S phase progression (Steiner, 1995). The proposed mechanism for this is not fully understood, but is believed to be the result of cdk inhibitor (p27) suppression (Vlach, 1996; Perez-Roger, 1997). In considering the mechanism by which Raf-1/MAPK activation may stabilize Myc, a possible explanation addresses the phosphorylation state of Myc. MAPK phosphorylation of Ser-62 may block the ubiquitin-mediated targeting of this protein that results in its degradation (Sears, 1999). This is highly consistent with our data showing marked reduction in both p-Myc and Myc in non-neoplastic PNECs pre-treated with PD98059 prior to NNK exposure. According to the stated mechanism, blocking MAPK activation would result in reduced Ser-62 phosphorylation which would decrease Myc half-life. This may subsequently lead to a lower Myc:MAD ratio and reduced transcriptional activation.

The c-myc protein and its overexpression has been previously associated with oncogenesis involving various cell types (Blackwood, 1992) including PNECs (Sekido, 1998). Our data suggest that a Myc dependent mechanism is involved in the cell signaling process of NNK stimulated PNECs. Further investigation is focusing on whether NNK stimulation of MAPK/Myc is sufficient to result in DNA transactivation or if other signaling events also play a role. Additionally, experiments will examine the possible role of NNK induced Myc stability as it relates to cell cycle regulation and PNEC proliferation.

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PART IV: RAF-1 AND MAPK 1\2 PROTEIN LEVELS IN PULMONARY NEUROENDOCRINE CELLS EXPOSED TO NNK

Chapter 1: Abstract

Pulmonary neuroendocrine cells (PNEC) contain numerous intracellular signaling pathways composed of various types of signaling mediators that are responsible for transmitting mitogenic signals from the cell surface to the nucleus where that information is used to regulate cell growth, quiescence, or death. Alteration of these signaling mediators may result in aberrant cell proliferation or transformation. Exposing normal PNECs to low concentrations of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) resulted in an increase in the levels of key protein kinases within the mitogen activated protein (MAP) kinase cascade, specifically Raf-1 and MAPK 1\2 (Erk). This NNK induced elevation of these kinases was altered by inhibition of the receptor mediated signal. Similar findings were not evident within the neoplastic PNECs which already demonstrate high levels of protein kinase prior to NNK treatment. As a result, these data show the responsiveness of Raf-1 and MAPK protein levels within normal and neoplastic PNECs to NNK exposure.

Chapter 2: Introduction

The MAP kinase cascade is a growth regulatory pathway in a wide variety of cells that is composed of specific signaling mediators responsible for transmitting a message from the cell surface to the nucleus (Guan, 1994). This in turn regulates cell growth or triggers cell death, depending on the initiating signal. Upon receiving a signal, these mediators are involved in a sequential series of highly specific phosphorylation and dephosphorylation reactions that transmit the signal to the nucleus where the cell cycle is regulated. Raf-1 and MAPK 1\2 are two critical, non-consecutive protein kinases that function within the MAP kinase pathway (Anderson, 1990; Ullrich, 1990; Cobb, 1991). Raf-1 is a 648 amino acid protein composed of three highly conserved regions (CR1, CR2, CR3) that are responsible for the regulatory and catalytic functions associated with this kinase (Williams, 1994). Although Raf-1 is located predominantly within the cytosol, it has also been found in association with the plasma membrane, the outer mitochondrial membrane, and less frequently within the perinuclear space (Prouty, 1998; Wang, 1996). Intracellular localization is highly dependent upon the specific initiating signal, and in part dictates the cellular response resulting from the signal (Wartmann, 1997; Fanger, 1999). It has been thoroughly demonstrated that activated Ras serves to recruit Raf-1 to the cell membrane for subsequent activation by autophosphorylated receptor tyrosine kinases

(RTKs) (Van Aelst, 1993; Warne, 1993; Zhang, 1993; Campbell, 1998). Additionally, Raf-1 relocalization to the outer mitochondrial membrane by Bcl-2 has been shown to be important in the regulation of apoptosis (Wang, 1996).

Until recently, Raf-1 was believed to be constitutively expressed in most cell types with little need for tight transcriptional regulation (Storm, 1990). However, it has since been demonstrated that extracellular growth factors, mediators, and chemicals alter Raf-1 transcription, stability, and movement which in turn alters Raf-1 protein levels (Schulte, 1995). The mechanism by which this occurs is not clear, but may be the result of one or more intracellular events. Possible mechanisms include: 1. stabilization/destabilization of Raf-1 complexes which alter protein half-life, 2. alteration of intracellular and membrane localiztion of Raf-1, 3. induced Raf-1 gene expression and protein synthesis. Likewise, these mechanisms could also account for receptor mediated variations in protein levels of MAPK 1\2, a highly conserved cytoplasmic kinase downstream of Raf-1. Similar to Raf-1, activation of MAPK may result in its translocation from the cytosol to the nucleus where it has been shown to phosphorylate a variety of nuclear transcription factors (Guan, 1994; Gupta, 1993; Gille, 1992).

Although various extracellular mitogens have been used to elicit a change in Raf-1 and MAPK protein levels, the results have been variable depending on the specific mitogen examined (Storm, 1990; Zmuidzinas, 1991; Hu, 2000). The tobacco-specific nitrosamine, NNK, has been shown to be a potent mitogen in normal and neoplastic PNECs (Schuller, 1994) via receptor mediated processes involving the α_7 nAChR and

serotonin secretion and uptake (Schuller, 1998; Codignola, 1994). Additionally, data indicate that NNK results in the stimulation of the mitogenic MAP kinase pathway via Raf-1 and MAPK 1\2 activation (Jull, Part II, Chapter 4). Therefore, it was our intention to examine the effect of NNK on Raf-1 and MAPK protein levels in both normal and neoplastic PNECs. Here we demonstrate that exposure to low concentrations of NNK results in the elevation of Raf-1 and MAPK levels in normal PNECs, but not the neoplastic cells. These findings further substantiate the mitogenic potential of NNK in these specialized cells, and point to the importance of the MAP kinase cascade as the pathway used by such a stimulus.

Chapter 3: Material and Methods

Cell Culture: Normal pulmonary neuroendocrine cells were obtained from the lungs of 15 day gestational Syrian golden hamster fetuses. The lungs were harvested and cultured as described in the materials and methods (Chapter 3) of Parts II and III. All cells were grown to 60-80% confluence in 25 or 75 cm^2 vented culture flasks prior to experimental procedures. All hamsters were maintained in a standardized housing environment with *ad libitum* commercial feed and water. The NCI-H69 (small cell carcinoma) cell line was obtained from the American Type Culture Collection. The H69 cells were maintained in RPMI 1640 medium supplemented with 10% FBS in a 5% CO_2 atmosphere prior to their use in experiments.

Cell treatment and lysate production: The NCI-H69 cells and fetal PNEC were placed into low serum DMEM media (Biofluids) containing 0.1% BSA, 210 mg L-glutamine, 500 units penicillin, and 500 mcg streptomycin for 12-24 hours prior to the experiment while maintained in 5% CO_2. Cells were washed with 1x Ca^{2+} free PBS and reintroduced to low serum media 30 minutes to 1 hour prior to the experiment. Competitive receptor inhibitors were added to the designated treatment group 10 minutes before the addition of NNK. Protein kinase inhibitors were placed on the designated cells 1 hour preceding the

addition of NNK. NNK and chemical inhibitors were made fresh within 3 hours of experimental use.

Collection of the fetal PNEC involved removal of the low serum DMEM media containing the designated treatment, washing the cells with ice-cold 1x Ca^{2+} PBS, and removal of adherent cells with 0.25% trypsin. The cells were washed twice with PBS and centrifuged at 500 rpms (30 g's) forming a cell pellet that was either immediately lysed or stored at -80° C. Cells were lysed by 600 µl RIPA solution (1x PBS, 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate) containing protease inhibitors (10µl/ml phenylmethylsulfonyl flouride (PMSF) [10 mg/ml], 30 ul/ml aprotinin [1.9mg/ml], 10 µl/ml sodium orthovanadate (Na_3VO_4) [100 mM]). The cell lysate was immediately passed three times through a 21 gauge needle with subsequent addition of 10 µl PMSF [10 mg/ml]. Following a 30 minute incubation on ice, the cell lysates were centrifuged at 14000 rpms (16000 g's) for 20 minutes at 4° C, and the protein containing supernate was separated from the pellet and collected for subsequent assays.

Following treatment of NCI-H69 cells, the cells in low serum media were placed in conical tubes, centrifuged at 500 rpms (30 g's) for 5 minutes, and washed twice with ice-cold PBS. The cells were subsequently processed in a manner similar to the fetal PNEC.

Western blot analysis of Raf-1 and Erk expression: The protein from the cells lysate was concentrated using Centricon™ 10 concentration columns (Milipore) centrifuged at 6000 rpms (4350 g's) for approximately 45 minutes to 1 hour. The protein concentration was

determined by placing 10 µl protein sample and 200 µl bicinchoninic acid (BCA™) reagent (Pierce) into individual microplate wells and incubating for 30 minutes at 37° C. The samples were quantified using an EL 312 microplate reader (Bio-Tek Instruments) at a 550 nm wavelength, and absorbance values were converted to µg/ml by plotting linear regression x from y values via Prism 2.01 (GraphPad Inc.). 20 µg protein from each sample was boiled in 2x sodium dodecyl sulfate (SDS) loading buffer (0.1 M Tris-Cl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) for 4 minutes and loaded onto a 10% Tris-HCl PAGE gel (Biorad) which was ran a 200 volts for approximately 30 minutes. The gel containing the protein was electrophoretically transferred to a nitrocellulose membrane (Midwest) using a standard transfer buffer (5.8 g Tris-hydroxymethyl-aminomethane, 2.9 g glycine, 3.7 ml 10% SDS, 200 ml methanol, 800 ml deionized water), and ran at 100 volts for 1 hour. The membranes were blocked with 5% nonfat dry milk in TBS containing 0.05% tween-20 (blocking solution) for 1 hour at room temperature. Following three separate 5 minute washes in TBS containing 0.05% tween-20, the membranes were incubated for 1 hour, at room temperature, in blocking solution containing polyclonal rabbit IgG for Raf-1 (200 µg/ml) or MAPK 1\2 (200 µg/ml) (Santa Cruz Biolabs) at a dilution of 1:2000. The wash steps were repeated, and the membranes were incubated in TBS/0.05% tween-20 containing anti-rabbit IgG-horse radish peroxidase labeled antibody (400 µg/ml) (Santa Cruz Biolabs) and streptavidin-horse radish peroxidase conjugate (Amersham), both at a dilution of 1:1000 for 1 hour at room temperature. The resulting band signals were detected by the use of a chemiluminescent

detection system (Amersham). Film exposure time varied according to the strength of the signal. To insure equal loading between protein sample, membranes were probed with polyclonal goat IgG actin antibody (Santa Cruz Biolabs) at a dilution of 1:200 using the same protocol as for Raf-1 and MAPK. The secondary antibody against the actin IgG consisted of anti-goat IgG labeled with horse radish peroxidase (Santa Cruz Biolabs) at a dilution of 1:20,000.

Antibodies attached to previously blotted membranes were removed by incubating the membrane in striping buffer (350 µl β-2-mercaptoethanol [100 mM], 8 ml SDS [2%], 3.125 ml Tris-Cl pH 6.7 [62.5 mM], 38.5 ml deionized water) for 30 minutes to 1 hour in a 50° C water bath with gentle agitation. Following antibody removal, membranes were washed twice in TBS/0.05% tween-20 for 20 minutes and stored in cellophane wrap at 4° C.

Densitometric analysis: Raf-1 and MAPK protein expression was quantified using SigmaGel™ gel analysis software V1.0 (SPSS Inc.). Threshold points were based on the degree of exposure for each film.

Statistical analysis: The results obtained from control groups and cells exposed to NNK were assessed via the unpaired t-test.

Chapter 4: Results

Raf-1 protein levels: Fetal PNEC cells were exposed to a low dose (100 pM) of NNK for short and long time intervals (5, 15, and 120 minutes). Western blots of cell lysates were probed for Raf-1 protein kinase and subsequently quantified by densitometric analysis with measurement of the entire width of each band to insure uniform quantification. Raf-1 protein has a molecular weight of 74 kilodaltons (kDa). These experiments revealed inducible changes in Raf-1 protein levels within fetal PNECs when exposed to a 100 pM dose of NNK in a 10% CO_2 environment ($p<0.05$). Depending on the level of basal expression in the PNEC, NNK exposure induced a mean 2.7 to 3.4 fold increase in Raf-1 within 15 minutes post-exposure and 2.5 fold over basal levels at 120 minutes (Figure 4-1). Upon examining figure 4-1, levels of Raf-1 increase to 3.4 fold by 5 minutes over the time 0 control (basal levels). This elevation declines slightly to a 2.7 fold increase by 15 minutes following NNK treatment, and remains at approximately this level up to the last time interval examined (120 minutes). This is represented in figure 4-2 where lane 2, 3, and 4 (5, 15, and 120 minutes post NNK exposure respectively) are increased compared to the time 0 control (lane 1). The less intense doublet band above Raf-1 is believed to

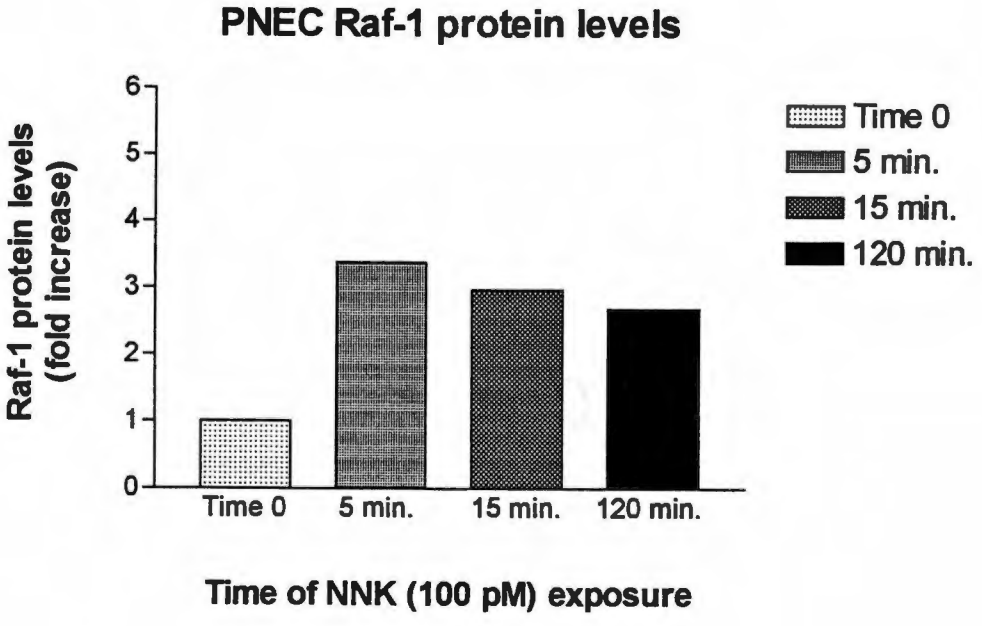

FIGURE 4-1. Raf-1 protein levels in non-neoplastic PNECs. Cells were exposed to NNK [100 pM] for 0, 5, 15, and 120 minutes in 10% CO_2. Western blot analysis of cellular Raf-1 protein is expressed as a fold increase in band intensity. Data are representative of two independent experiments with single samples per treatment group giving similar results.

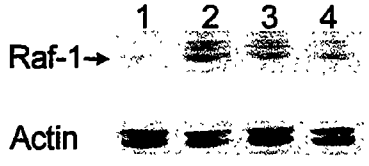

FIGURE 4-2. Representative western blot of Raf-1 protein levels in non-neoplastic PNECs following timed NNK exposure. The bands represent Raf-1 protein levels following 0 minutes (lane 1), 5 minutes (lane 2), 15 minutes (lane 3), and 120 minutes (lane 4) of NNK [100 pM] exposure. Actin controls demonstrate equal sample loading.

represent the slower migrating phosphorylated form of the protein kinase, and actin controls demonstrate equal loading between lanes. To examine the effects of extended (6 day/subchronic) NNK exposure, non-neoplastic PNEC were maintained in RPMI, full serum media, and treated twice a day with 100 pM fresh NNK. Prior to each treatment, the cells were washed in PBS. The last day of the experiment, the PNECs were placed in low serum media and harvested 2 hours after the last NNK treatment. At the end of the 6 day period, there was a 5 fold increase in Raf-1 protein levels over 6 day untreated controls (Figure 4-3). These results are demonstrated by the increased band intensity of Raf-1 in lane 2 compared to the untreated control band in lane 1 (Figure 4-4). Additionally, normal PNEC exposure to NNK for a longer time interval (6 days) resulted in greater elevation in Raf-1 protein levels compared to the earlier time periods examined. Therefore, NNK consistently induces both immediate (within 15 minutes) and long term (120 minutes and 6 days) Raf-1 protein kinase elevation in the non-neoplastic PNEC.

An additional experiment was conducted in 5% CO_2 to examine the effect of NNK on Raf-1 protein in the absence of a hypoxic environment. Normal PNECs were exposed to NNK and placed in a 5% CO_2 chamber for 5 minutes. In a manner similar to previous experiments, NNK induced a rapid 2.5 fold increase in Raf-1 levels over basal levels (Figure 4-5 and Figure 4-6). The increase in protein kinase was similar to the level observed in cells treated with 5-HT (1 μM) as a positive control. This concentration of 5-HT is similar to levels shown to exist in the lungs of patients with SCLC.

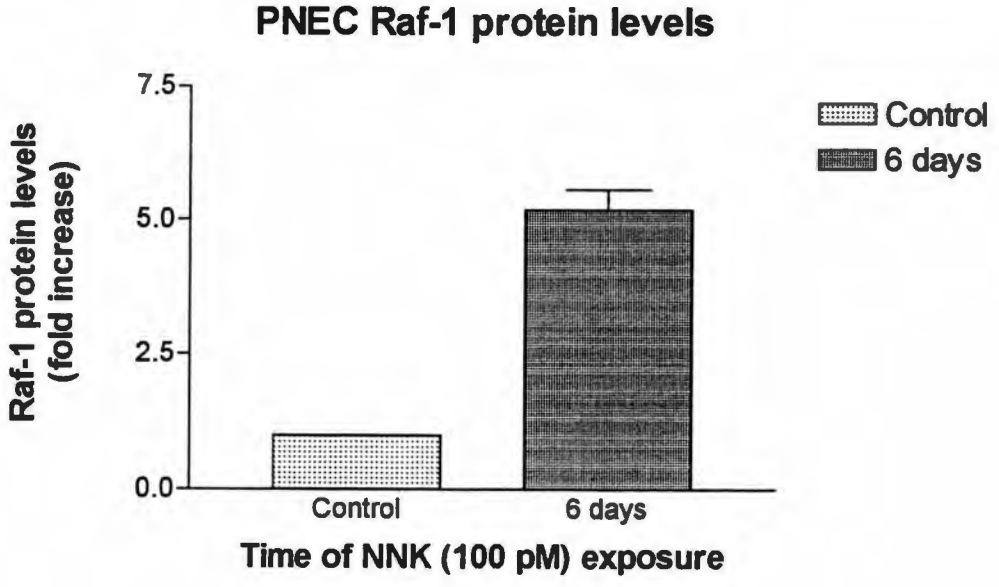

FIGURE 4-3. Raf-1 protein levels in non-neoplastic PNECs following subchronic (6 days) NNK exposure. (n=3).

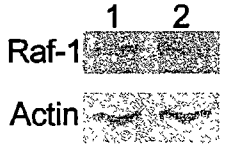

FIGURE 4-4. Western blot of Raf-1 protein levels following subchronic NNK exposure. Untreated control cells (lane 1) were maintained in same environment as NNK treated cells (lane 2).

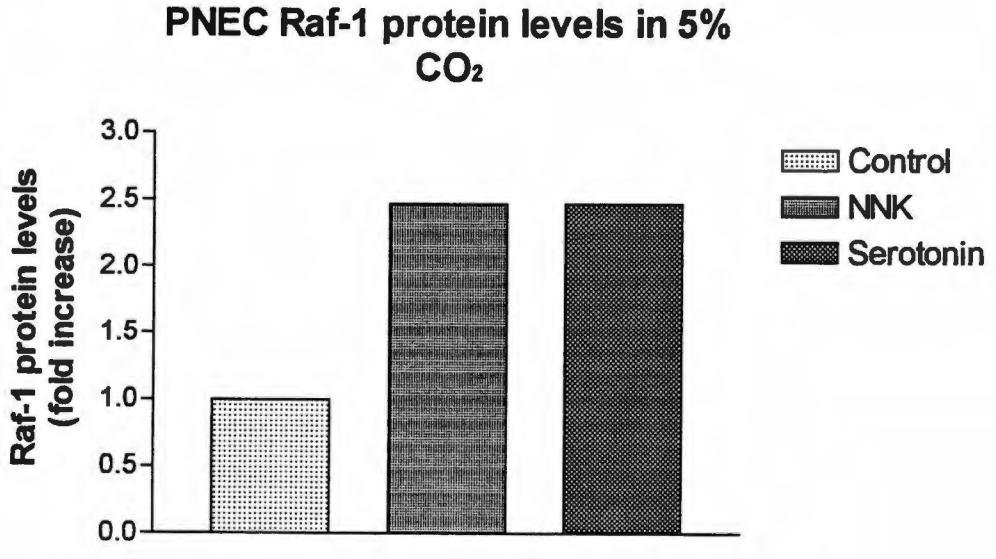

FIGURE 4-5. Raf-1 protein levels in non-neoplastic PNECs following NNK and 5% CO_2 exposure. Results demonstrate a change in Raf-1 protein levels following NNK [100 pM] treatment and introduction into a 5% CO2 environment for 5 minutes. Serotonin [1 μM] was used as a positive control. Data are representative of two independent experiments with single samples per treatment group giving similar results.

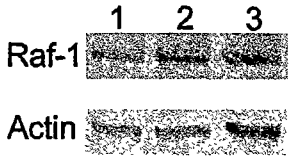

FIGURE 4-6. Representative western blot of Raf-1 protein levels in non-neoplastic PNECs exposed to NNK [100 pM] and 5% CO_2. The untreated control cells (lane 1), NNK treated cells (lane 2), and the positive control serotonin [1μM] treated cells (lane 3) were maintained in 5% CO_2 for 5 minutes following treatment. Bands representing the actin internal control demonstrate equal loading of sample.

In order to link this elevation of Raf-1 to the upstream α_7 nAChR and the 5-HT receptor, cells in a 10% CO_2 environment were exposed to either a 5-HT uptake inhibitor (imipramine [1 μM]), a broad spectrum nicotinic receptor antagonist (hexamethonium [1 μM]), or a site-selective α_7 nAChR antagonist (α-bungarotoxin [10 μM]) prior to NNK (100 pM) treatment. The imipramine had the most profound effect, completely blocking the NNK induced elevation of Raf-1 protein, and in some experiments further reducing the levels of basal Raf-1 by 42% (Figure 4-7). Blocking all nicotinic receptors with hexamethonium results in an 88% to 100% reduction in the amount of elevated Raf-1 protein due to NNK exposure. The amount of Raf-1 protein in these cells is similar to the levels within untreated control cells (Figure 4-7). Figure 4-7 also shows partial (30%) reduction of Raf-1 in cells treated with the α_7 specific antagonist α-bungarotoxin. Neither nicotinic receptor antagonist reduced kinase levels below control levels as did the 5-HT uptake inhibitor. A representative western blot (Figure 4-8) demonstrates the specific inhibition of the NNK induced receptor mediated Raf-1 protein kinase elevations refered to previously. The increased band intensity in lane 2 represents Raf-1 protein from cells exposed to NNK for a 2 hour time period. Cells pretreated with receptor antagonist were also exposed to NNK for the same time interval as the treated and untreated controls.

The effect of NNK (100 pM) on Raf-1 protein levels was subsequently examined in neoplastic pulmonary neuroendocrine cells, NCI-H69. Experiments using the H69 cells were conducted similar to those using the normal PNECs. As shown in figure 4-9, the amount of Raf-1 in cells exposed to NNK was not significantly elevated (less than a 1.6 fold increase) for any of the time intervals examined ($p<0.01$). In figure 4-10 this is clearly

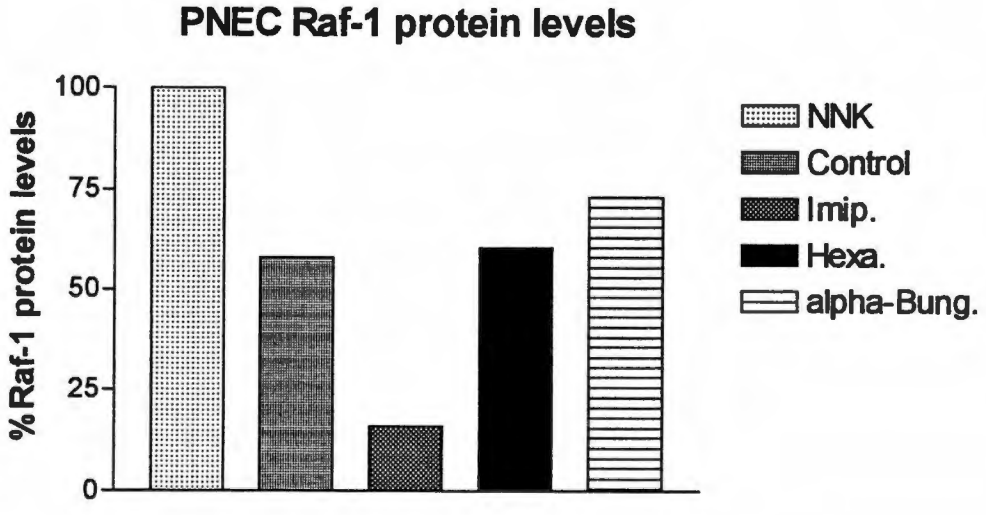

FIGURE 4-7. Raf-1 protein levels in non-neoplastic PNECs following receptor-mediated inhibition. NNK [100 pM] induced Raf-1 protein levels were normalized at 100%. Basal levels of Raf-1 and inhibition of NNK induced Raf-1 protein levels are expressed as a percentage of the total amount of Raf-1 protein in NNK treated cells. The 5-HT uptake inhibitor imipramine [1 μM], the broad-spectrum nicotinic receptor antagonist hexamethonium chloride [1 μM], and the site-selective antagonist for the α_7 nicotinic receptor, alpha-bungarotoxin [10 μM] were used to inhibit the effects of NNK. Data represent two independent experiments with single samples per treatment group giving similar results.

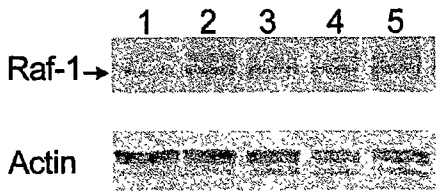

FIGURE 4-8. Representative western blot of Raf-1 protein levels in non-neoplastic PNECs following receptor-meditated inhibition. Raf-1 bands represent untreated control cells (lane 1), cells exposed to NNK [100 pM] alone (lane 2), and cells pre-treated with imipramine [1 μM] (lane 3), hexamethonium chloride [1 μM] (lane 4), or alpha-bungarotoxin [10 μM] (lane 5) prior to NNK exposure. Actin control bands demonstrate equal loading of protein sample.

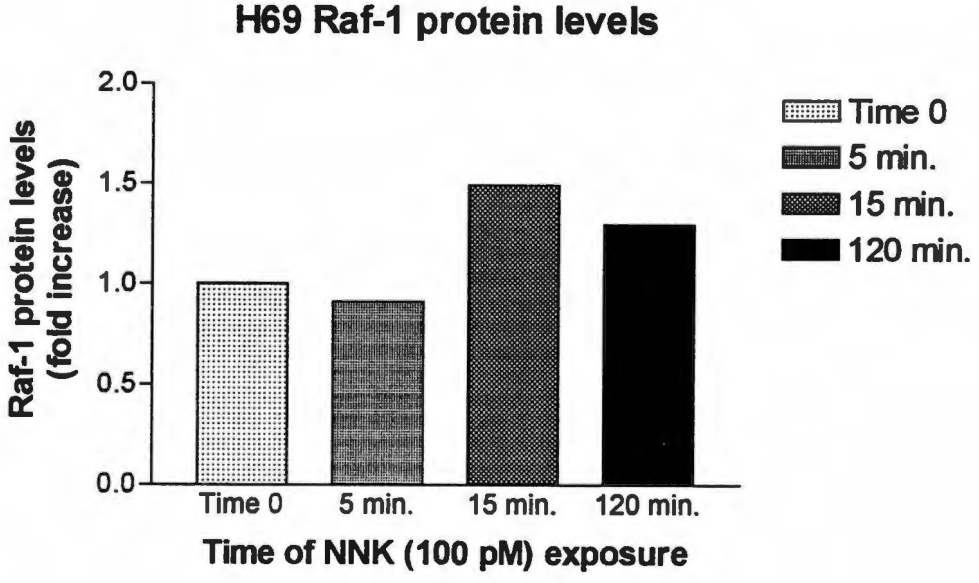

FIGURE 4-9. Raf-1 protein levels in neoplastic PNECs (NCI-H69 cells). Cells were exposed to NNK [100 pM] for 0, 5, 15, and 120 minutes. Western blot analysis of cellular Raf-1 protein is expressed as a fold increase in band intensity. Data are representative of two independent experiments with single samples per treatment group giving similar results.

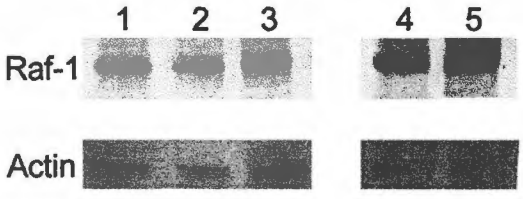

FIGURE 4-10. Western blot of Raf-1 protein levels in neoplastic PNECs (NCI-H69 cells) following timed NNK exposure. The bands represent Raf-1 protein levels following 0 minutes (lane 1 and lane 4), 5 minutes (lane 2), 15 minutes (lane 3), and 120 minutes (lane 5) of NNK [100 pM] exposure. Actin controls demonstrate equal sample loading.

evident via western blot analysis where there is equal band intensity between NNK treated cells at the 5, 15, and 120 minute time intervals (lanes 2, 3, and 5) and the time 0 control group (lane 1 and 4). H69 cells were also pre-treated with imipramine, hexamethonium, or α-bungarotoxin prior to NNK exposure in order to substantiate the lack of NNK control over intracellular Raf-1 protein levels in these neoplastic cells. Blocking α_7 and non-α_7 nicotinic receptors as well as 5-HT uptake did not change Raf-1 protein levels subsequent to NNK treatment (Figure 4-11). Furthermore, there was no difference in the Raf-1 protein levels of NNK treated cells compared to untreated cells which supports the previous data. Figure 4-12 represents the western blot for this experiment, in which it shows equal band intensity among all treatment groups and the untreated control. Therefore, it appears that the amount of Raf-1 protein within H69 cells is unaffected by NNK receptor mediated stimulation, unlike its non-neoplastic counterpart.

MAPK 1\2 (ERK) protein levels: To access the effect of NNK downstream of Raf-1, MAPK 1\2 levels were examined by way of western blot analysis using lysates from normal PNECs treated with 100 pM NNK for short and long time intervals (5, 15, and 120 minutes) in a 10% CO_2 environment. Western blots were probed with a MAPK polyclonal antibody which binds to the phosphorylated and non-phosphorylated MAPK 1 and 2 isoforms at 44 and 42 kDa respectively. The bands were subsequently quantified by densitometric analysis with measurement of its entire width to insure uniform quantification. In most cases, obtaining separate densitometric values for each isoform

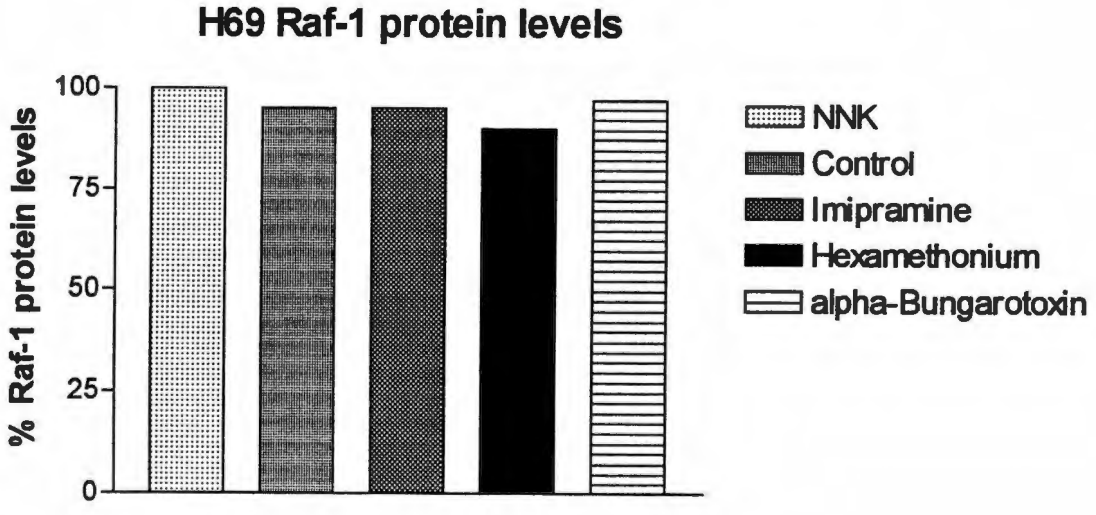

FIGURE 4-11. Raf-1 protein levels in neoplastic PNECs (NCI-H69) following receptor-mediated inhibition. NNK [100 pM] induced Raf-1 protein levels were normalized at 100%. Basal levels (control) of Raf-1 and inhibition of NNK induced Raf-1 protein levels are expressed as a percentage of the total amount of Raf-1 protein in NNK treated cells. Imipramine [1 μM], hexamethonium chloride [1 μM], and alpha-bungarotoxin [10 μM] were used to inhibit the effects of NNK. Data represent two independent experiments with single samples per treatment group that gave similar results.

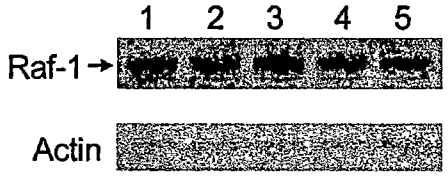

FIGURE 4-12. Representative western blot of Raf-1 protein levels in neoplastic PNECs (NCI-H69 cells) following receptor-mediated inhibition. Raf-1 bands represent untreated control cells (lane 1), cells exposed to NNK [100 pM] alone (lane 2), and cells pre-treated with imipramine [1 μM] (lane 3), hexamethonium chloride [1 μM] (lane 4), or alpha-bungarotoxin [10 μM] (lane 5) prior to NNK exposure. Actin control bands demonstrate equal loading of protein sample.

was not possible due to proximity of the bands. Therefore, these data represent densitometry readings taken together for both isoforms, unless otherwise stated. In non-transformed fetal PNECs, exposure to NNK induced a rapid change (greater than 1.6 fold increase) in the level of MAPK ($p<0.01$) similar to the change describing Raf-1. There was a 2 fold increase in the amount of MAPK protein within the cell by 5 minutes that was sustained beyond 15 minutes (Figure 4-13). At 120 minutes, levels remain elevated, although slightly lower (1.7 fold), compared to the time 0 control. MAPK at time 0 is considered to be basal level expression. These changes are represented by the increased band intensity in lanes 2, 3, and 4 of figure 4-14 as compared to time 0 control cells in lane 1 of the same figure. To examine the effect of NNK over an extended time interval, cells were exposed to NNK (100 pM) for 6 days (subchronic) in an experimental protocol similar to that used for evaluation of Raf-1 protein levels. As shown in figure 4-15 and figure 4-16, MAPK in normal PNECs was not significantly elevated over basal levels following this subchronic exposure. This extended exposure to NNK was conducted in a 10% CO_2 environment to insure PNEC viability during the course of the experiment. Therefore, to investigate the NNK effect on MAPK in the absence of high CO_2, cells were placed in a 5% CO_2 environment for 5 minutes subsequent to NNK treatment. This experiment resulted in attenuated elevation of MAPK compared to those levels observed at the same time interval in a hypoxic environment. A 1.5 fold increase in MAPK occurred in cells exposed to NNK, while under the same conditions, 5-HT resulted in a 1.6-1.7 fold increase in the protein level (Figure 4-17 and Figure 4-18). Therefore, the solitary effect of NNK appears to be diminished farther downstream within the MAP

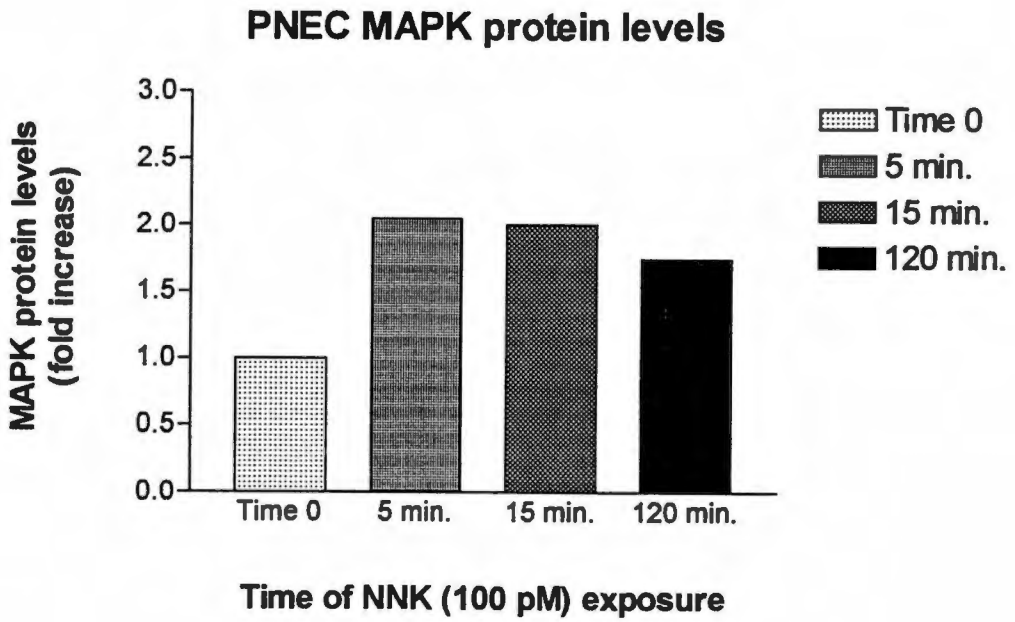

FIGURE 4-13. MAPK 1\2 protein levels in non-neoplastic PNECs. Cells were exposed to NNK [100 pM] for 0, 5, 15, and 120 minutes. Western blot analysis of cellular Raf-1 protein is expressed as a fold increase in band intensity. Data are representative of two independent experiments with single samples per treatment group giving similar results.

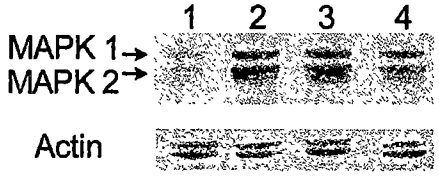

FIGURE 4-14. Western blot of MAPK 1\2 protein levels in non-neoplastic PNECs following timed NNK exposure. The bands represent MAPK 1 and MAPK 2 protein levels following 0 minutes (lane 1), 5 minutes (lane 2), 15 minutes (lane 3), and 120 minutes (lane 4) of NNK [100 pM] exposure. Actin controls demonstrate equal sample loading.

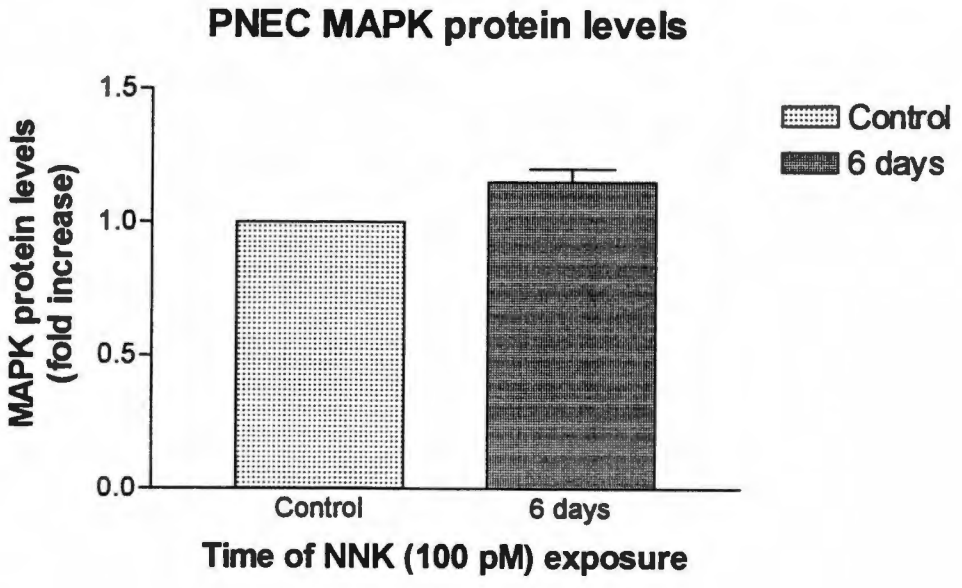

FIGURE 4-15. MAPK 1\2 protein levels in non-neoplastic PNECs following subchronic (6 days) NNK [100 pM] exposure. (n=3).

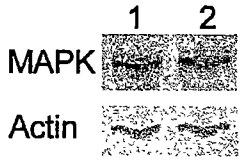

FIGURE 4-16. Western blot of MAPK protein levels following subchronic NNK [100 pM] exposure. Untreated control cells (lane 1) were maintained in same environment as NNK treated cells (lane 2).

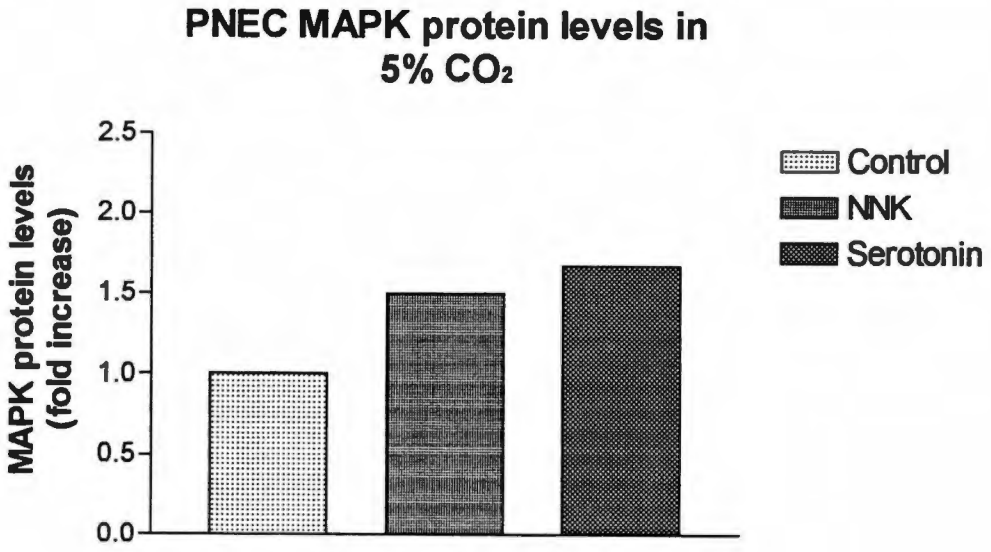

FIGURE 4-17. MAPK 1\2 protein levels in non-neoplastic PNECs following NNK [100 pM] and 5% CO_2 exposure. Results demonstrate a change in MAPK protein levels following NNK treatment and introduction into a 5% CO2 environment for 5 minutes. Serotonin [1 μM] was used as a positive control. Data are representative of two independent experiments with single samples per treatment group giving similar results.

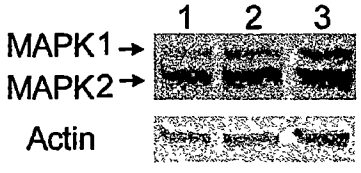

FIGURE 4-18. Western blot of MAPK 1\2 protein levels in non-neoplastic PNECs exposed to NNK and 5% CO_2. The untreated control cells (lane 1), NNK [100 pM] treated cells (lane 2), and the positive control serotonin [1 μM] treated cells (lane 3) were maintained in 5% CO_2 for 5 minutes following treatment. Bands representing the actin internal control demonstrate equal loading of sample.

kinase pathway when comparing these results with those representing Raf-1 protein levels. To further examine and substantiate the influence of NNK on the protein levels of the downstream kinase MAPK, receptor antagonists were used to block the initiation of the signal by the nitrosamine. Hexamethonium [1 μM] and α-bungarotoxin [10 μM] were used to directly block NNK binding to nicotinic receptors, and imipramine [1 μM] to prevent 5-HT uptake due to NNK induced 5-HT release. These data show that by blocking 5-HT uptake, imipramine reduced the NNK induced elevation of MAPK by 60% ($p<0.01$)(Figure 4-19). Furthermore, directly blocking all nicotinic receptors or specifically α_7 receptors by hexamethonium ($p<0.01$) and α-bungarotoxin ($p<0.01$) respectively, reduced the increase in MAPK levels by approximately 44%. Although complete inhibition of the NNK induced protein elevations did not occur as was the case for Raf-1, these data indicate a direct and indirect downstream response to NNK and 5-HT receptor-ligand binding. Additionally, these results are consistent with previous data demonstrating a 1.6 fold increase in MAPK levels after 120 minute NNK exposure. These changes are reflected in the western blot of figure 4-20.

Finally, MAPK levels were examined in H69 cells treated with NNK (100 pM) for 5, 15, and 120 minute time intervals. Neither NNK treatment or duration of exposure resulted in a significant elevation ($p<0.01$) of MAPK protein when compared to time 0 control cells (Figure 4-21). Western blots showed similar band intensity at 44 and 42 kDa among all time intervals and the time 0 controls (Figure 4-22). Also, figure 4-23 shows that the receptor inhibitors, imipramine, hexamethonium, and α-bungarotoxin, failed to

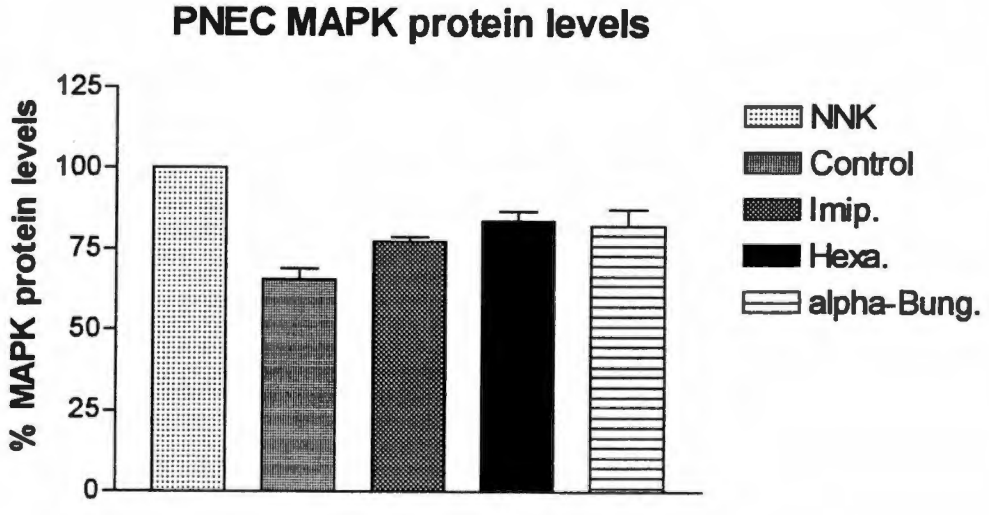

FIGURE 4-19. MAPK 1\2 protein levels in non-neoplastic PNECs following receptor-mediated inhibition. NNK [100 pM] induced MAPK protein levels were normalized at 100%. Basal levels of MAPK and inhibition of NNK induced MAPK protein levels are expressed as a percentage of the total amount of MAPK protein in NNK treated cells. Imipramine [1 μM], hexamethonium chloride [1 μM], and alpha-bungarotoxin [10 μM] were used to inhibit the effects of NNK. (n=3).

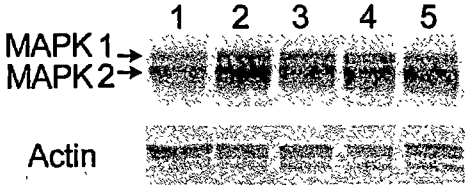

FIGURE 4-20. Representative western blot of MAPK 1\2 protein levels in non-neoplastic PNECs following receptor-meditated inhibition. MAPK 1 and MAPK 2 bands represent untreated control cells (lane 1), cells exposed to NNK [100 pM] alone (lane 2), and cells pre-treated with imipramine [1 μM] (lane 3), hexamethonium chloride [1 μM] (lane 4), or alpha-bungarotoxin [10 μM] (lane 5) prior to NNK exposure. Actin control bands demonstrate equal loading of protein sample.

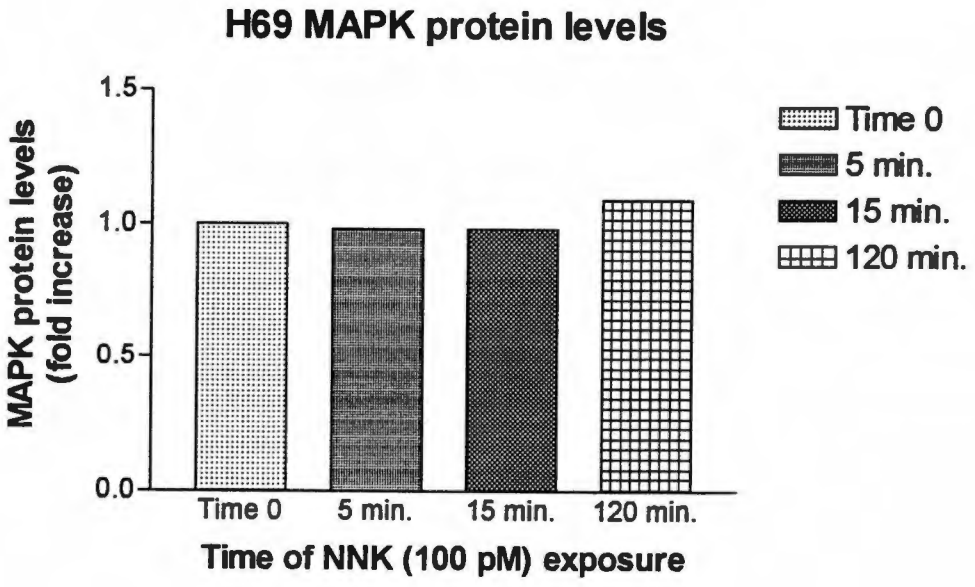

FIGURE 4-21. MAPK 1\2 protein levels in neoplastic PNECs (NCI-H69 cells). Cells were exposed to NNK [100 pM] for 0, 5, 15, and 120 minutes. Western blot analysis of cellular MAPK protein is expressed as a fold increase in band intensity. Data are representative of two independent experiments with single samples per treatment group giving similar results.

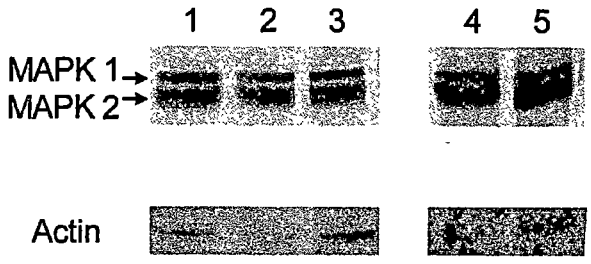

FIGURE 4-22. Western blot of MAPK 1\2 protein levels in neoplastic PNECs (NCI-H69 cells) following timed NNK exposure. The bands represent MAPK protein levels following 0 minutes (lane 1 and lane 4), 5 minutes (lane 2), 15 minutes (lane 3), and 120 minutes (lane 5) of NNK [100 pM] exposure. Actin controls demonstrate equal sample loading.

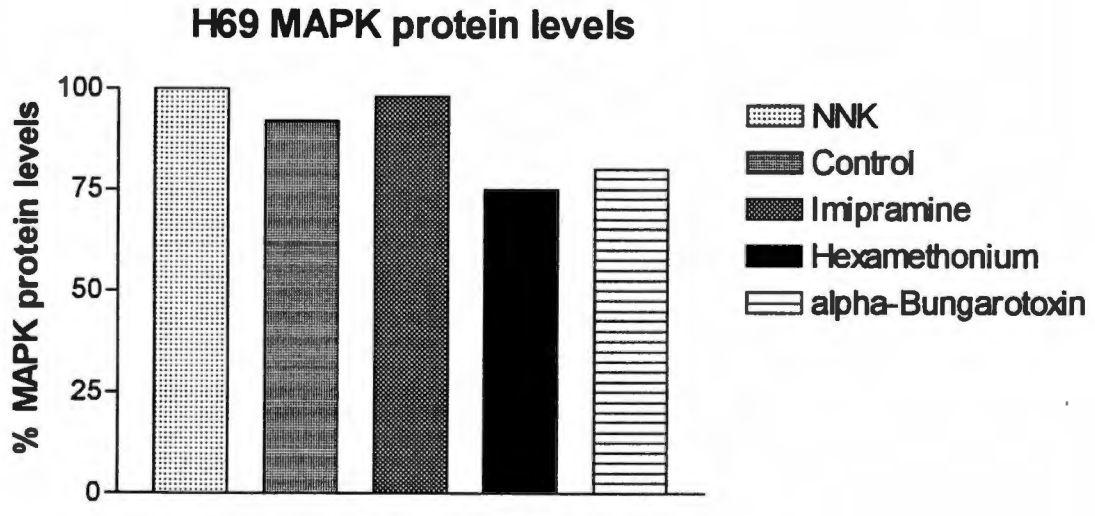

FIGURE 4-23. MAPK 1\2 protein levels in neoplastic PNECs (NCI-H69) following receptor-mediated inhibition. NNK [100 pM] induced MAPK protein levels were normalized at 100%. Basal levels (control) of MAPK and inhibition of NNK induced MAPK protein levels are expressed as a percentage of the total amount of MAPK protein in NNK treated cells. Imipramine [1 μM], hexamethonium chloride [1 μM], and alpha-bungarotoxin [10 μM] were used to inhibit the effects of NNK. Data represent two independent experiments with single samples per treatment group giving similar results.

consistently alter MAPK levels. Therefore, these findings are much alike those describing Raf-1 protein levels in neoplastic PNECs, were protein levels remained the same despite NNK exposure or receptor-mediated inhibition.

It should be noted that the Raf-1 protein level in NNK exposed fetal PNECs and H69 cells, and the MAPK level in H69 cells were not significantly altered following a 30 minute pre-treatment with the protein synthesis inhibitor cycloheximide [10 ug/ml]. However, western blots of fetal PNECs treated with cycloheximide followed by NNK exposure lacked any distinguishable 44 kDa (MAPK 1) band (data not shown). This change did not exist in the neoplastic PNECs.

Chapter 5: Discussion

There are a wide variety of intracellular signaling pathways made up of specific protein kinases that are responsible for growth, differentiation, and cell death. These proteins serve to transmit a signal, initiated through receptor ligand binding, from the cell surface to the nucleus where, among other functions, they regulate transcriptional and cell cycle activity. Subsequent to ligand binding, these signaling mediators are sequentially activated by the phosphorylation of highly conserved amino acid residues that result in a change in protein conformation. The altered protein conformation opens the catalytic domain for specific amino acid phosphorylation that in turn phosphorylates/activates other downstream protein kinases. The growth regulatory MAP kinase pathway is composed of sequentially activated kinases (Raf-1/MEK/MAPK) that may be stimulated via ligand binding to G-protein coupled receptors (GPCRs) (Porter, 1998) or autophosphorylated receptor tyrosine kinases (RTKs) (Gutkind, 1998) (Figure 4-24). These kinases are synthesized in the cytoplasm but located in various subcellular compartments depending on the mitogenic stimulus, their state of phosphorylation, and their stability/half-life (Wartman, 1997; Fanger, 1999).

We observed, by using western blot analysis, that exposure of PNECs to the mitogen NNK not only stimulated activation of MAP kinase cascade proteins (Raf-1 and

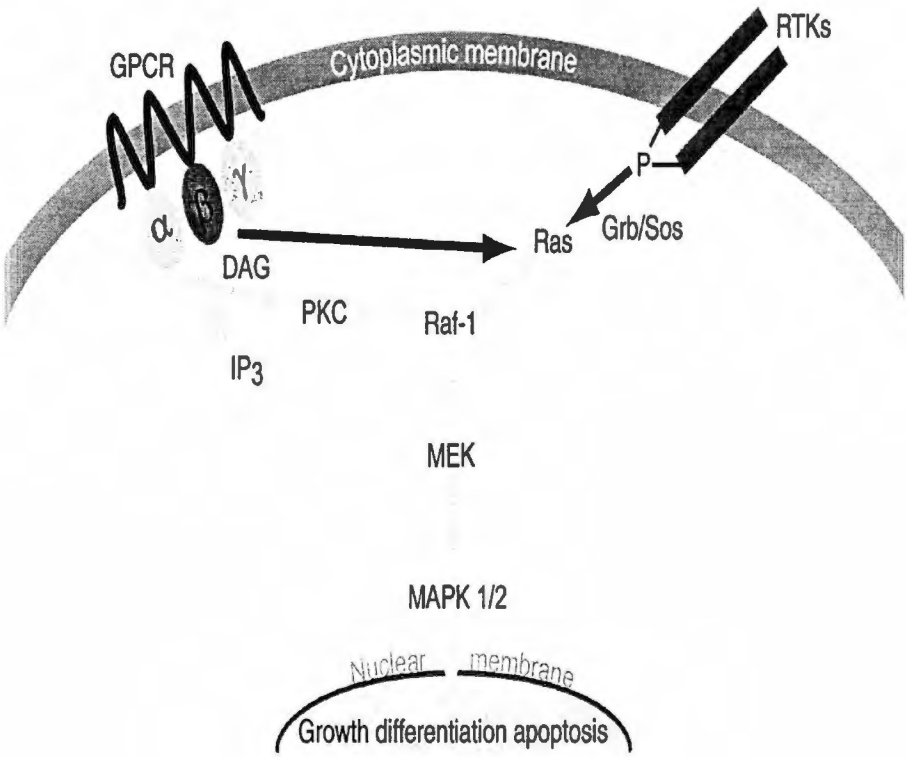

FIGURE 4-24. G-protein coupled and tyrosine kinase receptor mediated initiation of the MAP kinase pathway. GPCR activation of the signaling cascade occurs via phosphorylation of receptor subunits (α, β, γ) that subsequently activate Raf-1 through a PKC or Ras dependent mechanism.

MAPK), but also increased protein levels. Raf-1 and MAPK protein in normal PNECs were both rapidly elevated following NNK treatment, which was sustained as long as 120 minutes post-exposure. This suggests that NNK plays a direct role in regulating protein kinase levels in non-neoplastic PNECs. Relocalization of these kinases within the cell is one possible explanation for this NNK induced increase in cytoplasmic protein levels. Raf-1 exists predominantly as a cytoplasmic phosphoprotein that may be bound to various regulatory adaptor proteins such as 14-3-3 and heat shock proteins (Hsp90) (Wartman, 1994; Schulte, 1995; Vincenz, 1996). The ubiquitously expressed 14-3-3 protein serves as a regulatory protein via dimerization at multiple binding sites on Raf-1 (Campbell, 1998), and is known to form complexes with other proteins such as PKC (Vincenz, 1996). Disruption of this binding in Raf-1 mutants has been shown to alter the activation of this kinase depending on the specific 14-3-3 binding site inhibited (Rommel, 1997; Clark, 1997). Although all functional roles of this protein and the mechanisms by which these functions are carried out are not known, it is believed that the interaction of 14-3-3 with Raf-1 serves as a scaffolding mechanism which results in the recruitment/localization and subsequent interactions with other signaling components (Reuther, 1996; Fanger 1998). Recently, Prezeau (1999) reported data supporting an interaction between the 14-3-3/Raf-1 complex and GPCRs that was inhibited by phosphorylated Raf-1, but not non-phosphorylated Raf-1. Therefore, it is conceivable that inactive Raf-1 bound to 14-3-3 may be localized to the 5-HT GPCR and is subsequently released into the cytoplasm upon activation. This type of membrane localization may serve to bring Raf-1 into close

proximity to its immediate effector PKC. A similar functional role has been shown to exist as it relates to Ras recruiting of Raf-1 to the membrane where it is activated by autophosphorylated RTKs (Rommel, 1996). Interestingly, the signaling mediators generated by the GPCR induced hydrolysis of phospholipase C (PLC) ,diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), alter the localization of PKC to the subcellular membrane compartment where it is subsequently activated (Bell, 1988). Therefore, it is possible that activated PKC via DAG, IP_3, and 14-3-3 is complexed with or localized near non-phosphorylated Raf-1, which is in turn phosphorylated by PKC and released into the cytoplasm to activate downstream substrate kinases. This idea supports the emerging belief that signal transduction is in part achieved through complex formation rather than a simple linear signaling pathway (Campbell, 1998). Furthermore, Wartman (1997) demonstrated a decreased affinity of hyperphosphorylated Raf-1 for the plasma membrane with a corresponding increase in the cytoplasmic fraction of the kinase. Plasma membrane affinity was restored in this case by blocking this hyperphosphorylation of Raf-1. In addition to 14-3-3, other adaptor proteins have been implicated in the redistribution and trafficking of Raf-1 within the cells. Hsp90 has been shown to play a role in the formation of Ras-Raf-1 membrane bound complexes, which are disrupted when Hsp90-Raf-1 heterocomplex formation is blocked (Schulte, 1995). Although no link has been made between this chaperone protein and GPCRs, perhaps it functions in a manner similar to that of the 14-3-3 protein as it relates to membrane localization of Raf-1. Therefore, as illustrated in figure 4-25, it is reasonable to believe that inactive Raf-1 localized to GPCRs

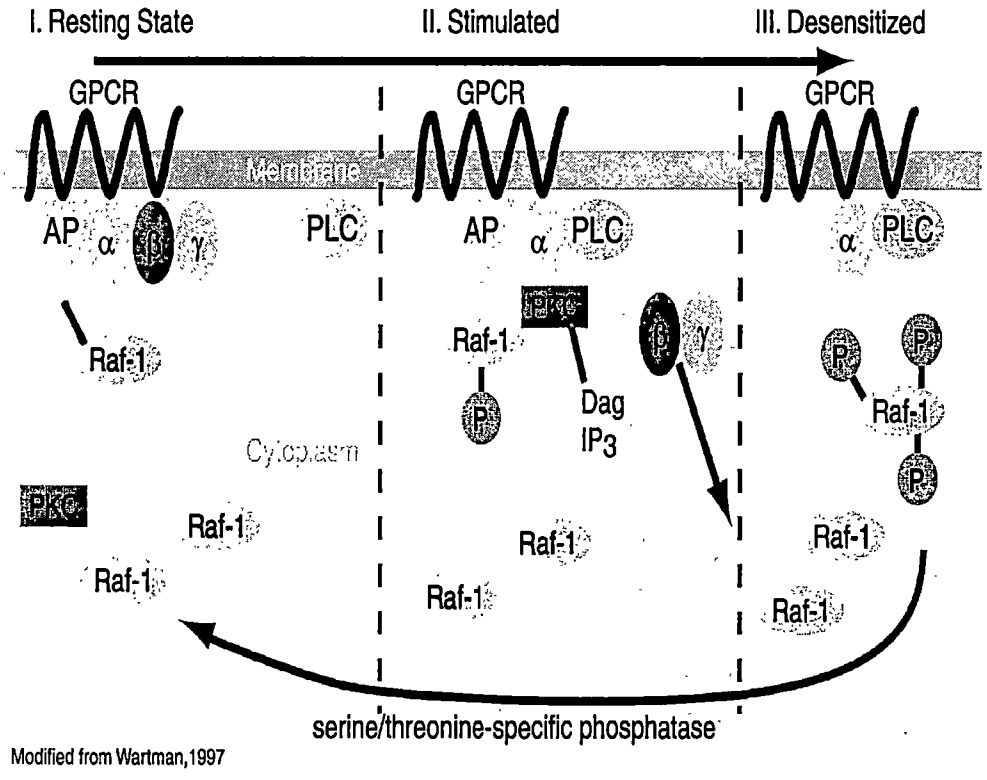

FIGURE 4-25. Proposed subcellular redistribution and relocalization of Raf-1 following NNK stimulated 5-HT receptor-ligand binding to G-protein coupled receptors. In the resting state, Raf-1 forms a complex with the G-protein coupled receptor via adaptor proteins. Following 5-HT receptor-ligand binding, Raf-1 is phosphorylated via a PKC dependent pathway. Continued phosphorylation of Raf-1 results in the release of the kinase from the membrane bound fraction into the cytosol.

via adaptor proteins (14-3-3 and Hsp90) is activated via phosphorylation by its complexed effector PKC, and subsequently propagates the signal through the activation of downstream substrates. Continued phosphorylation of Raf-1 may result in its dissociation from the plasma membrane associated complex; thus effectively preventing further activation and signal transduction. The hyperphosphorylation and resultant dissociation would serve as a negative self regulatory mechanism.

This proposed mechanism may explain the rapid increase in Raf-1 protein levels within the non-neoplastic PNECs following NNK stimulation via 5-HT release and uptake. Cell lysates collected from each experiment are likely to contain a predominant proportion of cytoplasmic Raf-1, while the remaining membrane is complexed with a smaller amount of the kinase. Nonsoluable cell components, particularly membrane fragments, are subsequently seperated and discarded away from the soluable cytoplasmic components. As a result, each sample examined is composed in large part of cytoplasmic protein. Therefore, according to the above proposed mechanism, Raf-1 redistributed away from the membrane following NNK induced activation could result in the elevation of cytoplasmic Raf-1 protein levels compared to either unstimulated or time 0 controls. This may account for this increase in the protein level which occurred in a similar amount of time as did the activation of this kinase.

Another explanation for this rise in cytoplasmic Raf-1 stimulated by NNK, may be related to the half-life of the kinase. It has been shown that the disruption or the stabilization of Raf-1 interactions with other adaptor proteins may result in a decrease or

increase in its half-life (Schulte, 1995). Specifically, inhibiting the interaction between Hsp90 and Raf-1 reduced the half-life of the kinase. Therefore, is it possible that NNK might actually stabalize such an interaction, increase the half-life of Raf-1 in PNECs, and subsequently increase the levels of cytoplasmic Raf-1 as demonstrated by our data? Although this potential mechanism accounting for the increase in kinase level can not be ruled out by our experimental data, it is believed to be a less likely explanation for the rapidly occurring observed response. Detectable changes in kinase half-life would not be expected to occur in the short period of time examined in our experiments. However, this mechanism may in part account for the higher protein levels observed after subchronic exposure to NNK. It is also likely that the accentuated increase in protein levels following 6 days of continuous NNK exposure is the end result of more than one mechanism. In addition to the increased half-life and the subcellular redistribution of Raf-1, the possibility of increased Raf-1 protein synthesis must be addressed.

Traditionally, Raf-1 was believed to be constitutively expressed in many cells with little need for tight transcriptional regulation (Storm, 1990). However, Schulte (1995) performed experiments in non-PNECs that demonstrated a chemically induced increase in the synthesis of Raf-1. Although the exact mechanism responsible for the increased protein expression is not known, these studies do establish an example of exogenous chemically induced transcriptional regulation. Therefore, Raf-1 synthesis may, in conjunction with other processes, increase the levels of Raf-1 kinase in PNECs subchronically treated with NNK. However, can increased protein synthesis account for

the rapid elevation of Raf-1 levels within the PNECs of our experiments? Studies using mouse hepatocytes treated with the mitogen 1,4-bis[2(3,5-dichloropyridyloxy)] benzene revealed markedly accelerated rates of protein synthesis compared to untreated hepatectomized stimulated cells (Ledda-Columbano, 2000). An 18 fold increase in the rate of cyclin dependent kinase (CDK2) protein synthesis was observed in treated cells with the highest level of expression occurring within 2 hours. Unfortunately, no time points less than 120 minutes were examined. Therefore, it is reasonable to believe that protein synthesis can occur in a very short amount of time following stimulation, and that NNK may stimulate this synthesis. This rapid synthesis may require constitutively expressed Raf-1 mRNA or may be the result of post-translational modification that unmasks the Raf-1 protein. However, in our studies, pre-treatment of normal and neoplastic PNECs with cycloheximide failed to inhibit the NNK induced elevation of Raf-1 and MAPK as measured by western blot analysis; thus lending support to the proposed theories of relocalization and/or protein stabilization. Interestingly in normal PNECs exposed to cycloheximide, there was no band at the 44 kDa (MAPK 1) position indicating a lack of this MAPK isoform. It is unclear whether this represents protein synthesis inhibition of this specific isoform or if the cycloheximide treatment altered the protein and its subsequent migration. Nevertheless, these plausible explanations relating to Raf-1 protein synthesis can neither be confirmed nor completely ruled out based on our experimental data. Investigation into the unique signaling pathways and specific signaling mediators responsible for NNK induced protein kinase synthesis are the focus of future experiments.

MAPK 1\2 was also mildly elevated in normal PNECs exposed to NNK for periods less than 120 minutes. Certainly the rapid rise in protein levels is consistent with intracellular protein redistribution as is the likely case with Raf-1. Although the relocalization of MAPK into the nucleus has been well documented in many cell types (Chen, 1992; Lenormand, 1993), other areas of subcellular localization and redistribution of this protein are less well understood. MAPK has been shown to phosphorylate and regulate a variety of other extranuclear substrates such as cell surface proteins, cytoskeletal components, and cytoplasmic kinases (Guan, 1994). This suggests interaction and possibly subcellular compartmentalization of MAPK. A mitogen induced conformational change of MAPK or bound adaptor proteins, could alter these interactions and result in shifts in subcellular location. This could also account for the multiple effect phenomenon MAPK has on either cell growth, differentiation, or death as reported in some studies (Pumiglia, 1997). Interestingly, although MAPK in the normal PNEC was rapidly elevated within 120 minutes, protein levels evaluated following 6 days of continuous NNK exposure did not show a significant increase over cells not treated with NNK. This is believed to be due to maintenance of the cells in 10% CO_2 for the 6 day time period in order to insure cellular viability. When comparing these results with the shorter time intervals and those obtained for Raf-1, there is suggestion that elevated CO_2 also resulted in increased protein kinase levels. Reintroduction of basally suppressed cells into a hypoxic environment in conjunction with NNK exposure likely leads to a

cumulatively induced increase in Raf-1 and MAPK levels. These levels appeared to decline slightly over 120 minutes which may suggest the reduction in the influence and acclimation to either NNK or the elevated CO_2. It is unlikely that there is acclimation to NNK since there is elevation in Raf-1 compared to untreated controls after 6 days of continuous NNK treatment while being maintained in 10% CO_2. Therefore, the lack of MAPK protein elevation after subchronic NNK exposure is suggestive of increased basal protein levels which may be the result of other growth factors and the integration of other non-NNK associated pathways at the point of MEK.

In order to circumvent the influence of the elevated CO_2, normal PNECs were exposed to NNK in a 5% CO_2 environment (similar to that found in the healthy lung). Levels of both Raf-1 and MAPK were increased, but to a lesser extent than when in combination with 10% CO_2. These results are consistent with our previous data and the proposed hypothesis explaining the data, and demonstrate an inducible effect on protein kinase levels by NNK without the influence of hypoxia.

To compare the effect of NNK on protein levels in neoplastic PNECs, similar experiments as those performed in non-neoplastic fetal PNECs were conducted in human SCLC cells (H69 cells). It was discovered that neither Raf-1 nor MAPK protein levels were increased significantly by NNK treatment irregardless of exposure time. The lack of difference between basal levels at time 0 and treated cells likely reflects constitutively high levels of expression in these cells that cannot be altered by NNK. These results are consistent with previous findings in which SCLC were shown to express very high levels

of normal size Raf-1 mRNA and protein (Pfeifer, 1989). This preferential overexpression was found to exist apart from the sporadic constitutive activation due to deletions in chromosome 3p25 which leads to the loss of the N-terminal regulatory region of the protein, as described by Naumann (1997). Our data are supported by these independent findings, as we also observed constitutive expression of both Raf-1and MAPK without concurrent constitutive activation. This change likely reflects amplification of each respective proto-oncogene, since these tumor cells rely in large part on the MAPK pathway as well as transcriptional and translational components for sustained growth. Using *in vitro* models, Pfeifer (1998) provides evidence that Raf-1 overexpression is required for the development of SCLC, and chemical induced gastric carcinomas have also been linked to amplification of ribosomal genes early in the course of carcinogenesis in tumors containing constitutively elevated levels of Raf-1 (Khanson, 1991). Nevertheless, an inciting cause of probable proto-oncogene amplification in SCLC has not been definitively determined. Some evidence eludes to profound upregulation of mitogenic receptors resulting in chronically intense stimulation of mitogenic pathways (Bencherif, 1995), while others implicate possible genetic deletions in non-coding DNA sequences (Patel, 1993). However, no specific evidence has been found in relation to SCLC, and previous work has failed to demonstrate specific mutations in SCLC proto-oncogenes that are responsible for Raf-1 and MAPK overexpression.

Upon demonstrating increased levels of Raf-1 and MAPK in non-neoplastic PNECs treated with NNK, we subsequently attempted to inhibit this induced response by

blocking suspected receptors involved, and therefore establishing a receptor-mediated link to the rise in protein kinase levels. The levels of Raf-1 protein observed in cells pre-treated with the 5-HT receptor uptake inhibitor, imipramine, were markedly reduced compared to all other treatment groups and the untreated controls. As illustrated in figure 2-11, 5-HT release and uptake are believed to be the result of concerted stimulation by NNK and CO_2. Blocking this centrally located receptor-mediated autocrine and paracrine effect would be expected to inhibit downstream signaling stimulated by numerous mitogens including NNK. As proposed in figure 4-24, blocking 5-HT GPCRs would be expected to prevent phosphorylation of Raf-1and may prevent subsequent relocalization into the cytoplasm. Higher Raf-1 levels in untreated control cells compared to imipramine treated cells are believed to reflect the basal levels of 5-HT release and uptake after 120 minutes in a hypoxic environment. Although the influence of the high CO_2 may have diminished slightly throughout this time period, other growth factors would likely have been elaborated over 120 minutes and subsequently caused 5-HT release and uptake. However, blocking all nicotinic receptors with hexamethonium should only eliminate the NNK induced 5-HT effect while leaving the 5-HT receptors intact for other mitogen induced 5-HT stimuli. In fact, our data supports this hypothesis, as Raf-1 levels in hexamethonium treated cells are similar to those in untreated cells. Furthermore, by blocking only the α_7 nAChR subtype via α-bungarotoxin, the NNK induced Raf-1 protein increase is partially inhibited when compared to cells treated with hexamethonium which totally blocked the effect of NNK. The difference in Raf-1 protein levels between these

two treatment groups is likely the result of NNK binding to unblocked non-α_7 nAChR. These latter results are consistent with the findings by Schuller (1998) concerning the specific high affinity binding of NNK to the α_7 nAChR. MAPK protein levels were found to be inhibited by these receptor antagonist in a similar manner as that of Raf-1. However, neither the receptor antagonist nor the 5-HT uptake inhibitor prevented the induced protein increase to the same degree as they prevented Raf-1 elevation. MAPK levels in imipramine pre-treated cells are similar to levels in untreated control cells, while the receptor antagonists, hexamethonium and α-bungarotoxin, only partially inhibited the induced protein elevation. Although the reason for this less pronounced inhibition of protein levels is not certain, it is highly possible that the influence of other signaling pathways on MAPK via other receptor-mediated processes attenuates such a dramatic shift in protein levels. The similar amounts of MAPK in imipramine treated cells and untreated control cells also likely points to the influence of other non-5-HT associated pathways that effect MAPK. Since MAPK serves as a more common integration point among multiple cell signaling pathways, it is reasonable that this protein be less responsive to a single inhibitor. Therefore, a link may be established between NNK ligand binding to specific receptors and increases in protein kinase levels. These data not only support the hypothesis of an NNK linked receptor mediated signaling pathway, but also exhibit the vital role of 5-HT in affecting the levels of Raf-1 and to a lesser extent MAPK. Additionally, these results are consistent with the proposed mechanisms of Raf-1 and

MAPK protein kinase relocalization, stabilization, or synthesis following NNK stimulation in non-neoplastic PNECs.

Pre-treatment of the H69 cells with the receptor antagonists and the 5-HT uptake inhibitor failed to change the the Raf-1 and MAPK levels when compared to cells treated with NNK alone or untreated controls. These protein kinases appear to be at constitutively high levels in the neoplastic PNECs and may not be influenced by receptor mediated inhibition. This certainly appears to be the case with Raf-1, and could implicate DNA amplification as a potential mechanism for this constitutively high kinase level. However, these levels may also be consistent with the upregulation of unidentified non-nicotinic and non-5-HT mitogenic receptors. Finally, the inability of NNK to stimulate a protein kinase increase in neoplastic PNECs is compatible with the earlier data described.

These data demonstrate a functional relationship between NNK receptor mediated binding and Raf-1/MAPK protein levels. The ability of this tobacco-derived nitrosamine to stimulate increases in the mitogenic pathway protein kinase levels of non-neoplastic cells lends further support for the mitogenic effect of this compound which could subsequently result in SCLC development. Future experiments should focus on the mechanisms by which these proteins are elevated following NNK treatment and the point at which protein levels within PNECs become unresponsive to NNK due to either gene amplification or receptor upregulation. These results in conjunction with our data will help elucidate the importance of kinase expression in SCLC carcinogenesis.

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PART V: GENERAL SUMMARY

Chapter 1: Summary

Lung cancer remains to be the most frequently encountered lethal malignancy in the United States with over a quarter of all cancer deaths associated with this group of neoplasms (American Cancer Society, 1998). Of these lung cancer related deaths, SCLC accounts for almost 90%, making it the most lethal form of pulmonary cancer (Alberg, 1998). Furthermore, of the diagnosed cases of SCLC, 90% have been epidemiologically linked to the act of smoking and COPD (Alberg, 1998). In fact, individuals that smoke and concurrently suffer from COPD, whether smoking related or not, are at higher risk to develop SCLC (Weiss, 1991). This has lead to a variety of investigations examining the mechanism by which individuals that smoke develop SCLC, particularly if they are diagnosed with COPD. It has been shown that SCLC cells and their non-neoplastic pulmonary neuroendocrine cell (PNEC) of origin proliferate when exposed to tobacco-specific carcinogens and/or 10% CO_2 (hypoxia) (Merryman, 1997; Schuller, 1994). Nicotine is one such carcinogen that in addition to stimulating cell proliferation was found to also result in the production of various bioamines, thus imitating the effects of acetylcholine (Tabassian, 1988). It has been well documented that nicotine exerts this effect via high affinity binding to the cholinergic receptors (Taylor, 1990; Schuller, 1998). In turn, it has been proposed that these released bioamines may act in an autocrine and

paracrine manner to facilitate a cellular response (i.e. proliferation). Although nicotine is the most abundant carcinogen present within tobacco smoke, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is known to be a highly potent derivative of nicotine that binds with much greater affinity to the α-bungarotoxin sensitive subtype (α_7) of the nicotinic acetylcholine receptor (nAChR) as compared to nicotine (Schuller, 1998). Of the many secretagogues stimulated by nicotine, NNK, or other mitogenic receptor-ligand binding, specifically 5-hydroxytryptamine (5-HT, serotonin) has been shown to be rapidly released and influence the proliferation of PNECs (Youngson, 1993; Merryman, 1997; Schuller, 1994).

In order to link the NNK induced receptor-mediated serotonergic signal to the proliferative response observed in PNECs, we examined the effect of NNK on a major cell regulatory signaling pathway, the MAP kinase cascade. This was achieved through the analysis of normal and neoplastic PNEC kinase activation and protein levels, specifically those of Raf-1 and MAPK 1\2, following NNK exposure.

The MAPK cascade is a growth regulatory pathway that is initiated by ligand binding to a variety of receptors, and is composed of specific protein kinases regulated by a series of phosphorylation and dephosphorylation reactions (Heidecker, 1992; Mgnusun, 1994; Mischak, 1996). A broad range of extracellular stimuli such as chemical compounds, growth factors, photons, and inflammatory mediators may bind to either tyrosine-kinase receptors (RTK) or heterotrimeric GTP-binding proteins that are coupled to receptors (GPCRs). The former, RTK, is composed of three major components which

include an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (Porter, 1998). Upon ligand binding, there is trans-autophosphorylation of the kinase binding domain which subsequently activates the G-protein Ras via the SH2 (src homology 2) containing adaptor protein Grb2. Although Ras acts on a wide variety of effector targets, it has been shown that activated Ras forms a high affinity complex with the serine/threonine kinase Raf-1 (Van Aelst, 1993; Moodie, 1993; Zhang, 1993; Warne, 1993). This complex formation results in the membrane localization of Raf-1 and subsequent activation of MAPK 1/2 . Alternatively, ligand binding to GPCRs can result in Raf-1/MAPK activation. GPCRs are composed of a seven transmembrane receptor linked to a heterotrimeric (α,β,γ subunits) G protein (Dohlman, 1987). Ligand binding results in the conversion of GDP to GTP on the α subunit which in turns dissociates from the $\beta\gamma$ subunits. The activated α subunit has been shown to activate phospholipase C-β (PLC) (Wu, 1992; Conklin, 1992), and subsequently hydrolyzes phosphatidylinositol into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Dhanasekaran, 1998). The generated diacylglycerol in conjunction with IP3 results in the membrane localization and activaton of protein kinase C (PKC) via a Ca^{2+} dependent mechanism (Dhanasekaran, 1998). An important effector of PKC is the protein kinase Raf-1.

Raf-1 (74 kDa) is a ubiquitously expressed 648 amino acid protein that is composed of three highly conserved regions within the N-terminal regulatory domain and the C-terminal kinase domain (Bonner, 1986). It has been demonstrated that

phosphorylation of specific serine residues (Ser259) results in the activation of this kinase (Isumi, 1991), while phosphorylation of other amino acid residues (Ser621) results in the inhibition of Raf-1 (Morrison, 1993). Phosphorylation of the appropriate amino acid is believed to conformationally alter the protein and expose the catalytic domain for subsequent substrate activation (Campbell, 1998). MEK (43-46 kDa) is the only known substrate of Raf-1 (Williams, 1994) which has been shown to form a relatively stable interaction at the Raf-1 C-terminus kinase domain (Alessi, 1994; Van Aelst, 1993). MEK activation is a result of phosphorylation at serine 217 and 221 of a highly conserved consensus sequence (Alessi, 1994), and acts as a dual specificity kinase which phosphorylates specific threonine and tryrosine residues within a characteristic TXY motif of MAPK 1\2 (Dhanasekaran, 1998). Activated/phosphorylated MAPK 1\2 may translocate to the nucleus where they in turn activate nuclear proteins involved in cell cycle regulation and cell death. The c-myc protein is a tightly regulated nuclear phosphoprotein that may be phosphorylated at specific amino acid residues (Thr-58 and Ser-62) by MAPK within the cytoplasm as well as within the nucleus (Lutterbach, 1994). In this way, a mitogenic signal, such as NNK, sends information from the cell surface to the nucleus where it may regulate the cell cycle.

From our experiments we were able to demonstrate the activation of both Raf-1 and MAPK in normal and neoplastic cells resulting from exposure to low concentrations of NNK that are consistent with those levels found in tobacco smoke. The role of 5-HT

was made evident by blocking its uptake which resulted in the reduction of NNK induced kinase activation. These findings supported the idea that 5-HT acts as a mitogen (NNK) induced secretagogue that subsequently acts in an autocrine manner to stimulate the activation of Raf-1 of the MAP kinase pathway. Since 5-HT receptors are characteristically transmembrane GPCRs (Hoyer, 1994), it is likely that they stimulate Raf-1 activation via a PKC dependent, non-RTK pathway. Furthermore, by inhibiting PKC activation with sphingosine our results demonstrated a profound reduction in Raf-1 activation. Therefore, alternative pathways involving GPCRs and Ras, that would normally circumvent PKC involvement, do not appear to play a major role in the NNK induced serotonergic activation of Raf-1. NNK may also contribute more directly to the activation of Raf-1 and augment the effect of serotonin. Since nicotinic receptors are calcium ion channels (Linstrom, 1995) and NNK binding to these receptors increases the Ca^{2+} influx into PNECs (Sheppard, unpublished), then elevated cytoplasmic calcium levels may enhance the localization and activation of PKC. The actual mechanism of NNK induced 5-HT release and the receptors involved in its uptake into PNECs are the focus of future experiments, but the role of 5-HT uptake in the activation of MAP kinase pathway components is evident.

The activation of downstream kinases within this cascade was also shown to be effected by NNK, as blocking the MAP kinase pathway at the level of MEK resulted in reduced MAPK 1\2 activation. This reduced activation of MAPK also resulted in a reduction in the NNK induced phosphorylation of Myc at the Ser-62 residue, an event that

has been found to be important in the stabilization of this protein and its ability to regulate the cell cycle (Sears, 1999). Our data represents the first to show an effect of NNK on Myc, and also opens the door for investigations into the effect of NNK on the cell cycle itself. These changes in activation/phosphorylation provide an explanation and an intracellular mechanism for the increased cell proliferation of both normal and neoplastic PNECs exposed to NNK. Although other mitogenic signals, such as elevated CO_2 and serum, have been shown to stimulate PNEC proliferation (Merryman, 1997; Schuller, 1994), measures were taken in our experiments to reduce the effect of these influences as much as reasonably feasible. Therefore, the activation of the MAP kinase cascade leading to the activation/phosphorylation of Myc is believed to predominately represent the effect of NNK. Nevertheless, elevated CO_2 may also effect the MAP kinase pathway in a similar fashion as the result of concurrent stimulation of 5-HT release and uptake. Increased CO_2 is the hallmark of COPD, and as previously mentioned is an important risk factor along with smoking that is associated with SCLC. The effect of CO_2 on the MAP kinase pathway, Myc, and 5-HT as well as the characterization of the oxygen sensitive receptor is the focus of future investigations.

The fact that NNK stimulates activation of the MAP kinase cascade and cell proliferation in both normally "dormant", non-neoplastic PNECs of adult lungs and neoplastic PNECs strongly suggests that this carcinogen is involved in the initiation (PNEC hyperplasia) and progression (tumor growth) of SCLC. The point at which transformation takes place and the events that constitute this change remains unclear.

Although various genetic alterations have been found to exist in some SCLCs, there incidence is sporadic and non-specific. Furthermore, the concentrations required to experimentally produce either bulky adducts (i.e. pyridyl oxobutanone) or smaller alkylating adducts (i.e. methyl diazoate) from NNK metabolites is much higher than those concentrations present within tobacco smoke. Therefore, it is believed to be unlikely that SCLC is a result of NNK induced DNA adduct formation with subsequent cellular transformation. Instead, our data suggests that the growth and proliferation of normal and neoplastic PNECs is responsive to NNK through a cell signaling mediated process that may result in the regulation of the cell cycle. Although cell cycle function in neoplastic PNECs may become dysregulated at some point in time, it is apparent through our findings and those demonstrating NNK induced proliferation of SCLC (Schuller, 1994) that these cells remain responsive to receptor-mediated signaling events initiated via NNK.

Our experiments produced an interesting finding when examining concurrent expression of Raf-1 and MAPK 1\2 in non-neoplastic PNECs following NNK exposure. NNK resulted in the rapid elevation of Raf-1 and MAPK 1\2 protein levels that was inhibited by blocking either the nicotinic AChRs or blocking 5-HT uptake. The increase in protein levels may be the result of Raf-1 and MAPK localization and redistribution within the cell which has been shown to stabilize and promote interactions with effector and substrate molecules (Reuther, 1996; Fanger, 1998; Guan, 1994). Although poorly understood, the redistribution and interaction of Raf-1 and MAPK may play a highly

orchestrated role in facilitating the activation of these kinases and may also be relocalized as a result of the activation. Therefore, the Raf-1 and MAPK protein levels observed in our experiments may be a function of there activation rather than a separate molecular event induced by NNK. However, increase protein synthesis can not be ruled out as a contributing factor also resulting in the elevation of protein levels, particularly those levels observed following subchronic exposure to NNK.

These changes were not apparent within the SCLC line, where levels of Raf-1 and MAPK 1\2 appeared to be independent of NNK receptor-ligand binding. The protein levels remained similar among all treatment groups examined, and likely represents constitutive expression, possibly overexpression, of these kinases in PNEC tumor cells. These data were consistent with previous findings documenting the overexpression of these kinases in SCLC (Pfeifer, 1989). The mechanism by which this constitutive overexpression occurs has not been completely elucidated. It can be proposed that the high levels of Raf-1 and MAPK may be the result of a primed growth pathway in which protein synthesis is accentuated (Pfeifer, 1998), and these high levels may overshadow more subtle changes in subcellular redistribution following kinase activation. Future experiments should be directed at clarifying the mechanism by which these protein levels are increased in mitogen stimulated cells. Nevertheless, NNK not only results in the activation of the MAP kinase pathway, but also increases the cytoplasmic protein levels of its major constituent kinases.

This study has demonstrated an important link between the tobacco associated carcinogen, NNK, and the proliferative cellular response it has been observed to cause. We have shown that the MAP kinase cascade serves as an important pathway for the NNK receptor-mediated signal in traveling to the nuclear cell cycling apparatus. Future investigations should focus on: a) the effect of NNK exposure on the cell cycle and its major regulatory components, b) the characterization of the 5-HT receptor and the mechanism by which 5-HT release and uptake is stimulated by NNK, c) the effect of COPD (elevated CO_2) on the MAP kinase pathway as it relates to NNK stimulation, 5-HT release and uptake, c-myc protein activation, and characterization of the oxygen sensitive receptors. These studies will help further explain the pathogenesis of smoking related SCLC, and advance the cause for the development of effective chemotherapeutic and chemopreventive measures.

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VITA

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