The Neurotensin-Induced Release Pathway of Vascular Endothelial Growth Factor in Human Mast Cells

An honors thesis for the Department of Biology

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ABSTRACT

Mast cells are immunocompetent cells located throughout the body, their function dictated by the specific microenvironments of the tissues in which they reside. Storing or synthesizing *de novo* a variety of chemicals, mast cells release mediators by selective secretion or compound exocitosis that are involved in host defense, allergy, inflammation, and in the growth of some tumors. One such mediator, Vascular Endothelial Growth Factor (VEGF), is crucial to both physiological angiogenesis and the angiogenesis associated with pathological conditions such as tumor growth. Our lab has previously shown that the neuroendocrine peptide neurotensin (NT) causes an increase in the expression of VEGF isoforms in HMC-1 cells mast cells and mediates VEGF release through an NT-specific NTS-1 receptor. This project sought to confirm and extend these findings by exploring additional 'downstream' components of the VEGF release pathway, identifying several enzyme mediators that, when selectively inhibited, block the release of VEGF following NT stimulation. Selectively inhibiting MEK1 MAPK, PKC, PKA, p38 MAPK, and PI3-Kinase have shown significant decreases in VEGF release following NT-stimulation of HMC-1 cells. In addition, a Zinc chelator blocked the release of VEGF. Western blotting was used to confirm these observations.

This research may be applied to the understanding of human cancers, as mast cells have been shown to accumulate around various tumors. In addition, various cancer cells have been shown to secrete NT. Thus, the release of a pro-angiogenic factor from mast cells by NT stimulation may be tied to the blood supply required by growing tumors. Knowledge of these cellular interactions may provide valuable insight into tumor growth and development.

INTRODUCTION

Mast Cells

Mast cells are immunocompetent cells that are derived from hematopoeitic cells in the bone marrow and express CD34, *c-kit* and CD13 surface markers (52). Mast cell precursors circulate via blood vessels and the lymphatic system as committed immature progenitors before maturing in peripheral tissue environments (100), where they express proliferative (and likely migratory) abilities, but no longer circulate (40). Mast cell development is in part due to the interactions of various stimuli with cell surface molecules, such as the interaction between the tyrosine kinase c-kit and its ligand stem cell factor (SCF) (40). In addition to SCF, interleukin (IL)-3, IL-4, IL-9, IL-10 and nerve growth factor (NGF) are also known to affect mast cell growth and proliferation (77).

Mast cells inhabit a diverse array of tissue environments, most frequently located around neurons, in vascular tissue, in the skin, and in the urogenital, respiratory, and gastrointestinal tracts (40). These locations enable mast cells to respond to numerous internal and external factors (40), such as blood-borne antigens, toxins from biting or stinging insects, neuropeptides, and other chemicals that may enter the body (30). Although mast cells are best known for their involvement in allergic and inflammatory responses, they also play roles in bone remodeling (30), in addition to many chronic inflammatory disorders such as asthma, multiple sclerosis, rheumatoid arthritis, cardiovascular disease, and irritable bowel syndrome (30, 43, 99). Increased numbers of mast cells have also been found closely associated with various types of cancers, including breast carcinoma (9, 42, 43), pulmonary adenocarcinoma, colorectal cancer (86), basal cell carcinoma, haemangioma and haemangioblastoma (34), and uterine cervix cancer (39),

among others (46, 51, 60). Increased mast cell presence around tumors may thus link mast cell mediators to the growth and development of cancers.

The tissue environment surrounding mast cells impacts the phenotype of the cells. Two primary types of human mast cells have been differentiated based on the neutral proteases expressed in their granules (94). Human mast cells expressing both tryptase and chymase are found predominantly in the skin, the small intestinal submucosa and in most types of connective tissue (76), while mast cells containing tryptase, but lacking chymase, are most prevalent in the gastrointestinal tract and lung tissue (85). The proteolytic activity of these surface enzymes allows mast cells to respond to various stimuli, such as environmental growth factors and disease. These enzymes have numerous effects on cytokine expression and release, in addition to various mast cell behaviors, such as migration, adhesion and differentiation. For example, tryptase has been shown to promote angiogenesis *in vitro* (7), which is linked to numerous processes throughout the body including: inflammation, allergy, and even potentially tumor growth and development. This characteristic helps to explain the positive correlation between tryptase-positive mast cell presence and the increase in vascularization as cancer cells in the uterine cervix progress from dysplasia to invasive cancer cells (3).

Mast Cell Activation

The most prominent mechanism of mast cell activation is mediated by the high-affinity immunoglobulin (Ig) E receptor, FcεRI. The binding of IgE promotes a cascade of biochemical and morphological changes known as anaphylactic degranulation (30), a form of compound exocytosis that leads to release of granule-associated mediators. In this process, mast cell granules rapidly fuse together before eventually forming a pore in the plasma membrane through

which an array of solubilized mediators is released to promote an allergic response (15). Some inflammatory molecules associated with mast cell stimulation and subsequent degranulation include histamine, IL-6, IL-8, IL-13, tumor necrosis factor-α (TNF-α), prostaglandin D_2 (PGD_2), tryptase, leukotriene C_4 (LTC_4), and vascular endothelial growth factor (VEGF) (8, 33, 40).

Mast cells also release mediators independently of IgE. They can be stimulated by non-immunologic mediators that include the complement fragments C3a and C5a, in addition to various neuropeptides, anaphylatoxins, and cytokines (99) without activation through the IgE receptor.

Neurotensin

Neurotensin (NT) is a 13 amino acid neuroendocrine peptide studied in our laboratory that promotes mast cell activation (34). NT was originally isolated from the bovine hypothalamus by Carraway and Leeman (12) and has been implicated in a number of central and peripheral functions through its interactions with NT-specific receptors (NTS-1, NTS-2, and NTS-3) (107). NT is reactive through the isoleucine and leucine residues of its Carboxy-terminal sequence (55).

Found in nerves and endocrine-like cells in the pituitary, pancreas, adrenal gland, and small bowel mucosa, NT plays roles in gastric motility and secretion, in the central nervous system, and in leukocyte chemotaxis and phagocytosis. Studies have also implicated NT in both the regulation of normal cell turnover and the growth of cancers, which secrete NT and express NT receptors (13). For example, Carraway and Ploma (2006) showed cancer cells from colon, breast, and prostate tumors to secrete NT and to express the NTS-1 receptor (13).

NT is formed from a 160 to 170 amino acid precursor molecule, which also contains the neuropeptide neuromedin N (NM-N) (72). NM-N is a hexapeptide that expresses the same C-

terminal amino acid sequence as NT, and thus exhibits similar effects to NT in the central nervous system (57). In the NT/NM precursor molecule, there are four sites present between basic arginine and lysine amino acid residues that may be cleaved by prohormone convertases (PC) in a tissue-specific manner (56) Previous work in our laboratory has shown HMC-1 cells to express of pro-NT/NM and the PC5 processing enzyme, in addition to the ability to secrete biologically active NT (16).

Mast Cell Mediators

As mentioned, mast cells serve in a multitude of roles throughout the body. This versatility is enabled by the numerous mediators that can be released from mast cells at selected locations following appropriate stimulation (101). Three major classes of mast cell mediators are released: pre-stored granule-associated mediators, lipid-derived mediators and peptides/cytokines (86).

Granule-associated mediators

Mast cell secretion, in part, consists of the regulated release of preformed mediators from granules by exocytosis; the granules fuse together before spilling their contents into the surrounding stroma. Mediators released in this manner include histamine (68, 76), proteases, proteoglycans, and cytokines such as TNF-β and IL-16 (68). These mediators perpetuate the role of the mast cell in allergy and inflammation.

Lipid-derived mediators

Mast cells also process precursors stored in the cell membranes and lipid bodies to synthesize and secrete lipid mediators (68). Examples include the arachidonic acid metabolites: PGD2, LTC_4, platelet-activating factor (PAF) (68) and thromboxanes (76). These molecules

have various roles beyond inflammation, including in wound healing, in blood clot formation and in the promotion of inflammation associated with asthma and allergy.

Cytokines and chemokines

Mast cells also secrete numerous cytokines, including TNF-α, SCF, GM-CSF, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IL-14 and IL-16, in addition to chemokines, such as macrophage inflammatory protein (MIP)-α, MIP-1β, T-cell activation gene 3, EGF, IFN-gamma, and VEGF (31, 32, 68, 70, 76). The specific set of cytokines produced by a mast cell is not constant; the production and release of this array of cell mediators is highly dependent on the physiological needs of the tissues and the phenotype of the mast cell (76). Specifically, cytokine release is dictated by factors present in the intercellular environment. For example, the presence of SCF triggers mast cells to predominantly produce pro-inflammatory cytokines (6). The addition of IL-4 enhances this effect, augmenting SCF's role in mast cell proliferation and shifting cytokine production to increase the release of IL-3, IL-5, and IL-13 (61), which stimulate T-helper 2 cells and may in turn augment the inflammatory response.

The mast cell may exhibit functions without the allergic and anaphylactic reactions associated with full mast cell degranulation through the 'differential,' or 'selective,' release of mediators (101). This gives the mast cell the ability to promote inflammation and implicates mast cells in the pathogenesis of various disorders (101). This concept was originally illustrated by serotonin, which can be released without histamine (98). Eicosanoid release without histamine was later reported (64). In addition, both corticotropin-releasing hormone (CRH) and prostaglandin E2 (PGE_2) have been shown to stimulate the selective release of VEGF (11). VEGF has been linked to the pathology of numerous chronic inflammatory conditions and the growth of cancers.

Mast Cell Mediators Involved in Tumor Growth

Mast cells are associated with tumors in numerous ways. Stimulated mast cells, while initially drawn to tumors by chemoattractants (such as MCP-1, RANTES and SCF), secrete mediators that both enhance and inhibit tumor growth (102).

Several cytokines released by perivascular, tumor-associated mast cells may be detrimental to cancer cells. For example, tryptase and TNF-α could induce tumor-cell death by inducing inflammation (18, 38). In addition, mast cell secretion of chondroitin sulfate may play a role in inhibiting tumor metastasis (58).

However, in spite of the release of these inflammatory molecules, the net impact of stimulated mast cells likely enhances tumor growth and development; mast cell accumulation at tumor sites positively correlates with increased tumor aggressiveness and poor patient prognosis (73, 102). For example, mast cells release numerous mediators including IL-6 and VEGF (50, 90), which may aid tumor growth by increasing blood vessel development around tumors through angiogenesis.

Mast Cells Promote Angiogenesis

Though angiogenesis is essential for would healing and inflammation (76), it has also been implicated in various pathological conditions, including chronic inflammation and the growth, invasion, and metastasis of solid tumors (76, 87). Some angiogenic molecules include VEGF, fibroblast growth factor-2 (FGF-2), IL-8, placental growth factor, transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and angiopoietins, among others (76, 86, 87). These molecules are exported from tumor cells, the extracellular matrix, or host cells, and may promote the establishment of a vascular network around the tumor (86). Such increased blood supply is essential for tumors to grow beyond 1 mm^3 in diameter (86).

In contrast to normal angiogenesis, tumor angiogenesis is highly disordered; the blood vessel growth generally lacks the order and efficiency of angiogenesis in healthy tissues, implying the inappropriate regulation of the normally tightly-controlled pro-angiogenic compounds (28, 93). Thus, mast cell presence and tumor growth and progression may be linked through the release of pro-angiogenic mediators, such as IL-6 (50), VEGF (90), TNF-α and IL-8, from mast cells (63, 97). This theory is supported by the association of mast cells with processes that involve neovascularization, such as tumor growth, wound healing, and inflammation (69). Kessler *et al.* (1976) showed experimentally heightened mast cell presence in induced tumors directly prior to the onset of angiogenesis (54). In addition, several studies reported a significant correlation between microvessel counts and mast cell density in B-cell non-Hodgkin's lymphomas, multiple myelomas and esophageal squamous cell carcinomas (88, 89). Furthermore, mice models examined by Starkey *et al.* (1998) and Dethlefsen *et al.* (1994) showed that tumors induced in mast cell-deficient mice models exhibited lowered levels of angiogenesis and decreased metastasis of the tumor cells (19, 96). In addition, VEGF secretion by mast cells has been demonstrated in laryngeal carcinoma (91), small lung carcinoma (44, 103) and melanoma (105). Ultimately, it appears VEGF secretion by mast cells may link mast cells to cancer growth and development through its angiogenic properties (23).

VEGF

Vascular endothelial growth factor (VEGF) is a protein family consisting of five isoforms (VEGFA, placental growth factor [PLGF], VEGFB, VEGFC and VEGFD) that have been tied to angiogenesis during embryogenesis, skeletal growth and reproductive functions (24). VEGF is produced and secreted by various cell types, including: smooth muscle cells (21), fibroblasts, epithelial cells (81), keratinocytes (10), macrophages (5), and tumor cells (21). Though mast cells

express several VEGF isoforms (40), $VEGFA_{165}$ is the most prevalent and the most potent pro-angiogenic factor *in vivo* (1). Thus, $VEGFA_{165}$ was thus studied in this project.

Following appropriate stimulation, mast cells synthesize VEGF *de novo* (46). Both oxygen demand and various growth factors up-regulate VEGF mRNA expression, including: epidermal growth factor (EGF), TGF-α, TGF-β, keratinocyte growth factor and insulin-like growth factor-1, among others (25, 74). Moreover, inflammatory conditions also enhance the mast cell synthesis of VEGF *in vivo* (40). The inflammatory cytokines IL-1α and IL-6 induce VEGF expression and release in mast cells, among other cell types (24), thus tying VEGF's angiogenic properties with the increased vascular permeability of inflammatory disorders (74). Mast cells have also been shown to synthesize and secrete VEGF in response to allergic stimulation (8) and in association with newly formed blood vessels within tumors (4, 111). This link may thus connect mast cell release of VEGF to tumor growth in numerous instances.

VEGF in Tumor Growth

The presence of VEGF has been highly associated with tumor growth and development. VEGF mRNA is up-regulated in many malignant human tumors (20, 25) and in cell lines derived from multiple myeloma, Burkitt lymphoma, T-cell lymphoma, acute lymphoblastic leukemia, histiocytic lymphoma and chronic myelocytic leukemia (35).

From a treatment perspective, this information is relevant as decreasing the presence of VEGF should be beneficial in terms of impeding tumor growth and development. For instance, data from Phase 2 clinical trials has shown evidence that rhuMab VEGF (a humanized, monoclonal antibody to VEGF) administered alongside conventional chemotherapy resulted in an increase in time to progression, and even survival, in patients suffering from metastatic

colorectal carcinoma (48) and renal-cell carcinoma (110). Ultimately, targeting the mode of action by which VEGF acts on tumor cells may provide treatment options for cancer patients in the future.

Mast Cell Angiogenesis: VEGF Release by NT Through the NTS-1 Receptor

NT has been implicated in the growth and metastasis of cancer cells in numerous instances (13). Carraway and Ploma (2006) showed NT to significantly increase tumor growth *in vivo* in rats, while NT antagonists have been shown to inhibit the growth of engrafted tumors (13). As previously mentioned, one potential mechanism by which NT may be linked to cancer growth is through its stimulation of mast cells to release pro-angiogenic molecules; the stimulation of tumor-associated mast cells by NT could induce VEGF release and thus enhance the vascular supply to tumors by increased angiogenesis (13).

NT has been shown to stimulate mast cells *in vitro* and *in vivo* through the neurotensin-specific NTS-1 receptor (52, 71). Past results in our laboratory have shown the g-protein coupled NTS1 receptor to be present on the HMC-1 mast cell (16). In addition, SR 48692 (Sanofi Recherche), a non-peptide, highly specific NTS-1 antagonist, competitively inhibits NT binding to the receptor, and thus blocks the release of mast cell mediators both *in vitro* and *in vivo* (23, 52, 71). NTS1 receptors are expressed in the brain and intestine primarily, though they are also present in cells throughout the body (108). Furthermore, NTS-1 receptors are present on several types of tumor cells.

Potential 'Downstream' Components

Knowing that NT acts through the NTS-1 receptor to induce VEGF release (52), I aimed to identify additional 'downstream' components of the VEGF signaling pathway. Using past results as a guide, I specifically inhibited various common components of similar g-protein coupled molecular pathways and observed VEGF release in the presence and absence of inhibitors.

PL-Cβ

Phospholipase Cβ (PL-Cβ) is an intracellular enzyme involved in a multitude of eukaryotic cell processes (109). PL-Cβ functions as a key step in the transduction of various intracellular signals, catalyzing the cleavage of membrane-bound phosphatidylinositol 4,5-bisphosphate (IP2) to generate inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (27). DAG remains bound to the cell membrane while IP3 is released into the cytosol, triggering an increase in cytosolic calcium crucial to various signal-transduction pathways (29). PL-Cβ has been implicated in the NT-induced release of histamine from isolated rat mast cells, both *in vitro* and *in* vivo, and may thus be a component of the VEGF-release pathway (2).

p38 MAPK

p38 MAPK is a mitogen-activated protein kinase (MAPK) common to many eukaryotic signal transduction and cellular regulation pathways (14). MAPKinases consist of several subgroups, including extra-cellular signal-regulated protein kinases (ERK1 and 2), the p38 MAPK, the C-JUN N-terminal kinase (JNK1 and 2), and other related kinases, and function through a cascade of MAPKinase (MAPK), MAPK kinase (MapKK), and MapKK Kinase (MAPKKK) (14, 47). MAPKs connect receptors on the cell surface to targets within the cell, regulating almost all cellular processes (14). They have been implicated in the regulation of IL-1,

TNF-α, IL-4, and VEGF (62, 92, 104), among other cytokines, in addition to gene expression, cell proliferation and programmed cell death (14). In addition, the neuroendocrine peptide corticotrophin releasing hormone (CRH) has been shown to trigger the activation of p38 MAPK in VEGF release by HMC-1 cells (11). Thus, it may also be involved in the NT-activated pathway evaluated in this project.

MEK 1 MAPK

Since CRH has been shown to trigger the activation of p38 MAPK in its stimulation of VEGF release by HMC-1 cells (11), other MAPKinases may also be involved in the NT-activated pathway evaluated in this project. Thus, I inhibited the MEK1 MAPKinase to investigate its potential role in the VEGF-release pathway of HMC-1 cells following NT stimulation.

PKC-β

Protein Kinase C (PKC) is another class of enzymes potentially involved with this signal transduction pathway. PKC family molecules have multiple effects in the regulation of exocytosis and endocytosis (79). An 80-kDA multifunctional protein kinase, PKC-β is activated by signals such as increases in intracellular diacylglyercol or calcium concentrations (95). PKC-β's calcium and phospholipid-dependent activity involves phosphorylating hydroxyl groups on the serine and threonine components of target proteins that control both growth and cellular differentiation, among other processes (14, 80). The various PKC isozymes also play important roles in many signal transduction cascades including smooth muscle contraction in the gastrointestinal tract, reproductive, urinary, and integumentary systems, in the bronchi, adipocytes, hepatocytes, sweat glands, and platelets (14). NT stimulation of histamine release from rat mast cells has been shown to involve PKC-β (2).Thus, a PKC may be a component of

the signal transduction cascade evaluated in this study.

PI3-Kinase

Phosphatidylinositol 3- Kinases (or PI3-Kinases) are a family of enzymes involved in cell growth, differentiation, survival, proliferation, and motility, and also have ties to various forms of cancer (83). VEGF release in macrophages has been shown to be regulated through PI3-Kinase, and could thus be a potential mediator involved in the release of VEGF by NT stimulation in HMC-1 mast cells (85).

NF-kB

Nuclear Factor-kB (NF-kB) is a transcription factor that is often involved in pathways involving MAPKs (22). NF-kB generally exists in the cytoplasm as a homo- or hetero-dimer bound to inhibitory proteins known as I-kBs (66). During signal transduction, the IkB is degraded and NF-kB is released to bind the kB sequence on DNA (most commonly GGGACTTCC) (66, 78). There are eight proteins identified thus far in the NF-kB family, and they have been shown to be involved in the expression of numerous immune-and inflammatory-response genes (66). I inhibited NF-kB to determine if it is involved in VEGF release from mast cells following NT stimulation.

The role of Zinc

Zinc (Zn^{2+}) is an essential nutrient; its deficiency promotes growth retardation, immunodeficiency, and neuronal degeneration (59, 83). Zn^{2+} is involved as a catalytic and structural cofactor in numerous metalloproteins and signaling molecules (66, 84, 106), and is essential in multiple modes of mast cell activation (49). Kabu *et al.* (2006) showed Zn^{2+} to be required for both degranulaton and cytokine production in mast cells, and to be essential for

granule translocation to the plasma membrane (49). Zinc is also essential for FcεRI-mediated translocation of PKC to the plasma membrane and NF-κB activation (49). Since PKC is also involved in cytokine production in mast cells (77, 89), *in vitro* use of a Zinc chelator strongly inhibited the release of histamine, β-hexosaminidase, cytokines, and leukotrienes (49), and may thus also inhibit VEGF release. I used cell membrane permeant Zn2+ chelator N,N,N0,N0-(2-pyridylethyl)ethylenediammine (TPEN) (84) to determine the potential role of zinc in this signaling.

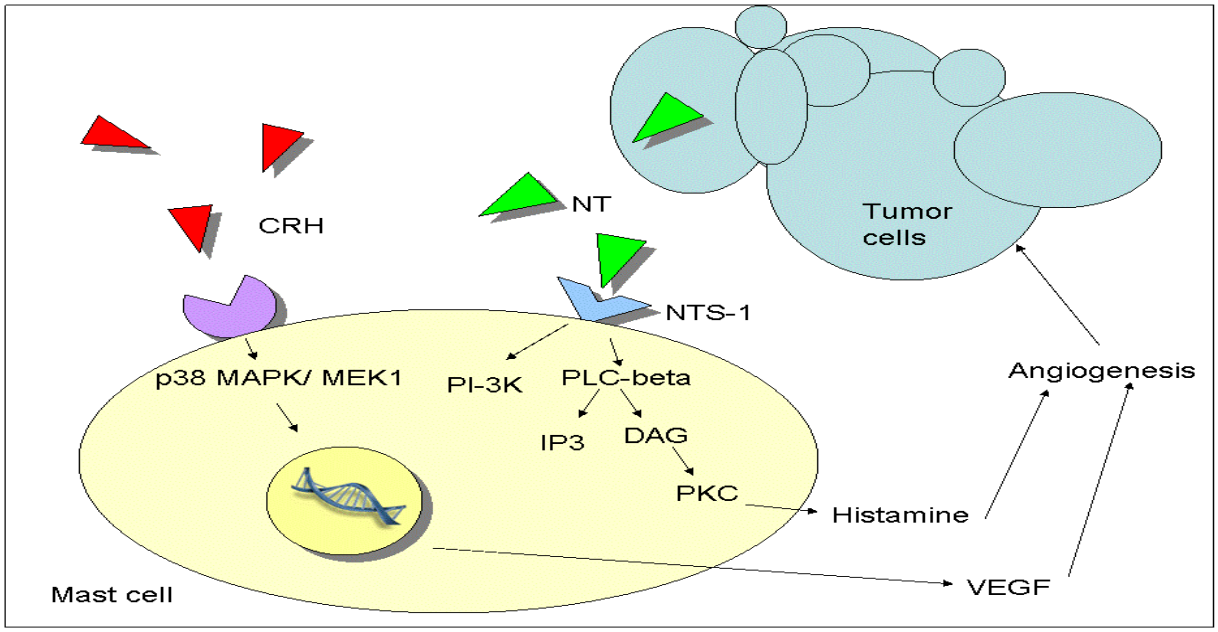

Figure 1. A potential interaction between mast cells and tumor cells. Molecules released by tumor cells and inflammation (such as NT and CRH) stimulate mast cells to release numerous mediators, such as VEGF and histamine, by stimulation through specific receptors and signal transduction pathways. These mediators promote angiogenesis, and may thus contribute to cancer growth and development.

MATERIALS AND METHODS

Human Mast Cell Culture

HMC-1 cells are a line of human leukemic mast cells that exhibit many of the characteristics of human mast cells. Phenotypically immature, they express only portions of the IgE receptor complex, but display several related receptors, such as c-kit, the receptor for SCF (75). HMC-1 mast cells also express prohormone convertases, which are capable of processing peptides (75).

The HMC-1 cells used in this project were provided by Dr. Butterfield of the Mayo Clinic in Rochester, MN and cultured in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA) with 1% fetal calf serum (FCS). The cells were maintained as suspended in 10 mL populations in 5% CO_2 balanced, humidified air at 37 ° C, and split 1:4 by sterile technique every 4 to 6 days.

Mast Cell Culture Treatments

1)Determining VEGF Release by NT Stimulation

Mast cells were removed from the 10% FCS medium by centrifugation (1400 rpm for 5 minutes) and re-suspended in 1% FCS culture media at a $2.5x10^6$ cells/mL concentration (determined by hemocytometer under phase contrast microscopy). Aliquots (1 mL) of HMC-1 cells were then added to each well of a 24-well plate, and stimulated with NT diluted in FCS-free medium to 10, 1, and 0.1 μM concentrations.

The cells were incubated in the 24-well plates at 37 ° C for varying amounts of time (4, 8, and 24 hours). The cells and media were then removed from the 24-well plate, centrifuged at

2000 rpm, and the supernate fraction frozen at -80 ° C for later analysis by an ELISA (Enzyme-linked Immunosorbent Assay).

2) Efforts to Decrease Variations in VEGF Release

My results have shown NT to stimulate HMC-1 cells to synthesize and secrete VEGF (52), consistent with past observations of increased VEGF RNA following NT stimulation (22). Though NT stimulated a significant VEGF release under these conditions, the VEGF concentrations generated were quite variable. Thus, I made efforts to decrease this variability by both re-suspending the cells in fresh media directly following stimulation to remove proteases and evaluating VEGF-release based on the passage number of the cells.

Centrifugation

As suggested by previous work in our laboratory, VEGF was shown to be degraded over time by mast cell populations (22). To attempt to avoid this variability, I re-suspended cells in 1% FCS media following 15 minutes of NT-stimulation. This would remove any proteases released by the cells that would degrade VEGF.

Evaluation of Passage Number

Past studies involving HMC-1 cells only tested up to 10 passages, at which point the 5-lipoxygenase gene pathway was observed to be deficient (65). Thus, I sought to observe whether passage number impacted VEGF release as well. Compiling data from several tests, I monitored the number of passages performed prior to each ELISA, and then plotted the fold increase of VEGF production in NT stimulated samples. The data was expressed as the fold increase in VEGF concentration over basal VEGF release, due to varying basal VEGF release between the trials.

3) NTS-1 Receptor Presence: SR 48692 Inhibition of the NTS-1 Receptor

I used the highly-specific NTS-1 inhibitor SR 48692 (Sanoffi Recherche) to show that NT stimulates mast cells through the NTS-1 receptor. The SR 48692 was stored at 6 ° C at a 5 mM in DMSO, shielded from light by aluminum foil. It was added to cells at 50 μM (diluted in FCS-free media immediately prior to the test) and incubated at 37 ° C for 15 minutes prior to the addition of 10 μM NT. The cells were incubated for 4, 8 and 24 hour time periods, and the VEGF release by mast cells analyzed by an ELISA. I sought to confirm past results, which showed an 18% decrease in VEGF release following inhibition of the NTS-1 receptor (22).

Testing for Downstream Mediators

Past results indicated that NT stimulates mast cells through an NT-specific receptor (22). I sought to extend these findings by inhibiting potential 'downstream' mediators of VEGF release to determine if their inhibition impacted VEGF release. In the following tests, I observed cells after stimulation by 10 μM of NT and following 8 hours of incubation at 37 ° C, as these conditions induced the highest VEGF release in my previous results (52, 53).

1) PL-C*β Treatment*

One mL aliquots of HMC-1 cells were added to 24-well plates and incubated for 1 hour at 37 ° C with 10 μM of the PL-C*β* inhibitor U73122 (CALBIOCHEM, La Jolla, CA) dissolved in DMSO and diluted in FCS-free media. FCS-free media was used to standardize the volumes of control treatments. Samples were then stimulated with 10 μM of NT and incubated for 8 hours at 37 ° C. The supernate was then frozen at -80 ° C prior to analysis by an ELISA.

2) Additional Treatments

I also inhibited: p38 MAPK with 10 μM of SB202190 (CALBIOCHEM, Irvine, CA), MEK1 MAPK with 10 μM of U0125 (CALBIOCHEM, La Jolla, CA), PKC with 20 μM of Bisindohylmaleimide I (CALBIOCHEM, La Jolla, CA), PI-3K with 1 and 10 μM of Wortmannin (CALBIOCHEM, Irvine, CA), and NF-kB with 1 μM of *CAY 10512* (CALBIOCHEM, Irvine, CA). All of the inhibitors were dissolved in DMSO and diluted in FCS-free media. I also used a Zinc chelator to investigate the involvement of Zinc in this pathway. Aliquots (1 mL) of HMC-1 cells were incubated with 50 μM of Zn^{2+} chelator N,N,N0,N0-(2-pyridylethyl)ethylenediammine (TPEN) (Calbiochem, La Jolla, CA, U.S.A) dissolved in DMSO and diluted in FCS-free media. The cells in all of these samples were then treated as described in the PL-C*ß treatment*.

VEGF ELISA

The concentrations of VEGF released in the cell treatments were quantified following the manufacturer's instructions of an R+D Systems ELISA. First, a 96-well plate was coated with a monoclonal antibody specific for $VEGFA_{165}$ and allowed to incubate at room temperature for approximately 24 hours. It was then washed three times with 0.05 % Tween® 20 in filtered PBS (pH 7.2-7.4), removing capture antibody that did not adhere to the well. An identical wash was performed following each subsequent step of plate preparation to remove excess chemical from the wells.

A nonspecific protein solution (1% Bovine Serum Albumin in PBS, pH 7.2-7.4) was then added for a 1-hour incubation at room temperature to block nonspecific receptors. Excess BSA was removed by an additional set of washes following the incubation. Samples from cell

cultures, or standards with known VEGF concentrations, were then added to the wells for a 2-hour incubation at room temperature. This enabled the VEGF to bind to the VEGF-specific monoclonal antibodies. Again, the wells were washed 3 times with wash buffer. Detection antibody was then pipetted into each well and allowed to bind to the VEGF during an additional 2-hour incubation at room temperature. Following an additional set of 3 washes with the wash buffer, Streptavidin-HRP (diluted 1:200 μL), was pipetted into the wells and allowed to bind for 20 minutes at room temperature while shielded from direct light. After another wash, a 1:1 mixture of H_2O_2 and Tetramethylbenzidine (a substrate solution specific to Streptavidin-HRP), was added to the wells. The generation of a blue color indicated the presence of VEGF. Following another 20-minute incubation (avoiding exposure to direct light), a stop solution (2N sulfuric acid) was added to terminate the reaction.

The optical density of the samples was determined by an endpoint analysis with a Microplate reader (at 450 nm). A standard curve was generated with each separate test to measure the absorbance in wells with known VEGF concentrations. These curves were averaged to create the standard curve used to quantify unknown VEGF concentrations.

Western blotting

Cell Treatments

HMC-1 mast cells were removed from 10% FCS media by centrifugation (at 1400 RPM for 4 minutes), and then re-suspended in FCS-free media. One-mL aliquots were then pipetted into microfuge tubes and inhibited by 10 μM of the MEK1 MAPK inhibitor U0126, 20 μM of the PKC inhibitor Bisindohylmaleimide I, 10 μM of PI-3Kinase inhibitor Wortmannin, and 10 μM of the p38 inhibitor Sb202190 for 1 hour at 37 ˚C. The samples were then centrifuged (2000

rpm for 30 seconds) and re-suspended in Hank's Solution (pH 7.2-7.4). Selected samples were then stimulated with 10 μM of NT for 2 minutes before being centrifuged at 2000 rpm for 3 minutes, and re-suspended in 100 μL of ice-cold lysis buffer with phosphatase inhibitor cocktail. The samples were then placed at -80 ˚C.

Determining Protein Concentration in Samples

The concentration of protein in a sample was determined by BCA™ Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. The concentration of unknown samples of protein (from each sample above) was determined in comparison to known protein concentrations established in a standard curve of Bovine Serum Albumin (BSA) solutions. The standard curve is created by serial dilutions of BSA, dissolved in lysis buffer (which was diluted 1:3 with pico water). Known protein samples ranged from 25 μg/mL to 2000 μg/mL.

To measure protein concentration, 25 μL of a control or unknown sample was placed in a microplate well and mixed with 200 μL of Working Reagent (50:1 mixture of BCA Reagent A and BCA Reagent B, as provided in the kit). The absorbances of each well were then measured (after a 30 minute incubation at 37 ° C) at 562 nanoM by Microplate reader. Unknown samples were then diluted with pico water to have equal protein concentrations (approximately 1 μg/μL), and stored at -80 ˚C prior to western blotting.

Western Blotting

Western blotting was performed on the samples by Dr. Robert Carraway at the University of Massachusetts Medical School.

Statistics

The data are represented as the mean +/- SE, and analyzed with two-tailed, independent Student's t–test. Statistical significance was considered for $p< 0.05$.

RESULTS

Establishing a VEGF Standard Curve

Known concentrations of VEGF (0 pM, 250 pM, 500 pM, and 1000 pM) were measured by an ELISA to establish a standard curve (Figure 2). Figure 2 represents 9 trials, with the absorbance of the solutions measured by Microplate reader at 450 nm. A standard curve was performed in each test to assure the assay was measuring VEGF release. All of the standard curves were fairly consistent, creating a line (R^2 value of 0.9836). The equation of the line ($y=0.0008x+0.18$) was utilized to determine the concentration of VEGF in the sample, based on the absorbance measured. However, the equation derived from the standard curve derived made it difficult to calculate low concentrations of VEGF, such as the basal level released from mast cells without NT treatment. This was thought to be due to the somewhat increased variability in the readings involving the 1000 pM concentrations. Thus, low concentrations were estimated using an equation calculated after removing these upper values.

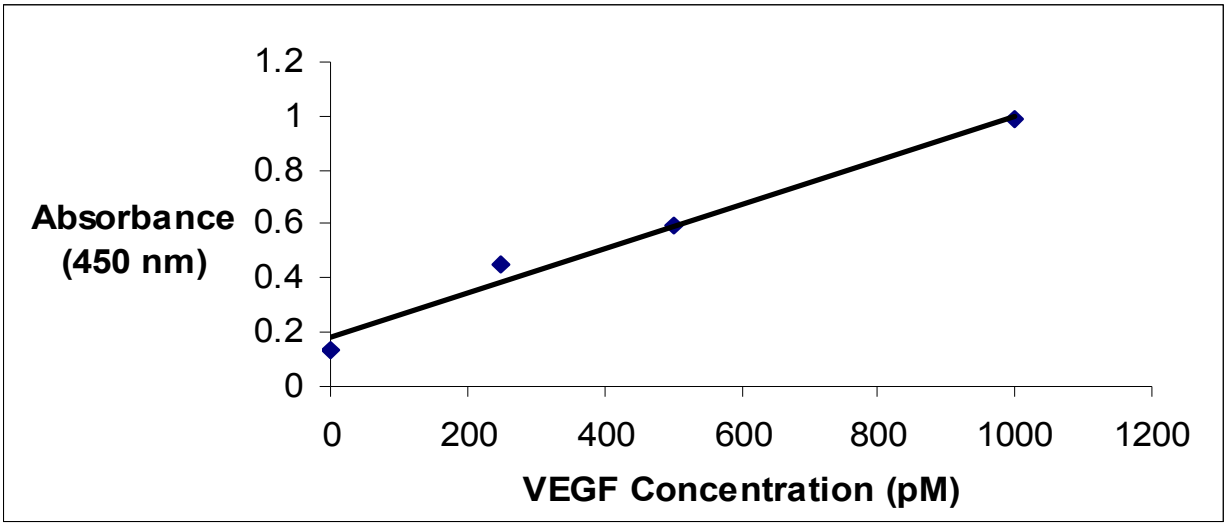

Figure 2. The VEGF standard curve was generated by assaying known concentrations of VEGF and measuring the absorbencies at 450 nm in 9 trials. This curve was utilized to determine the concentration of VEGF in samples of treated cells with unknown concentrations.

Dose-Dependent VEGF Release by NT Stimulation

I sought to replicate past trends of VEGF release in relation to NT concentration, measuring VEGF release from treatments stimulated with 0.1, 1, and 10 μM of NT for 8 hours, (the time point previously shown to have the greatest release of VEGF) (22). Although early results seemed to show an increase in VEGF release correlating positively with an increase in NT concentration, these results were not reproduced in various later duplicate experiments. While treatments stimulated with NT consistently showed greater VEGF release than basal levels recorded in samples not treated with NT, the amount of variation present in the results made it difficult to find any dose-response correlation between NT concentration and VEGF release. Based on this variation, none of the treatments showed a significant VEGF release over untreated controls, or different from each other.

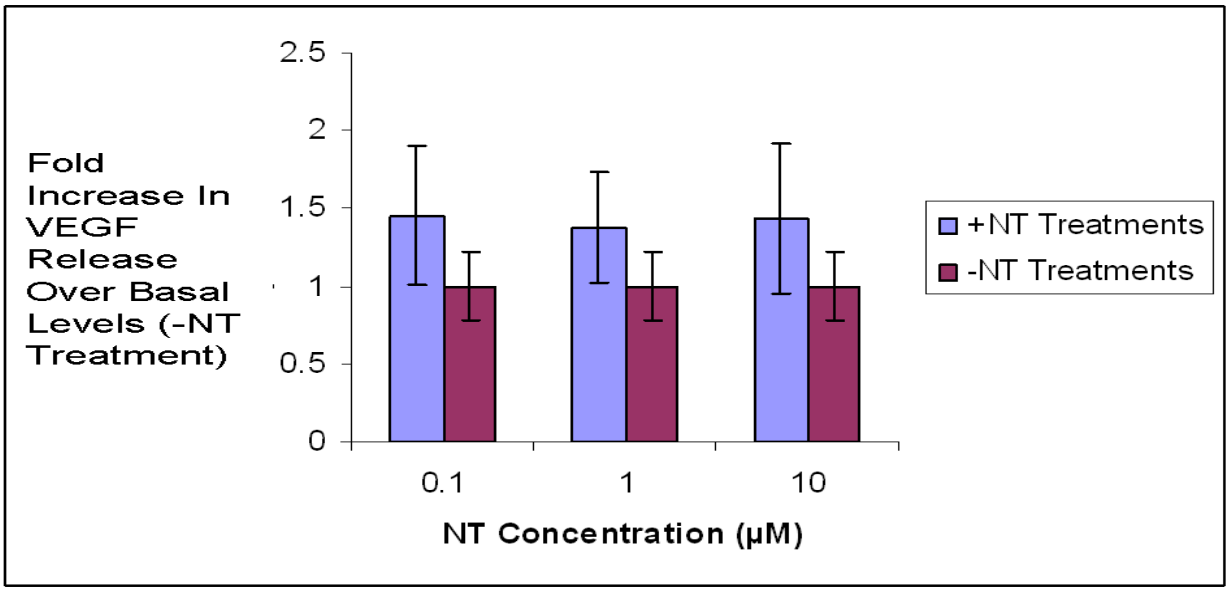

Figure 3. VEGF release measured compared to untreated control by varying concentrations (0.1, 1, and 10 μM). Each treatment represents 4 trials, incubated for 8 hours at 37 ° C. None of the values differ significantly. Error bars represent standard deviation in the experimental series. The average for the untreated controls was utilized for the calculations.

Effect of NT on secretion of VEGF by HMC-1 Cells

Past results in our laboratory have illustrated that NT stimulates HMC-1 cells to synthesize and secrete VEGF and that mast cells degrade VEGF over time (22). This was confirmed by measuring VEGF concentrations in HMC-1 cell cultures incubated with 1 µM NT over 4, 8 and 24 hour time periods. VEGF concentration was shown to peak at 8 hours (Figure 3). The VEGF release in samples incubated for 4 and 8 hours was shown to be significantly greater than the control (n=3, p=0.024, and n=3, p= 0.0016, respectively). The concentration of VEGF released following 8 hours and 24 hours was also found to be significantly different (n=3, p=0.02351).

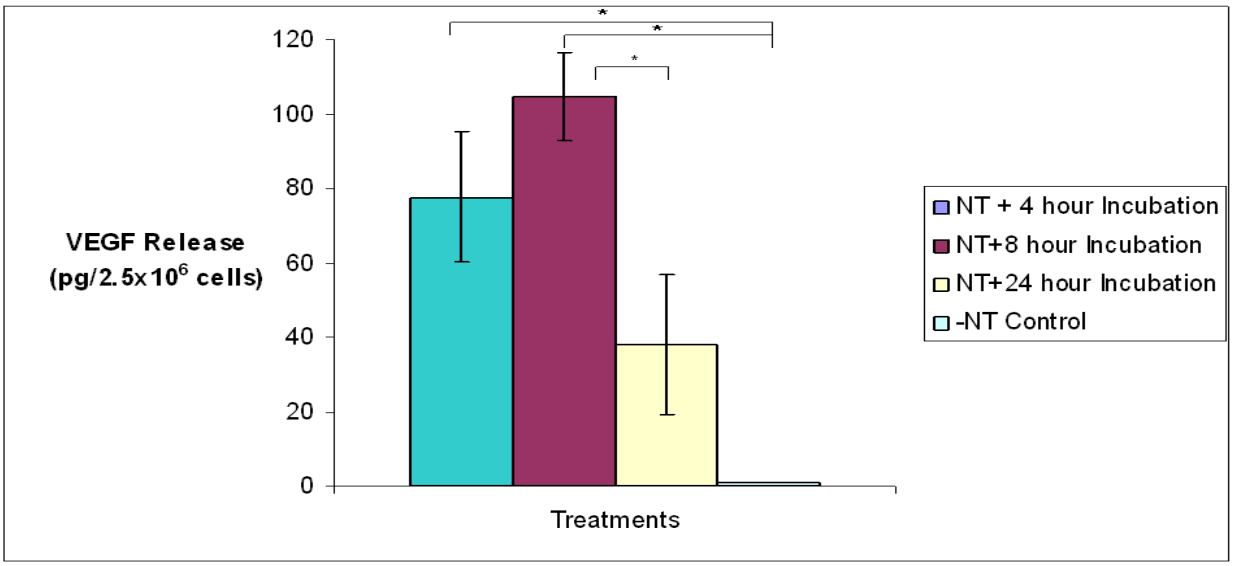

Figure 4. VEGF degradation over time in mast cell samples. Mast Cells were treated with 1 μM NT and incubated for 4, 8 and 24 hour periods. The media was then isolated and assayed by ELISA. The 8 hour treatment represents 4 tests while the 4 and 24 hour treatments represent 3 tests each. The control treatment represents the average of three tests (one corresponding with each time point), which did not differ significantly. VEGF release was significantly greater than untreated controls in the 4 and 8 hour treatments (n=3, p= 0.024; n=3, p=<0.002). The bars represent standard error.

Re-suspending Cells to Remove VEGF-degrading Proteases

Mast cells are known to release both endo- and exo-proteases capable of degrading VEGF. In addition, past work in our laboratory showed VEGF to be degraded by mast cells (13). Additional tests were performed to overcome variability due to the potential presence of these factors (Figure 5). The cells were stimulated by 10 μM of NT, and then incubated for 4 hours, with one population of cells centrifuged and re-suspended on 1% FCS media 15 minutes after NT-stimulation. Since duplicate tests without NT stimulation did not show consistent release of VEGF, the data was plotted as fold increase of the stimulated cells over the average VEGF release of the un-stimulated control. Cells that were re-suspended showed significantly less VEGF production compared to un-stimulated cell samples (n=2, p=0.00578). The re-suspended

cell population did not show significantly different VEGF release compared to the cells not stimulated with VEGF.

Based on the results of Figure 5, one continued testing samples without re-suspending the cells.

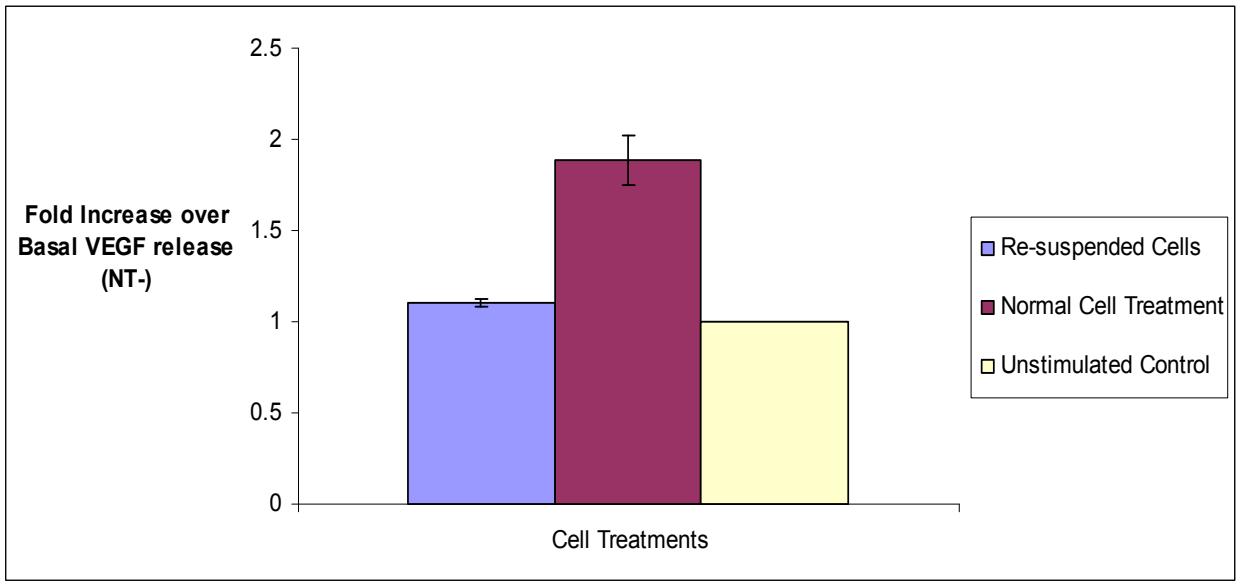

Figure 5. VEGF release by re-suspended cells compared to normal cell treatments. VEGF concentration is shown as the fold increase of the NT treated samples compared to the corresponding control treatments. Performed in duplicate, 2 samples were stimulated with 10 µM NT, incubated for 15 minutes, centrifuged, and then re-suspended in fresh 1% FCS medium. The other sample was also treated with 10 µM NT. Both samples were incubated together for 4 hours, and then the absorbances determined at 450 nm following a VEGF-specific ELISA. The normal cell treatment showed significantly greater VEGF release than the untreated controls (n=2, p=0.006), while re-suspended samples did not.

Observing VEGF Release Variation Based on Passage Number

Past studies involving HMC-1 cells tested cells only up to 10 passages, at which point changes were observed in the 5-lipoxygenase gene pathway (65). Thus, I investigated whether passage number impacted VEGF release. Compiling data from several tests, I monitored the number of passages performed prior to each ELISA, and then plotted the fold increase of VEGF production in NT stimulated samples (blue) over un-stimulated controls (red) (Figure 6).

The results showed the increase in VEGF release from NT-stimulated cells over un-stimulated cells to decrease as the number of passages increased. The data was expressed as the fold increase in VEGF concentration over basal VEGF release, due to varying basal VEGF release between the trials. The sample number in each trial varied since the data was compiled from multiple experiments.

Cells below 9 passages showed a significant increase in VEGF release when stimulated by 10 μM of NT. However, the VEGF release after 3 passages was found to be significantly greater than the VEGF release after 5, 6, 8, and 9 passages (p=0.040, 0.0269, 0.0057, and 0.00001). Though VEGF release was greatest after 4 passages, this result was not significantly different from the other points due to a lack of trials (n=2). While VEGF release after 5, 6, and 8 passages was similar, VEGF release from these samples was significantly greater than VEGF release after 9 passages (p= 0.00132, 0.0000935, and 0.001043). No data was available for cells after 7 passages.

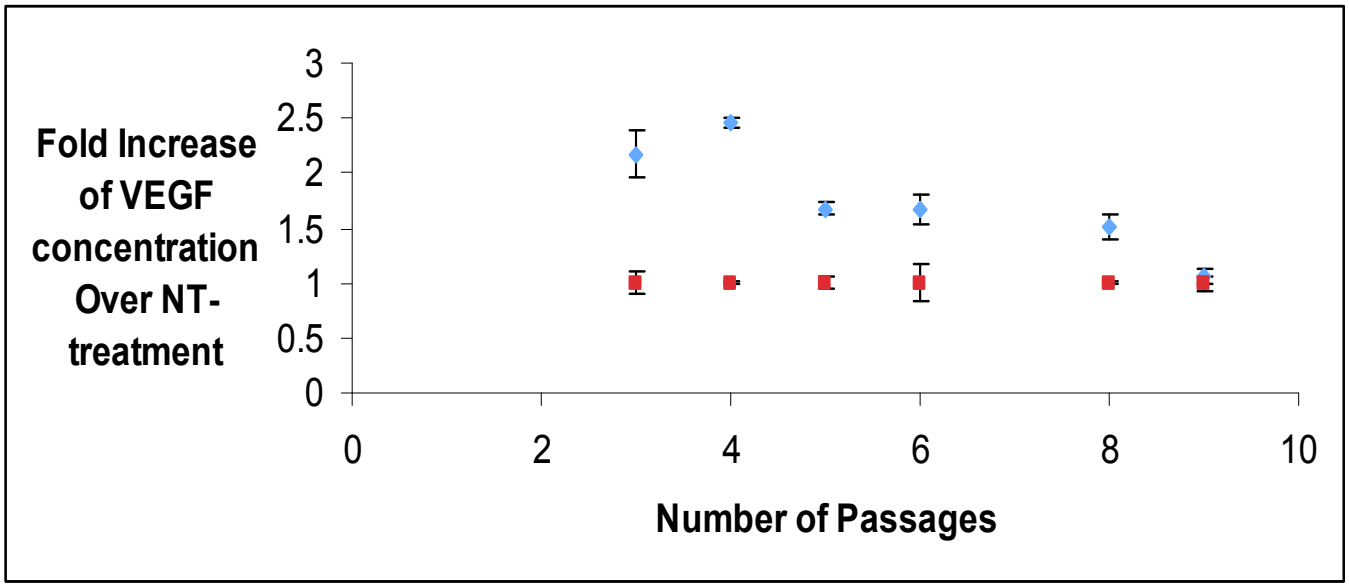

Figure 6. NT stimulation of HMC-1 cell VEGF release. HMC-1 cell populations were stimulated with 10 μM concentrations of NT for 8 hours at 37 ° C. VEGF release, measured by an ELISA, was shown to be significantly greater than the VEGF release of cells not treated with NT for samples that had undergone 3, 4, 5, 6, and 8 passages. Though the number of trials varied for each point, the VEGF release after 3 passages was found to be significantly greater than the VEGF release after 5, 6, 8, and 9 passages (p=0.040, 0.0269, 0.0057, and 0.00001). The data is shown as fold increase in VEGF release over basal VEGF release. The bars present show standard error.

NTS1 Antagonist SR48692 on VEGF Secretion from HMC-1 Cells

NT has been shown to bind the NTS-1 on mast cells to stimulate the release of various mediators, such as VEGF (22). SR48692 is a highly-specific, non-peptide NTS1 antagonist, shown to compete with NT to bind to the receptor and inhibit the release of VEGF. SR48692 was added at 50 μM concentrations to the cells and incubated for 15 minutes (enabling the inhibitor to bind to the NTS1) prior to the addition of 10 μM of NT. The use of this antagonist decreased NT's ability to stimulate VEGF release from HMC-1 cells. Based on the variability in releases of the cells between tests, the fold increase of VEGF production (compared to the controls), was evaluated in these figures to normalize the results attained relative to one another.

The fold VEGF increase over basal VEGF concentrations was not found to be significantly different from untreated controls for all of the SR48692-inhibitted samples. While all of the fold increases in the uninhibited samples (1.261, 1.534, and 1.503 for the 4, 8 and 24 hour samples, respectively), were found to be greater than the untreated control, only the VEGF release in the 8 hour time point was found to be significant (p=.02214, n=3). This lack of significance was due to the high amount of variability in the samples, which can be seen by the fairly large standards of deviation calculated.

The results also indicate a similar VEGF release over time as shown in Figure 4. However, this effect was muted by SR48692 exposure.

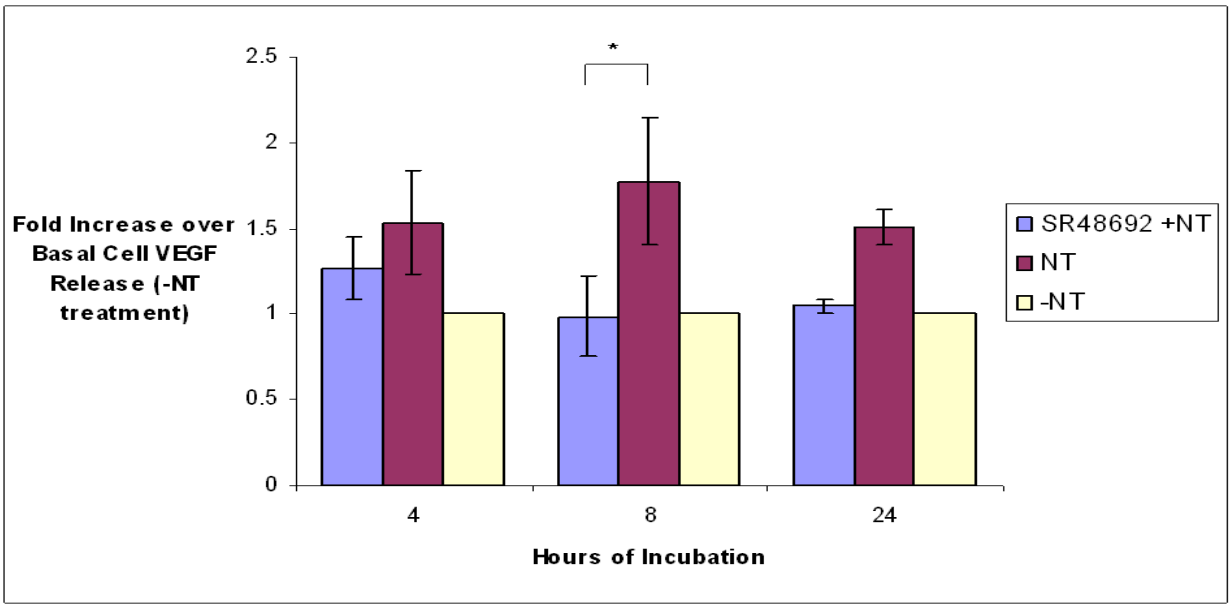

Figure 7. The impact of competitive NTS-1 antagonist SR48692 on NT stimulation of HMC-1 cells was observed by the concentration of VEGF released. Cells were incubated in 1% FCS medium, with the experimental cultures pretreated with 50 μmolar concentrations of SR48692 for 15 minutes prior to NT stimulation. After 4 and 8 hour incubation times, the cells were centrifuged at 2000 rpm, and the supernatant removed. It was then analyzed by a VEGF ELISA. Each data point represents 3 trials. The VEGF release (measured by Microplate reader at 450 nm), was released by its fold increase over un-stimulated controls. The error bars indicate standard deviation. The 8 hour time point was significantly greater than both the control and the inhibited sample.

PL-Cβ Inhibition on VEGF release

As past research has potentially implicated PL-Cβ as a component of NT-stimulation of histamine release (2, 22), I studied the role of this enzyme on VEGF release by NT-stimulation by inhibiting it with U73122. Figure 8 represents samples incubated for 1 hour with 10 μM of U73122 followed by 8 hours with 10 μM of NT. While NT-/U73122- and NT-/U73122+ samples were not significantly different from each other, both NT+/U73122- and NT+/U73122+ showed significantly greater VEGF release than the NT- samples (p=0.001 and p=0.011). VEGF release from NT+/U73122- and NT+/U73122+ samples were not shown to be significantly different (p=0.0667). The lack of any significance over several trials suggests that PL-Cß may not be involved in this pathway.

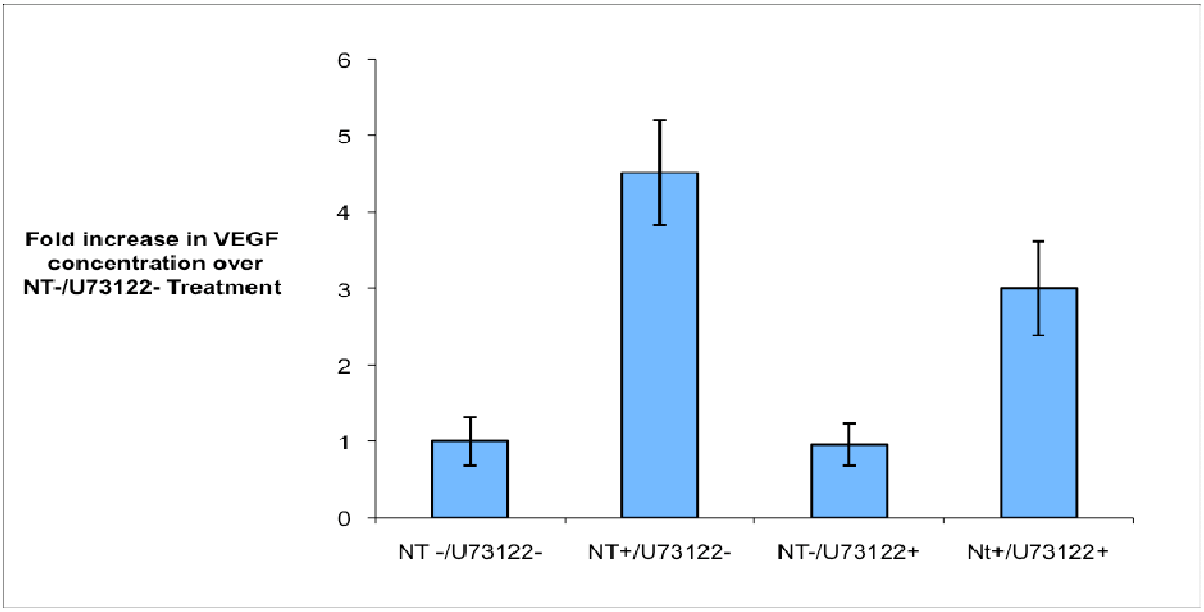

Figure 8. PL-CB inhibitor U 73122 does not inhibit NT stimulation of HMC-1 cell VEGF release. HMC-1 cells were incubated in 10 μM of U73122 for 1 hour, and then stimulated with 10 μM of NT for 8 hours at 37 ° C. VEGF release was measured by ELISA. NT-/U73122- and NT-/U73122+ samples were not significantly different, while NT+/U73122- and NT+/U73122+ showed significantly greater VEGF release from the NT-/U73122- samples (p=0.0012 and p=0.011). VEGF release from NT+/U73122- and NT+/U73122+ samples were not shown to be significantly different (p=0.067). The NT+/U73122- and NT+/U73122+ each represent 6 trials, while the NT-/U73122- and NT-/U73122+ represent 5 trials each. The data is shown as fold increase in VEGF release over basal VEGF release. Standard error is shown.

P38 MAPK Inhibition on VEGF release

As past research has implicated the p38 MAPK as a component of the VEGF release pathway from HMC-1 cells by CRH stimulation (11), I studied the impact of inhibiting this MAPK on VEGF release. Figure 9 represents samples incubated for 1 hour at 37 ° C with 10 μM of p38 MAPK inhibitor, and then 8 hours with10 μM of NT. The NT+/ SB202190- treatment showed a significant increase in VEGF release over NT-/ SB202190- treatments (p=0.00296, n=20), the NT+/ SB202190+ samples (p=0.00133, n=20), and the NT-/SB202190 + samples (p=0.00549, n=20). The NT-/SB202190-, NT-/SB202190+, and NT+/SB202190+ samples were not significantly different. Thus, the p38 MAPK inhibitor SB202190 was shown to significantly decrease VEGF release from HMC-1 cells stimulated with NT.

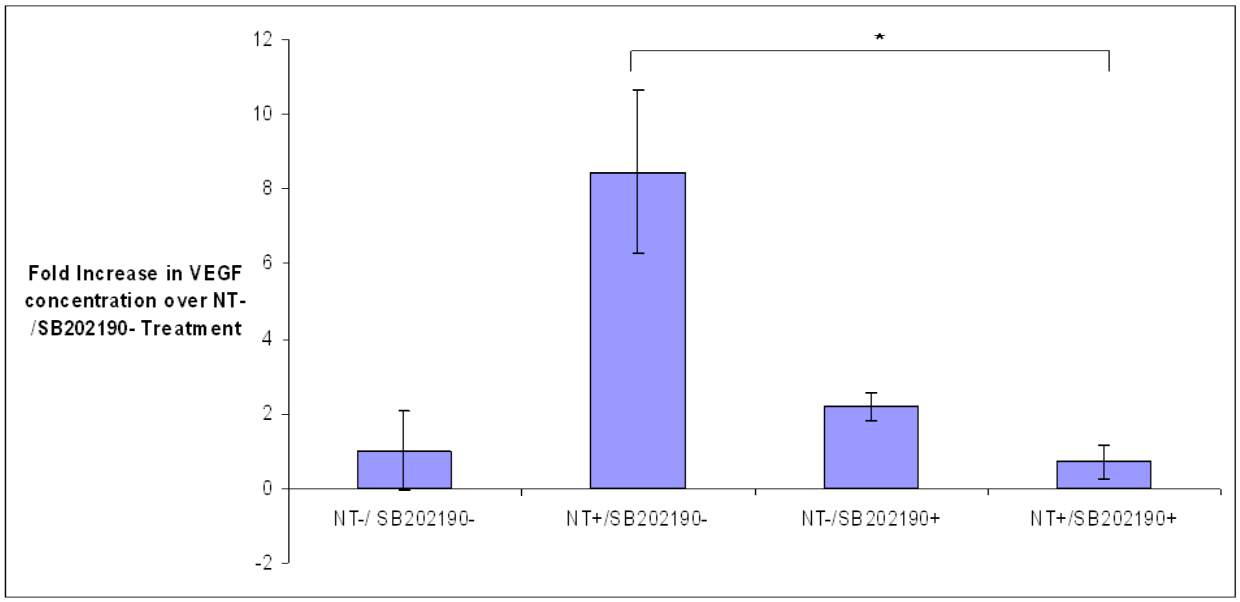

Figure 9. p38 MAPK inhibitor SB202190 inhibits NT stimulation of HMC-1 cell VEGF release. HMC-1 cells were incubated with 10 μM of SB202190 for one hour, and then stimulated with 10 μM of NT for 8 hours at 37 ° C. VEGF release was measured by ELISA. The NT+/ SB202190- treatment showed a significant increase in VEGF release over the NT-/ SB202190- treatments (p=0.00296, n=20), the NT+/ SB202190+ samples (p=0.00133, n=20), and the NT-/SB202190 + samples (p= 0.00549, n=20). The VEGF release by the NT-/SB202190-, NT-/SB202190+, and NT+/SB202190+ samples were not significantly different. The data is shown as fold increase in VEGF release over basal VEGF release. Standard error is shown.

MEK1 MAPK Inhibition Blocks VEGF release

Since p38 MAPK is a component of CRH-stimulated VEGF release from HMC-1 cells, other MAPKinases may be involved in this pathway (11, 14). Thus, I inhibited the MEK1 MAPK to see if it is involved in VEGF release following NT stimulation. Figure 10 shows samples incubated for 1 hour at 37 ° C with 20 μM MAPK inhibitor U0125, and then 8 hours with 10 μM of NT. Nine NT-/U0125- and NT-/ U0125+ samples and 24 NT+/ U0125+ and NT+/ U0125- samples were tested. The NT+/U0125- treatment showed a significant increase in VEGF release over NT-/ U0125- samples (p=0.004) and NT+/ U0125+ samples (p=0.002). NT+/U0125+ samples did not show a significant increase in VEGF release over NT-/ U0125- samples, and there was no significant difference observed between NT-/ U0125- and NT-/ U0125+ samples. Thus, inhibition of the MEK1 MAPK was shown to significantly decrease VEGF release from samples stimulated with NT.

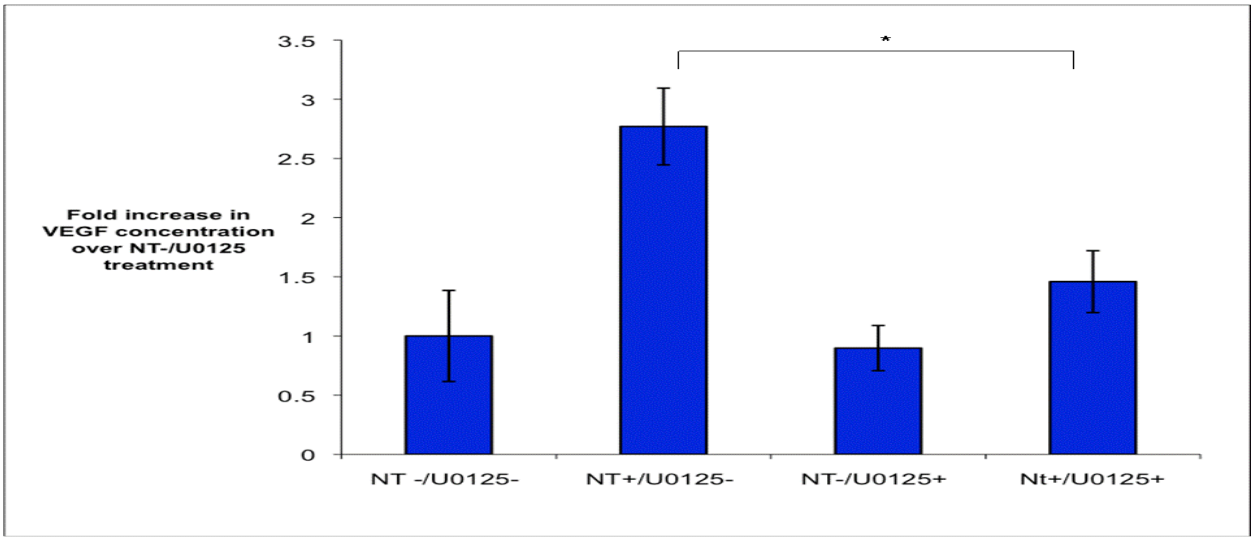

Figure 10. MAPK inhibitor U0125 inhibits NT stimulation of HMC-1 cell VEGF release. HMC-1 cells were incubated in 10 μM of U0125 for one hour, and then stimulated with 10 μM of NT for 8 hours at 37 ° C. VEGF release was measured by ELISA. The NT+/ U0125- treatment showed a significant increase in VEGF release over NT-/ U0125- samples (p=0.0043) and NT+/ U0125+ samples (p=0.00224). NT+/ U0125+ samples did not show a significant increase in VEGF release over NT-/ U0125- samples, and there was no significant difference observed between NT-/ U0125- and NT-/ U0125+ samples. The NT+/ U0125- and NT+/ U0125+ bars represent 24 samples each, while the NT-/ U0125+ and NT-/ U0125- bars represent 11 samples each. The data is shown as fold increase in VEGF release over basal VEGF release. Standard error is shown.

PKC Inhibition Blocks VEGF release

Past research has identified PKC as a component of CRH-induced VEGF release from HMC-1 cells (2, 22). Thus, this enzyme could be involved in the NT-stimulation of VEGF release studied in this project. The data in Figure 11 represents samples incubated in 10 μM of PKC inhibitor, Bisindohylmaleimide I, for 1 hour at 37 ° C, and then stimulated 10 μM of NT for an additional 8 hour incubation. Six NT+/Bis.- and NT+/Bis.+ samples and 5 NT-/Bis.- and NT-/Bis.+. The NT+/Bis.- treatment showed a significant increase in VEGF release over NT-/Bis.- samples ($p<0.001$). While NT+/Bis. + samples also showed a significant increase of VEGF release over NT-/Bis.- samples ($p<0.001$), NT+/Bis. + samples showed a significantly lower VEGF release than NT+/Bis.- samples ($p=0.0015$). There was no significant difference observed between NT-/Bis.- and NT-/Bis.+ samples. While earlier tests in which the samples were incubated with inhibitor for 30 minutes did not show PKC inhibition to block VEGF release, PKC inhibition was shown to significantly inhibit VEGF release following 1 hour of incubation with the inhibitor.

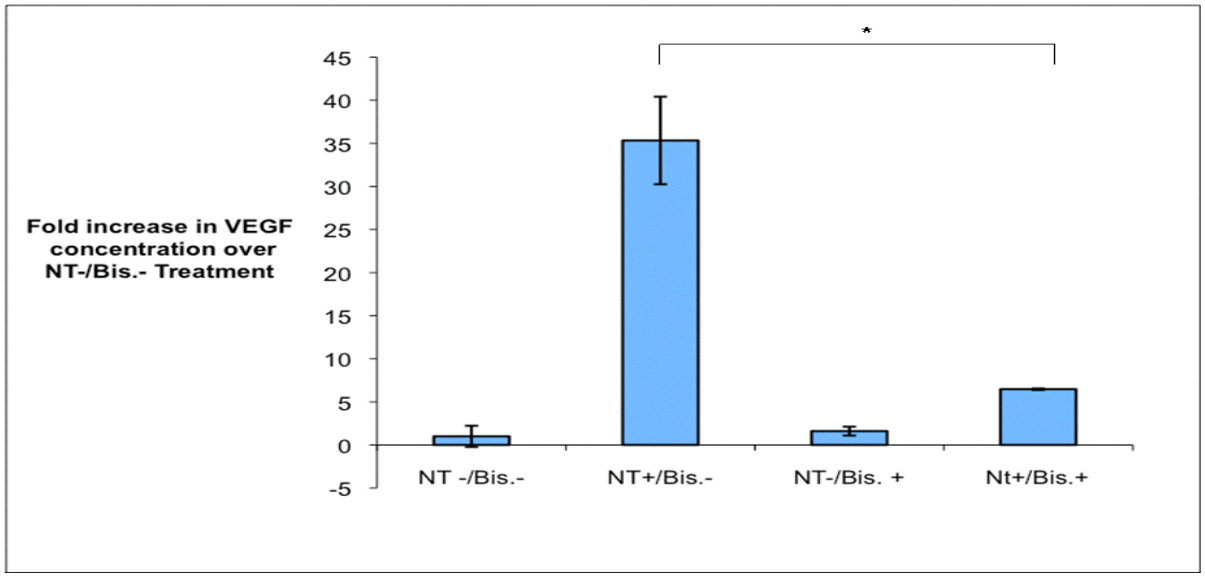

Figure 11. PKC inhibition significantly decreased HMC-1 cell VEGF release by NT stimulation. HMC-1 cell populations were incubated in 10 μM Bisindohylmaleimide I for one hour at 37 ° C and then stimulated with 10 μM of NT for 8 hours at 37 ° C. VEGF release in the NT+/Bis.+ sample, measured by an ELISA, was shown to be significantly greater than the VEGF release of cells not treated with NT ($p<0.001$), but significantly less than the VEGF release from the NT+/Bis.- sample ($p=0.0015$). The data is shown as fold increase in VEGF release over basal VEGF release. 5 NT-/Bis.- and NT-/Bis.+ samples, and 6 NT+/Bis.+ and NT+/Bis.- samples were tested. Standard error is shown.

PI3-Kinase Inhibition on VEGF release

VEGF release in macrophages was shown to be regulated through the PI3-Kinase (86). Thus, Wortmannin, a common PI3-K inhibitor, was used to determine if PI-3 K could also be a potential mediator in the NT-induced release of VEGF from HMC-1 cells. HMC-1 cells were incubated in 10 μM of Wortmannin for 1 hour, and then stimulated with 10 μM of NT for 8 hours at 37 ° C. VEGF release in the NT-/wortmannin-, NT-/wortmannin+, and NT+/wortmannin+ treatments were not significantly different, while VEGF release from NT+/wortmannin- was significantly greater than all of these samples ($p=0.015$, 0.007, 0.0097, $n=18$).

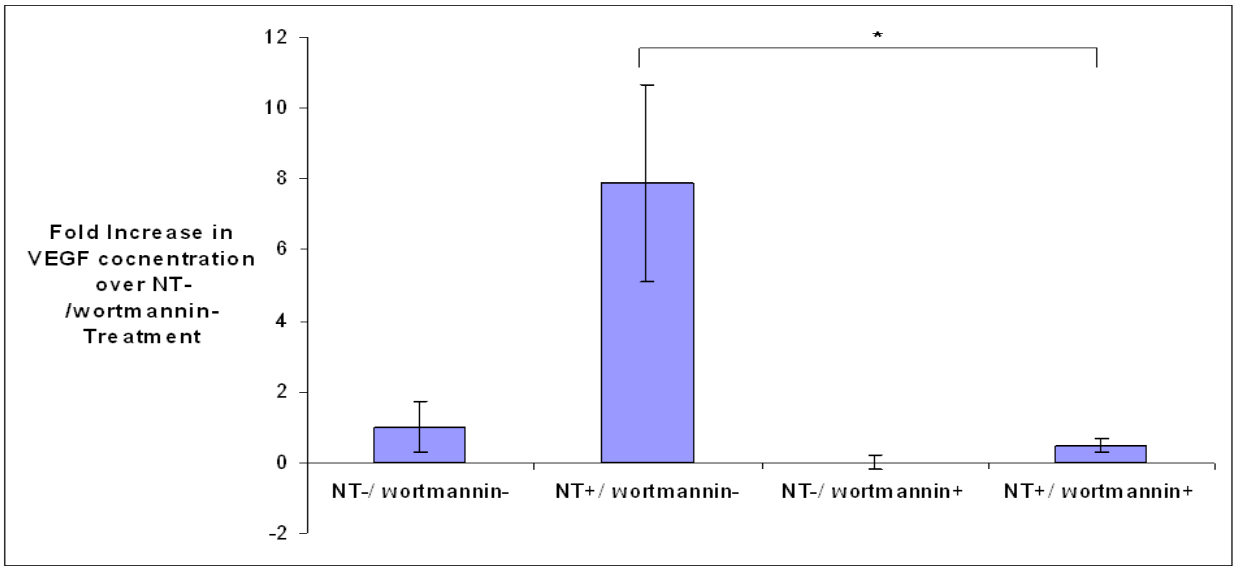

Figure 12. PI3-Kinase inhibitor Wortmannin inhibits VEGF release from HMC-1 cells by NT-stimulation. HMC-1 cells were incubated in 10 μM of Wortmannin for 1 hour, and then stimulated with 10 μM of NT for 8 hours at 37 ° C. VEGF release was measured by ELISA. VEGF release in the NT-/wortmannin-, NT-/wortmannin+, and NT+/wortmannin+ treatments were not significantly different, while VEGF release from NT+/wortmannin- was significantly greater than all of these treatments (p=0.015, 0.007, 0.0097, n=18). Standard error is shown.

However, inhibiting HMC-1 cells with 10 μM of Wortmannin showed levels of VEGF release that were lower than the NT-/wortmannin- treatment (Figure 12). Thus, I inhibited HMC-1 cell release of VEGF using 1 μM of Wortmannin to ensure that the observed impact on VEGF release was not due to unintended effects from a high concentration of inhibitor (Figure 13). While the NT-/wortmannin-, NT-/wortmannin+, and NT+/wortmannin+ samples were not significantly different from each other, VEGF release from NT+/wortmannin- was significantly greater than all of these samples (p=0.0005, 0.043, 0.0097, n=18). Thus, even at 1 μM, Wortmannin still inhibited VEGF release from HMC-1 cells.

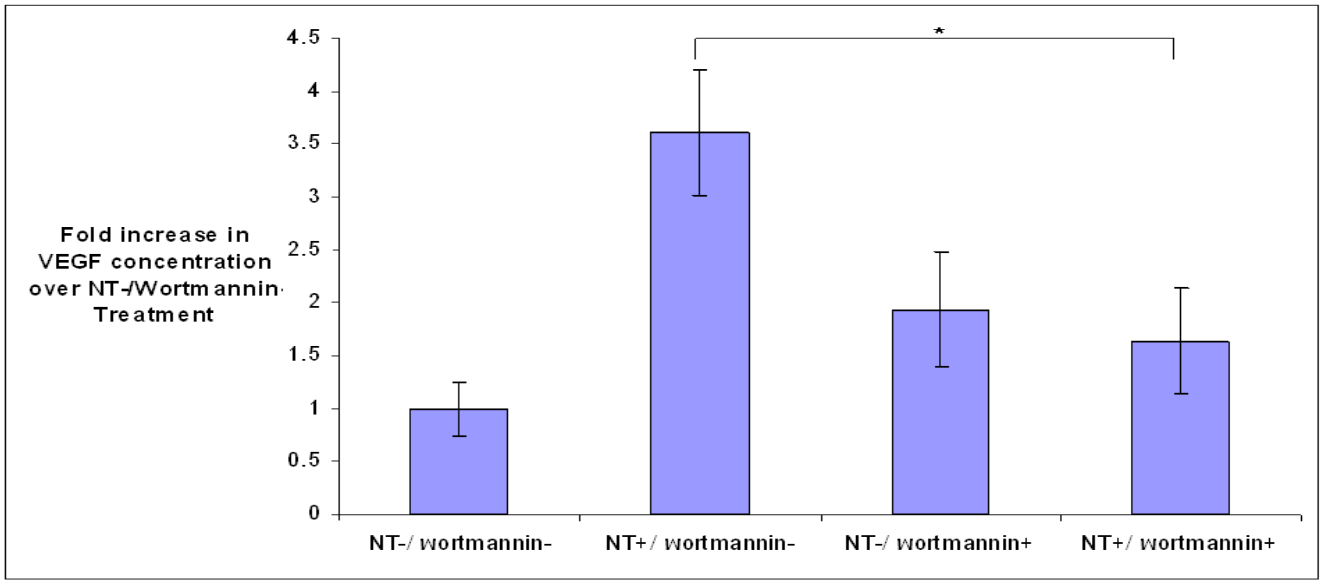

Figure 13. PI3-Kinase inhibitor Wortmannin inhibits VEGF release from HMC-1 cells by NT-stimulation. HMC-1 cells were incubated with 1 μM of Wortmannin for 1 hour, and then stimulated with 10 μM concentrations of NT for 8 hours at 37 ° C. VEGF release was measured by ELISA. NT-/wortmannin-, NT-/wortmannin+, and NT+/wortmannin+ samples were not significantly different, while VEGF release from NT+/wortmannin- was significantly greater than all of these samples (p=0.0005, 0.043, 0.0097, n=18). The data is shown as fold increase in VEGF release over basal VEGF release. Standard error is shown.

NF-kB inhibition of VEGF release

NF-kB is a common transcription factor in many cell-signaling pathways, shown to be involved in the expression of numerous immune-and inflammatory-response genes (66). Thus it was examined as a potential component of this pathway. CAY10512 was used as an NF-kB inhibitor. In Figure 14, the NT+/ CAY10512- and NT+/CAY10512+ treatments showed a significant increase in VEGF release over the NT-/ CAY10512- treatment (p=0.0034, 0.0000225, n= 18, respectively) and the NT-/CAY10512+ treatment (p=0.004425, 0.000528, n= 18, respectively). The NT+/ CAY10512+ treatment did not show a significant difference in VEGF release compared to the NT+/ CAY10512- samples, and there was no significant difference observed between NT-/ CAY10512- and NT-/ CAY10512+ samples. Ultimately, NF-kB

inhibitor CAY10512 did not cause a significant difference in VEGF release from HMC-1 cells stimulated with NT.

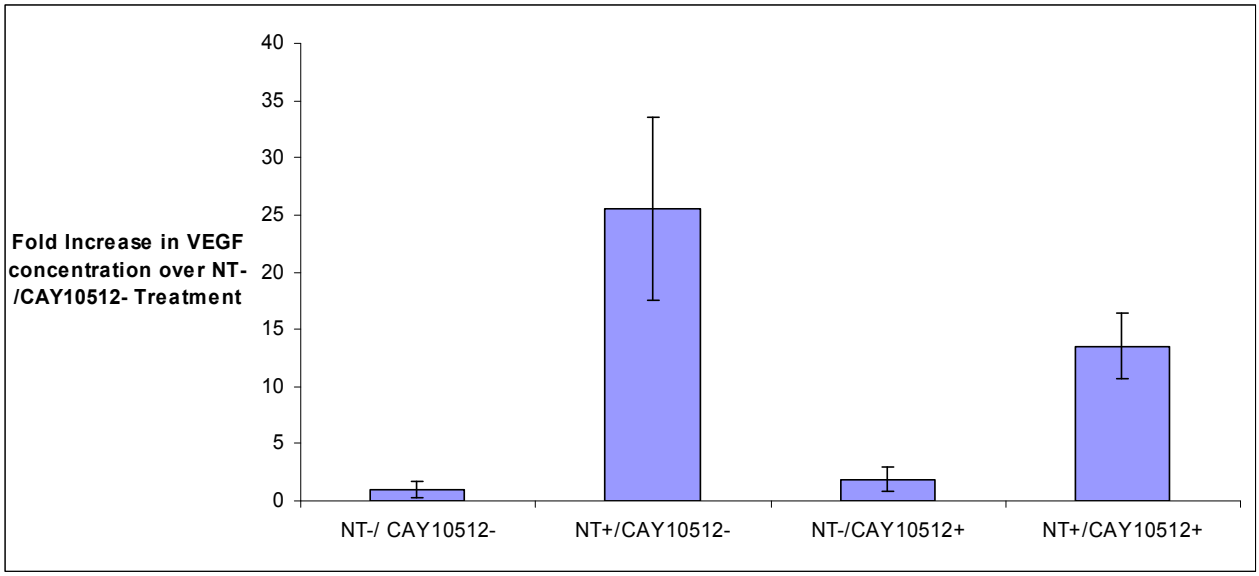

Figure 14. NF-kB inhibitor CAY10512 does not inhibit NT stimulation of HMC-1 cell VEGF release. HMC-1 cells were incubated in 1 μM of CAY10512 for 1 hour, and then stimulated with 10 μM of NT for 8 hours at 37 ° C. VEGF release was measured by ELISA. The NT+/ CAY10512- and NT+/CAY10512+ treatments showed a significant increase in VEGF release over the NT-/ CAY10512- (p=0.0034, 0.0000225, n= 18, respectively) and the NT-/CAY10512+ samples (p=0.004425, 0.000528, n= 18, respectively). NT+/ CAY10512+ samples did not show a significant difference in VEGF release compared to NT+/ CAY10512- samples, and there was no significant difference observed between NT-/ CAY10512- and NT-/ CAY10512+ samples. The data is shown as fold increase in VEGF release over basal VEGF release. Standard error is shown.

Zinc Chelator TPEN inhibits VEGF release

(Zn^{2+}) is an essential nutrient, involved as a catalytic and structural cofactor in numerous metalloproteins and signaling molecules (67, 84, 106). Kabu *et al.* (2006) showed Zn2+ to be required for both degranulaton and cytokine production in mast cells, and essential for granule translocation to the plasma membrane (49). *In vitro* use of a Zinc chelator strongly inhibited the release of histamine, β-hexosaminidase, cytokines, and leukotrienes (49). In addition, unpublished work in our laboratory has shown that zinc chelator TPEN significantly inhibited histamine release from mast cells in response to NT (16). Thus, based on the observed role of

Zinc in mast cell signaling, I used cell membrane permeant Zn^{2+} chelator, N,N,N0,N0-(2-pyridylethyl)ethylenediammine (TPEN) (85), to determine the potential role of zinc in this signaling pathway. Figure 15 represents samples incubated for 1 hour at 37 ° C with 50 μM of TPEN, and then 8 hours with 10 μM of NT. The NT+/ TPEN- treatment showed a significant increase in VEGF release over NT-/ TPEN- treatments (p=0.0195, n=16) and the NT+/ TPEN+ samples (p=<.00005, n=16). The NT-/TPEN-, NT-/TPEN+, and NT+/TPEN+ samples were not significantly different. Thus, TPEN was shown to significantly inhibit VEGF release from HMC-1 cells.

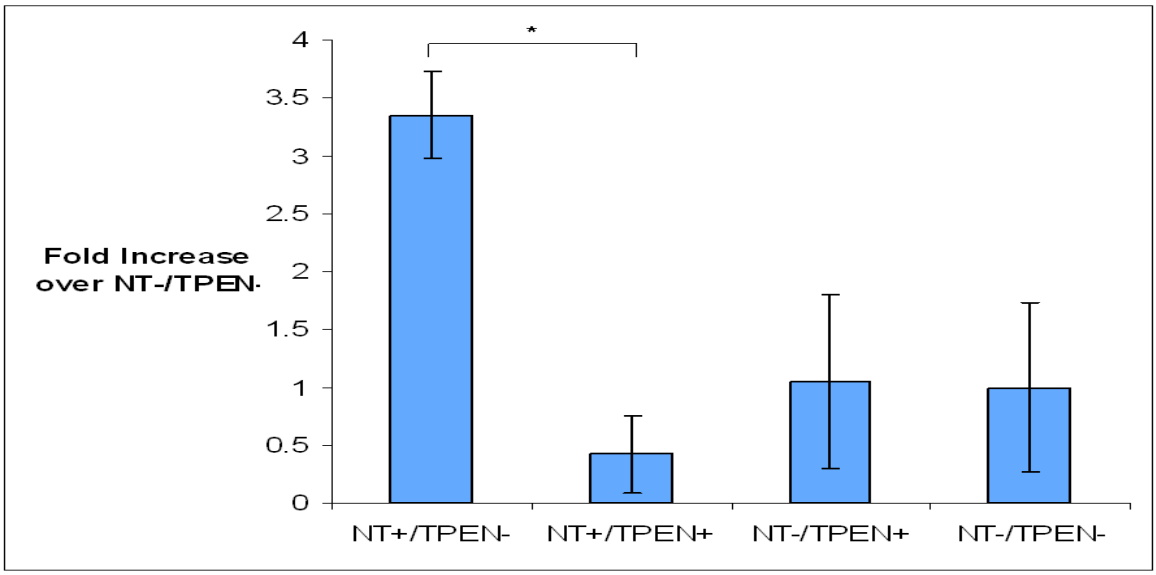

Figure 15. Zinc Chelator TPEN inhibits NT stimulation of HMC-1 cell VEGF release.
HMC-1 cells were incubated in 50 μM of TPEN for 1 hour, and then stimulated with 10 μM of NT for 8 hours at 37 ° C. VEGF release was measured by ELISA. The NT+/ TPEN- treatments showed a significant increase in VEGF release over the NT-/ TPEN- (p=0.0195, n=16) and the NT+/ TPEN+ samples (p=<.00005 n=16). NT+/ TPEN+ samples did not show a significant difference in VEGF release compared to the NT-/ TPEN- samples, and there was no significant difference observed between NT-/ TPEN- and NT-/ TPEN+ samples. The data is shown as fold increase in VEGF release over basal VEGF release. Standard error is shown.

DISCUSSION

Mast cells are a heterogeneous classification of cells found throughout the body. They have a variety of roles due to their production of numerous biologically active mediators following appropriate stimulation. Based on my past results, in addition to previous findings in our laboratory, the neuroendocrine peptide neurotensin (NT) stimulates significant VEGF release from HMC-1 mast cells (22, 52, 53). I investigated this signaling pathway, stimulating cells with NT and measuring the concentration of VEGF released under several conditions.

As in the past, VEGF release was highly variable, making it difficult to attain consistent levels of VEGF release. This was especially evident during my efforts to determine the effect of NT concentration on VEGF release. Although VEGF release was consistently greater in the samples stimulated with NT than in untreated samples, the differences in VEGF release dependent on concentration were not deemed significant due to the variability expressed over numerous trials (Figure 3). To attempt to overcome this difficulty, I investigated potential sources of variation related to VEGF degradation, passage number and cell clumping.

Previous work in our laboratory showed VEGF to be degraded in the presence of mast cells (22). My results also showed that VEGF is degraded by mast cells over a 24 hour time frame (Figure 4). This decrease in VEGF concentration over time is most likely due to proteases released by the mast cells following stimulation (76). For example, HMC-1 cell granules contain trypsin, chymase, and carboxypeptidases, which are released upon the activation of mast cells (76) and known to degrade neuropeptides involved in inflammation (27). Thus, it is highly likely that these proteases also degrade VEGF. It is possible that NT may also be degraded by these proteases. Cochrane *et al.* (1991) showed mast cells to release NT-degrading compounds following stimulation by compound 48/80 (16). Ultimately, these proteases may have

contributed to the inconsistencies in VEGF release observed in this project, and may have made it difficult to fully replicate previous research performed in our laboratory (22).

I attempted to address this potential complication by re-suspending the cells shortly after NT stimulation in order to remove any proteases released. However, resuspended mast cells did not exhibit the significant increase in VEGF release over unstimulated controls that I observed in the NT-stimulated cells that were not resuspended (Figure 5). Thus, I continued performing tests without resuspending the cells following NT stimulation.

Another source of variation may be related to the passage number of the HMC-1 cell lines. Macchia *et al.* (1995) showed the degradation in the 5-lipoxygenase pathway in HMC-1 cell cultures after 10 passages (65). Thus, I sought to observe whether there were also changes in VEGF release based on the passage number. Using samples that had undergone between 3 and 9 passages, my results showed the greatest significant VEGF release after 3 passages (Figure 6). The observations after 4 passages were quantitatively larger, but were not significant due to a smaller number of trials. VEGF release in samples that had undergone 5, 6, and 8 passages was also shown to release significantly more VEGF than unstimulated controls (no data was available for cells that had undergone 7 passages), though the release after 3 passages was significantly greater than these samples as well. These observations indicate the importance of using cells after a low number of passages to achieve optimal VEGF release.

A third potential source of the variability could be due to clumping observed in HMC-1 cell populations. Although I attempted to maintain cell concentrations of 2.5×10^6 cells per 1 mL sample (as measured by hemocytometry), cell clumping could have skewed these estimates. Variations in cell density could have led to inconsistent results since higher concentrations of cells may release greater concentrations of VEGF. This factor could explain why some wells of

NT-stimulated cells illustrated dramatic release of VEGF, while others generated VEGF concentrations similar to basal levels of release. In future testing, I will attempt to more accurately estimate cell concentration by performing multiple estimates using hemocytometry and by gently pipetting cells down the side of the flask to dissociate the clumps in solution.

Overall, to more precisely illustrate trends in VEGF release from HMC-1 cells, it will be important to standardize the levels of VEGF release by evaluating these, and other, factors. Efforts to use cells at a constant number of passages within any particular test and experimenting with different cell population densities could make results more precisely reproducible.

Beyond simply measuring VEGF release, I conducted experiments to identify potential components of the signaling pathway through which NT promotes VEGF release. Past results have shown NT to stimulate VEGF secretion from HMC-1 cells by activating the NTS-1 receptor; VEGF release was significantly decreased by treatment with SR 48692, a non-peptide, competitive inhibitor of the NTS-1 receptor (22, 52). I confirmed these results (Figure 7). I also looked to extend these findings by identifying 'downstream mediators' beyond the NTS-1 receptor that may be involved in VEGF release. I tested for the involvement of MEK 1 MAPK, p38 MAPK, PL-Cβ, NF-kB, PI-3K, PKC, and Zinc by the use of selective inhibitors prior to NT stimulation. To attempt to minimize the variation in my results, I tested HMC-1 cells under the conditions that I found to yield the largest VEGF release (10 μM NT, 8 hours of incubation at 37 ° C, and cells under nine passages) (52, 53). A significant decrease in VEGF release was observed following inhibition of PKC, MEK I MAPK, p38 MAPK, PKC, and PI-3K, potentially implicating these enzymes in this VEGF release pathway. Use of a zinc chelator also blocked VEGF release, indicating the potential role for ZN^{2+} in the secretion of VEGF. This finding is similar to the inhibition of NT-stimulated release from isolated rat mast cells by TPEN (16). In

continuing this research, I expect to confirm (through Western Blotting) that the phosphorylation of MEKI MAPK, p38 MAPK, PI-3K, and PLC-β was prevented by the inhibitors utilized in the above-mentioned tests, illustrating that the inhibitors have the intended effect on the target enzymes.

Ultimately, through this project, I both identified and eliminated several potential components from the many possible mediators involved in this release pathway. Continuing to evaluate this pathway is important; a greater knowledge of the enzymes involved in mast cell release of VEGF following NT stimulation may yield effective techniques for preventing the VEGF-induced angiogenesis that promotes cancer growth and malignancy, and thus enable the treatment of certain types of human cancers. Additional potential mediators to test include PKA and additional MAPKinases, among other signal transduction components involved in the VEGF release pathway from HMC-1 cells by CRH stimulation (11). In addition, although the inhibition of PL-Cβ (an enzyme commonly involved in stored mediator release from mast cells [2]) was not shown to be significant in my project ($p=0.067$) (Figure 8), deceased variation in VEGF release could potentially show it to be involved in this pathway.

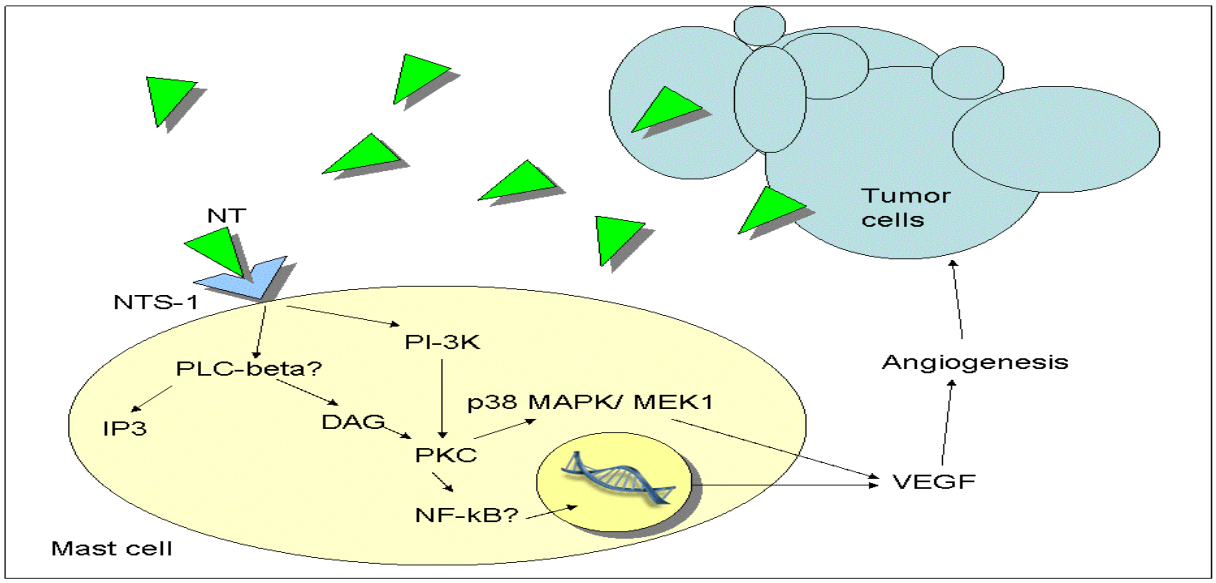

Figure 16. Based on the results of this project, I proposed a potential pathway that incorporates the enzymes that I evaluated into VEGF release. NT released from tumor cells stimulates mast cells through the NTS-1 receptor to release VEGF. Potential mediators involved include: PI-3K, PKC, and various MAPKinases.

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