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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
The aims are (a) to determine the mechanism by which neurotensin (NT) enhances prostate Cancer (PC3) cell growth and (b) to test if NT released by eating mediates the cancer-Promoting effects of high fat diets. Experiments conducted here show that NT enhanced Arachidonic acid metabolism generating primarily lipoxygenase-derived products. NT Stimulated DNA-replication and its effect was blocked by lipoxygenase inhibitors, by MAP-kinase inhibitors, byPI3 kinase inhibitor, by Ca^{2+}- channel blocker and by tyrosine Kinase inhibitors. NT caused the accumulation of EGF-like ligand(s) and transactivation Of the EGF receptor. We conclude that NT promotes growth by a coordinate stimulation Of MAP kinase and PI3 kinase by a mechanism involving lipoxygenase(s).

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Table of Contents

Introduction-

The overall goal of this work is to examine the involvement of the intestinal hormone neurotensin (NT) in the regulation of prostate cancer cell growth *in vivo*. The initial hypothesis under study is that NT, which is released from the intestine by ingestion of fatty meals, contributes to the enhanced growth of prostate cancer cells in animals consuming high fat food, and that the effects of NT may explain the worldwide association between high fat intake and growth / incidence of prostate cancers in humans. Since NT has been shown to exert important effects on growth of both androgen-independent (PC3) and androgen-dependent (LNCaP) human prostate cancer cells *in vitro*, a key goal is to understand the mechanism(s) involved in its growth promoting effect(s) in these two cell lines (1).

To examine the mechanism(s) involved in NT-induced growth promotion, we will perform experiments using PC3 and LNCaP cells in tissue culture, and we will study the interaction of NT with major growth factor pathways. EGF will be the primary focus but TGFβ and PDGF may be examined. Particular attention will be given to growth regulation by the MAPK-, Akt- and CDK-pathways. We will identify NT inputs into the phosphorylation of the key enzymes in these pathways and we will investigate relationships to inositol phosphate formation, Ca^{2+}-release, Ca^{2+}-influx, PKC-activation and cAMP formation (2, 3).

To examine the contribution of NT to prostate cancer cell growth *in vivo*, we plan to utilize an animal model involving PC3 and LNCaP cells xenografted into nude mice. The animals will be maintained on high and low fat diets and the effects of NT antagonist SR48692 on tumor growth will be measured.

Body

Task #1—To compare the effects of NT antagonist on the growth of PC3 and LNCaP cells in nude mice (months 1-30).

Initial studies were performed using PC3 cells and the preliminary data showed that daily injections of NT antagonist SR48692 slowed the growth rate of the PC3 tumors in nude mice. However, we were not completely satisfied with the test system and we thought it best to improve on the method. In the prior report, we described our quandary as to whether xenografting was best achieved by the injection of cancer cells or the implantation of cubes of tumor tissue using a trocar syringe. We are still dissatisfied with these methods and we are still studying this question before embarking on these critical and costly experiments. In our hands, the trocar method is too invasive for these delicate mice. For us to do it properly, we must make a small incision, and this requires anesthesia and the placement of a wound clip. With the attendant problems in immune compromised animals, we decided to return to the cell injection method. In order to improve our performance, we are receiving instruction from another cancer laboratory (Dr. Timothy Wang, Dept of Gastroenterology, UMMS) where the cell injection method has been optimized to avoid the difficulty of multiple tumor

4

development. We believe that delaying these studies was the correct action to take and we think that this phase of the work will soon be underway. The success of these studies depends heavily on the precision of the method, particularly since only a fraction of tumor growth is determined by the fat intake and may relate to NT secretion. Therefore, it is essential that we have uniformity of tumor size and composition at the start of the experiment. Tumor cubes may be heterogeneous depending on site of origin in the original mass. Because of these considerations and difficulties, we would like to extend the time for this task assignment from the prior 1-30 months to 1-36 months.

Task #2—Perform dietary studies to correlate NT levels with fat intake and cancer cell growth (months 18-36).
   These studies will be done in the final year of this project.

Task #3—To develop NT knock-out / nude mouse strain from our NT knock-out and nu/nu purchased from Jackson Labs (months 1-24).
   To evaluate the importance of endogenous NT for the growth of prostate cancer tumors, we crossed NT knockout mice with mice carrying the nude mutation ($foxn1^{nu}/foxn1^{nu}$) to generate immuno-deficient nude mice that are also deficient in NT. We initially attempted to generate these mice by crossing homozygous nude males with homozygous NT knockout and wild type females; however, these crosses were unsuccessful probably due to greatly decreased fertility of nude males (females are infertile).

   To circumvent this problem, we crossed heterozygous nude females ($+/foxn1^{nu}$; congenic C57BL/6T) with heterozygous NT knockout males ($+/NTKO$; 5N C57BL/6J) to generate compound heterozygotes ($+/foxn1^{nu}$; $+/NTKO$) on a C57BL/6 background (6N). These were subsequently intercrossed to generate heterozygous nude, homozygous NT knockout ($+/foxn1^{nu}$; NTKO/NTKO), and heterozygous nude, NT wild type ($+/foxn1^{nu}$; $+/+$) mice. These mice have now been intercrossed to generate mice for tumor growth experiments ($foxn1^{nu}/foxn1^{nu}$; NTKO/NTKO and $foxn1^{nu}/foxn1^{nu}$; $+/+$). Thus, we have succeeded in completing this task and now we will proceed to the testing of cancer cell growth in these animals.

   We anticipate having sufficient numbers of mice that are >12 weeks of age to begin tumor cell injections for a pilot study by January. Initially, we will compare tumor growth in nude/NT knockout and nude/wild type male mice that are fed a high fat diet. The rational being that dietary fats are a potent stimulus for NT release into the circulation from enteroendocrine NT cells in the gastrointestinal tract. We anticipate having the results of this initial study by the beginning of March. If tumor growth is attenuated in NT-deficient nude mice, we will then expand the study to systematically investigate whether dietary fats play a role by comparing high fat and low fat diets. We anticipate completing this aspect of the study by the end of the funding period.

5

The generation of the animals for these studies will require a moderately sized breeding colony, since the expected number of progeny with the desired genotype and sex represents only $1/8^{th}$ of the total number of progeny. Thus, to generate approximately 10 mice for each group that are a similar age at the onset of the study (12-24 weeks) we anticipate needing approximately five breeding pairs of each type (+/foxn1^{nu}; NT/KO/NTKO) x (+/foxn1^{nu}; NTKO/NTKO) and (+/foxn1^{nu}; +/+) x (+/foxn1^{nu}; +/+). This should result in the production of approximately 17-18 male progeny/month for each set of crosses and 1/4 of these (~4, or ~12 during the 3 month time window) will have the desired genotypes. The housing of the progeny during genotype analysis will require maintaining approximately 20-25 cages/month in addition to the breeding cages (10). During the study, mice will be individually housed following tumor cell injection (~20-25 cages). Thus we anticipate that approximately 50-60 cages will be required to maintain the colony during the duration of these studies.

Task #4—Test effect of fat on cancer growth in NT-knockout/nude mice (months 16-36).
These studies will be performed in the final year of the project.

Task #5—Examine effects of NT on arachidonic acid metabolism and on lipoxygenase expression in prostate cancer cells (months 1-12).
We have extended the observations reported earlier regarding the effects of NT on arachidonic acid (AA) metabolism. PC3 cells, labeled with [3H]-AA and washed, were stimulated and release of [3H]-AA was monitored. Stimulation with EGF and with NT increased the release by 2- and 3-fold respectively. The effect of NT was inhibited by PKC inhibitor (staurosporine), PI3-kinase inhibitor (LY294002 and wortmannin), MEK inhibitor (U0126), calcium channel blocker (nifedipine) and EGFR-tyrosine kinase inhibitor (AG1478). The radiolabeled products in the release media were separated by HPLC and NT was found to increase by 2-3 fold the sizes of the peaks which eluted at the position of AA, as well as peaks for the lipoxygenase metabolites, 5-, 12- and 15-HETE. These results support our contention that NT activates MAP-kinase and that this enzyme, which is known to activate PLA2, liberates AA from the phospholipid pool. Subsequent action by lipoxygenase gives rise to HETE and leukotriene.

Task #6- Test NT for ability to alter growth responses to EGF in PC3 cells (months 13-24).
We got an early start on this task and reported some progress in the first year report. We demonstrated the stimulatory effect of NT on DNA-replication in PC3 cells (dose-response and time-course were completed). The effect of NT was nearly equal in magnitude to that of EGF. Together, their effects were less than additive, suggesting that they shared key steps or their pathways merged. NT caused an accumulation of EGF-like agonist in PC3 cell cultures at 24-48 hrs after its addition, suggesting that NT might cause transactivation of EGF-receptor. Both the effect of EGF and that of NT were inhibited by tyrosine-kinase inhibitors (eg., AG1478 and genistein), inhibitors of PKC (staurosporine, rottlerin),

inhibitors of Mek (U0126, PD98059), PI3 kinase inhibitor (wortmannin), lipoxygenase inhibitors (ETYA, NDGA) and L-type Ca2+-channel blocker (nifedipine). Indomethacin had little effect on the NT growth response.

To extend these results, we examined ^{125}I-NT binding to PC3 cells and measured the formation of inositol phosphates in response to NT (the first step in its Gq-mediated signaling). The Kd in the binding assay (Kd, 0.9 nM) related well to the EC50 for NT-induced IP-formation (EC50, 1.0 nM). This also correlated to the EC50 for the activation of EGFR and ERK1/2 (EC50, 1 nM). Interestingly, the binding of ^{125}I-NT to the cells was enhanced in the presence of PKC inhibitors, suggesting that this might explain the PKC-dependence of the growth-promoting effects of NT.

To extend our results to other prostate cancer cell lines, we tested NT for growth effects in the LNCaP cell line. NT dose-dependently (EC50, 0.2 nM) stimulated DNA synthesis in LNCaP cells, with a maximal increase of 2-3 fold. Similar to PC3 cells, the response in LNCaP was associated with transactivation of EGFR and activation of ERK1/2 (see below). In contrast to results in PC3 cells, where binding of ^{125}I-NT was easily demonstrated and where western blotting indicated expression of NT-receptor (NTR1), LNCaP cells did not exhibit ^{125}I-NT binding and did not test positive for NTR1. Since NTR1 is Gq-linked, NT stimulated inositol phosphate formation in PC3 cells, activating PKC and thus, the growth response to NT was PKC-dependent. In contrast, NT did not stimulate IP-formation in LNCaP cells and the growth effects of NT were PKC-independent. It is strange to have a potent growth response to NT in LNCaP cells which appear to lack NTR1. It may be that a different NT-receptor is present and that NT and ^{125}I-NT are rapidly degraded by LNCaP after interaction and stimulation of the receptor. It may be that NTR1 is present, but that it is modified such that the C-terminal directed antibody cannot recognize it. We are pursuing answers to these questions.

Additional results concerning the growth effects of NT are reported under "Task #7" to facilitate presentation, relating the growth studies to the effects of NT on EGF binding and to the activation of specific growth-promoting pathways.

Task #7—Test NT for ability to activate MAP-kinases and to induce Krox-24, a ubiquitous indicator of mitogenic stimulation (months 25-36).

To extend these studies in PC3 cells, we performed Western blotting to assess the activation (phosphorylation) of specific growth-promoting pathways. NT dose-responsively (0.1 to 30 nM) enhanced the activation (phosphorylation) of EGFR, ERK1/2 and Akt, but it did not affect JNK/SAPK and p38 MAPK. NT stimulated the phosphorylation of these proteins within 3 min and the stimulation lasted up to 15 min. The NT-induced transactivation of EGFR and the enhancement in DNA synthesis that followed were blocked by heparin (10μg/ml) but were unaffected by neutralizing anti-EGF (10μg/ml), indicating the

7

involvement of HB-EGF instead of EGF itself. Since HB-EGF can be liberated from the cell surface by PKC-dependent steps, we examined the involvement of PKC. The EGFR transactivation, the ERK activation and the stimulation of DNA synthesis in response to NT were all blocked by PKC inhibitor (staurosporine) and by downregulation of PKC with overnight treatment of PMA (1μM), indicating a mediator role for PKC.

These effects of NT in PC3 cells were Ca^{2+}-independent. The NT-induced EGFR transactivation and ERK phosphorylation were not blocked by intracellular calcium chelator (BAPTA-AM), nor by extracellular chelator (EGTA). There was also a lack of effect of Ca^{2+}-calmodulin kinase inhibitor on these processes. As has been shown for EGF, the stimulatory effect of NT on DNA synthesis was diminished by increasing cell density (contact inhibition). EGFR transactivation as well as ERK phosphorylation in response to NT were contact inhibited. These responses were also diminished by increasing the volume of the medium, which was consistent with a mechanism involving release of mediator (HB-EGF) into the extracellular space.

The activation of EGFR and ERK in response to NT was also inhibited by agents that elevated intracellular cAMP levels (dibutyryl-cAMP, isoproterenol), which is consistent with the inhibitory effect of these agents on DNA synthesis in PC3 cells. Interestingly, NT was found to have opposite effects on DNA synthesis, dependent on the cellular milieu. In the presence of a Gs-stimulator (such as isoproterenol), NT further inhibited DNA synthesis and growth; however, in the absence of any cAMP inputs, NT was growth promoting. Further experiments proved that PC3 cells contain adenylyl cyclase (subtypes 2 and 4), which can be super-activated by PKC. Thus, NT activates PKC which enhances the responses of AC2 and AC4 to Gs-stimulation, generating more cAMP and producing more growth inhibition. This enhancement of cAMP formation occurred in response to other Gq-coupled receptor agonists (bombesin, ATP). Thus, we concluded that tumors with neuroendocrine foci (involving Gq-coupled receptors) may display an enhanced growth-inhibitory response to cAMP stimulation by giving Gq-coupled agonists (NT, bombesin, ATP) along with a Gs-coupled agonist (isoproterenol, PGE2) or forskolin.

The above results suggested that NT transactivation of EGFR was ligand-mediated, ie., involving release of HB-EGF. To further address this point, we determined whether NT altered the binding of ^{125}I-EGF to PC3 cells. NT treatment for 5 min decreased ^{125}I-EGF binding at 37°C by 20%, and this involved a decrease in both cell-surface binding and internalization. This effect was PKC-dependent. These results are consistent with a PKC-mediated stimulation of HB-EGF processing from the cell-surface and the formation of EGF-like ligand(s) that activate EGFR. This is reflected by a decrease in EGF binding due to competition with ^{125}I-EGF and/or enhanced EGFR internalization and/or enhanced desensitization of EGFR. All of the above would be expected to follow ligand-mediated activation of EGFR.

KEY RESEARCH ACCOMPLISHMENTS-

- Developed NT-knockout/nude mouse strain.
- Demonstrated that NT stimulated DNA synthesis in PC3 and LNCaP cells at near physiologic concentrations (0.1 to 1 nM range).
- Demonstrated that NT transactivated EGFR in PC3 and in LNCaP cells.
- Demonstrated that formation of HB-EGF in response to NT led to the activation of EGFR and to downregulation of ^{125}I-EGF binding.
- Demonstrated that NT activated ERK in PC3 and in LNCaP cells.
- Demonstrated that NT activated Akt (PI3-kinase) in PC3 cells.
- Demonstrated that NT-induced activation of EGFR, ERK and Akt in PC3 cells was Ca^{2+}-independent, PKC-dependent and contact inhibited.
- Demonstrated that cAMP inhibited NT-induced activation of ERK, and that NT could enhance cAMP formation in response to Gs-stimuli.
- Demonstrated ^{125}I-NT binding to PC3 cells and the presence of NTR1 by western blotting. Related the binding Kd to the EC50 for IP-formation and to activation of EGFR and ERK.
- Demonstrated the absence of NTR1 in LNCaP cells by western blotting and the inability of LNCaP to bind ^{125}I-NT and to respond to NT by increasing IP-formation.

REPORTABLE OUTCOMES:

Abstracts have been written and presented at meetings:

1.- S. Hassan and R.E. Carraway. Involvement of MAP-kinase, PI3-kinase and EGF receptor in the Stimulatory Effect of Neurotensin on DNA-replication in PC3 Cells. Reg Pep 2002 Meeting, Boston MA, Aug 31 - Sept 3,2002.

2.- R.E. Carraway, S. Hassan, P.R. Dobner. Positive and Negative Effects of Neurotensin on DNA synthesis in PC3 caells Mediated by EGF receptor and Adenylyl Cyclase, respectively. 9th Prouts Prostate Cancer Meeting, Nov 7-10, 2002.

Manuscripts are in preparation:

1.- S. Hassan and R.E. Carraway. Involvement of EGF-receptor, MAP-kinase and PI3-kinase in the Stimulatory Effect of Neurotensin on DNA-replication in PC3 Cells.

2.- R.E. Carraway, M. Sanderson and X. Gui. Role of Ca^{2+} and PKC in the cAMP-enhancing Effect of Neurotensin in PC3 cells.

3.- R.E. Carraway and S. Hassan. Role of Lipoxygenase-mediated Arachidonic Acid Metabolism in PC3 Cell Growth Responses to EGF and Neurotensin.

4.- R.E. Carraway and S. Hassan. Role of PKC in ^{125}I-neurotensin (NT) Binding and Biologic Effects leading to Growth of Prostate Cancer PC3 cells.

CONCLUSIONS:

The importance of the completed work is in regards to the mechanism by which NT enhances the growth of prostate PC3 and LNCaP cells in culture. Our work indicates that in PC3 cells, NT binds to NTR1, transactivating EGFR by liberating within 3 min HB-EGF from the cell-surface by a PKC-dependent mechanism. In LNCaP, the mechanism is PKC-independent and a different receptor may be involved. Temporally associated with activation of EGFR is an activation of MAP-kinase and PI3-kinase, which is followed a day later by a stimulation of DNA synthesis. These responses depend on the hormonal milieu. In the presence of a Gs-stimulus, NT can enhance cAMP formation, resulting in an inhibition of DNA synthesis. Since we found that lipoxygenase inhibitors can reduce basal and NT-stimulated DNA replication, it seems likely that NT & EGF both act by way of lipoxygenase(s). Thus, growth enhancement by NT & EGF involves a coordinate enhancement of lipoxygenase(s) and protein kinase(s), and the growth response can be influenced by the activity of adenylyl cyclase(s).

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P-19. Involvement of map kinase, PI3 kinase and EGF receptor in the stimulatory effect of neurotensin on DNA replication in PC3 cells

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The signal transduction mechanism(s) involved in trophic effects of neurotensin (NT) are not fully elucidated. This study uses prostate cancer epithelial PC3 cells, which express high levels of the type 1 NT receptor (NTR1), to examine the involvement of MAPK (ERK1/2, JNK/SAPK, p38), PI3 kinase, PKC and EGFR in the mitogenic effect of NT. NT dose-dependently (0.1 to 30 nM) enhanced phosphorylation of EGFR, PKB and ERK1 2, reaching maximal levels within 3 min as measured by Western blotting. This was followed in 24 h by an accumulation of EGF like substance(s) in the medium (assayed by EGFR binding) and a twofold increase in DNA synthesis (assayed by [^{3}H]thymidine incorporation). The enhancement of DNA replication and EGFR/MAPK phosphorylation in response to NT were inhibited by EGFR tyrosine kinase inhibitors (AG1478 and PD153035) and by heparin, but not by neutralizing anti EGF antibody. Thus, transactivation of EGFR by NT appeared to involve heparin binding EGF rather than EGF itself. The effect of NT on MAPK activation and DNA replication were non-additive with those of EGF. These effects were also attenuated by PLC inhibitor (U73122), PKC inhibitors (rottlerin and bisindolylmaleimide), MEK1/2 inhibitor (U0126) and PI3 kinase inhibitors (wortmannin and LY 294002). We conclude that NT stimulates DNA replication via transactivation of the EGFR/MAPK cascade and that the mechanism involves PLC, PKCδ and PI3 kinase.

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ABSTRACT
9th Prouts Neck Prostate Cancer Meeting, November 7-10, 2002

Positive and Negative Effects of Neurotensin on DNA synthesis in PC3 cells Mediated by EGF-Receptor and Adenylyl Cyclase, respectively

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Prostate cancer incidence increases with high fat / calorie intake. Neurotensin (NT), a gut hormone released by ingestion of fat, stimulates DNA replication and growth of PC3 cancer cells. Molecular biologic analysis and inhibitor studies indicated that the effect was mediated by G-protein coupled NT receptor (NTR1). By Western blotting, NT (0.1-30nM) dose-dependently caused a rapid (3min) phosphorylation of EGFR, Akt and ERK1/2, followed in 24 hrs by accumulation of EGF-like substance(s) in the medium (assayed by EGF binding) and a 2-fold increase in DNA synthesis (assayed with [3H]-thymidine). The enhanced DNA synthesis and the EGFR & MAPK phosphorylations were suppressed by U71322, rottlerin, U0126, wortmannin, LY29002, AG1478 and heparin, but not by anti-EGF antibody, indicating that NT transactivates the EGFR/MAPK cascade via heparin-binding EGF and involving PLC, PKC and PI3-kinase. Since NT is co-stored with catecholamines and releases histamine and eicosanoids, we wondered how these might influence its growth effects. To our surprise, NT gave growth inhibition when used with a threshold dose of catecholamine (ISO). Growth inhibition was due to NT's ability to enhance cAMP formation in response to G_s-agonists (ISO, PGE2) and to forskolin (F), causing a 2-fold increase in efficacy without altering EC50s. By itself, NT did not alter cAMP levels, but it induced a 5-fold increase in inositol phosphate (IP) formation and a 15-fold rise in $[Ca^{2+}]i$. The inhibitor of PI-metabolism (quercetin) blocked NT's effects on IP, $[Ca^{2+}]i$ and cAMP, and the PLC inhibitor (U-73122) blocked the IP and cAMP responses. Elevators of $[Ca^{2+}]i$, (thapsigargin, ionomycin) mimicked NT. Work with specific inhibitors showed that the cAMP effect of NT was not mediated by Ca^{2+}/ calmodulin but did require PKC and was mimicked by PKC-activator PMA. Western blotting with phospho-specific (pan) antiserum to PKCα, βI, βII, δ showed that NT induced a rapid PKC phosphorylation; however, translocation of PKC from cytosol to membrane was difficult to demonstrate. Conditional sensitization of AC by PKC is a property shown for ACII. Western blotting indicated presence of ACII and absence of ACI, ACV, ACVI and ACVIII. These results suggest that NT sensitizes ACII via a PLC/Ca^{2+}/PKC pathway. Since cAMP inhibits DNA replication in PC3 cells, sensitization of ACII would enhance the growth inhibitory response to an AC-stimulator. Thus, ACII could act as a coincidence detector in PC3 cells, giving (for example) growth enhancement to NT alone and growth inhibition to NT in presence of catecholamine, PGE2 or histamine.