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Prostate Cancer Cell Growth: Stimulatory Role of Neurotensin and Mechanism of Inhibition by Flavonoids as Related to Protein Kinase C

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14. ABSTRACT

We investigated the mechanisms by which flavonoids (FLAV) alter neurotensin receptor (NTR) function in PC3 cells in order to shed light on the role of NT in the negative effects of fat intake on PC incidence and the positive effects of Asian diets. We found that NTR is subject to heterologous regulation by PKC. Conventional PKCs appear to exert negative effects, whereas novel PKCs exert positive effects. Our results suggest that FLAV modulate NTR by a number of mechanisms, one of which could be to alter the balance between conventional and novel PKCs. Other potential mechanisms supported by experiments reported here involve receptor tyrosine kinases, lipoxygenases and cellular metabolism. We also show here that NTR is localized to membrane caveolae, and that the agonist NT caused a decrease in the level of NTR in these signaling modules. β-cyclodextrin, which disrupts caveolae by removing cholesterol from membranes, displaced NTR from caveolae and produced FLAV-like effects on NTR. Although FLAV did not displace NTR from caveolae, this suggested that FLAV might interact with membranes to induce similar conformational changes in NTR that alter its function. These findings have implications regarding mechanisms that regulate NTR function and the design of agents to block NT-induced growth signaling in PC.

15. SUBJECT TERMS
neurotensin, PC3 cells, prostate cancer, growth, PKC, flavonoids, metabolism, caveolae

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4. INTRODUCTION

Neurotensin (NT), an intestinal growth factor, has a potential role in prostate cancer (PC) since NT receptor (NTR) is over-expressed in PC, and since NT stimulates growth of cultured human PC cells. The levels of NTR are dramatically higher in androgen independent cell lines and the effects of NT are more remarkable in androgen dependent LNCaP cells after androgen withdrawal. Fat intake, which correlates with PC incidence around the world, is the strongest stimulus for NT secretion in humans, and NT promotes the conversion of arachidonic acid (AA) into growth-promoting eicosonoids, particularly leukotrienes. Our work in PC3 cells shows that inhibitors of phospholipase A2, DAG lipase and lipoxygenase (LOX) block NT-induced growth signaling, supporting the idea that the effects of NT involve the liberation of AA and its conversion to eicosanoids. Thus, NT and fatty acids could work in concert to mediate fat-induced PC growth. In contrast, a diet rich in antioxidant flavonoids (FLAVs) correlates with decreased PC incidence in humans. One possibility is that FLAVs inhibit the cancer promoting effects of NT. Our work shows that FLAVs inhibit NT growth signaling in PC3 cells, which provides a rationale for the beneficial effects of the Asian and Mediterranean diets. A key goal in this grant is to understand the mechanism(s) by which FLAVs inhibit NT signaling, to identify the most potent FLAVs and to evaluate the overall significance to the inhibition of PC growth *in vivo*. Since FLAVs have been reported to inhibit PKC, LOX and receptor tyrosine kinases (RTKs), the mechanism for inhibition of NT could be quite complex. However it is intriguing that FLAVs, like PKC inhibitors and LOX inhibitors, exert upstream effects on NTR, altering its binding properties in live cells. For example in PC3 cells, FLAVs caused a three fold increase in NTR binding affinity, while inhibiting the ability of NT to stimulate IP production and to induce DNA synthesis. Based on this, we have hypothesized that FLAVs inhibit enzyme(s) that mediate NT effects (e.g., PKC, LOX and RTKs) and that this somehow feeds back to regulate NTR binding. Indeed, we have shown that PKC can regulate NTR function, and we have identified some of the PKC isotypes that are involved. However, our findings indicate that FLAVs do not exert remarkable effects on the endogenous PKC activity in live cells. This led us to investigate the involvement of LOX and RTKs, as well as the possibility that FLAVs disrupt the integrity of caveolar modules where NTR interacts with G proteins and effector molecules to promote signaling. The proper functioning of these signaling modules requires cellular energy to maintain the specific membrane architecture. Indeed, our results demonstrate that the effects of FLAVs on NT binding in live cells can be mimicked by metabolic inhibitors, as well as agents known to diminish PLC, LOX and RTK activity. Defining the mechanism(s) by which FLAVs inhibit NT-stimulated growth could shed light on general aspects of G-protein receptor action, signal transduction and pathway relationships, which could have a widespread impact due to the number of growth stimuli involved.

5. BODY

The work accomplished during this period is presented below according to the revised "Statement of Work" presented in the first year report submitted on January 31, 2007.

Task 3. Identify PKC isotypes that are activated by NT-induced growth signaling.

A. PKC isotype phosphorylation-

To initiate the work on task 3, we first examined the expression of PKC isotypes in the human prostate cancer cell lines PC3 and LNCaP cells. Western blotting was performed with specific antisera from Santa Cruz to detect various PKC isotypes in extracts of PC3 and LNCaP cells. Our results indicated that both cell lines expressed primarily the following PKC isotypes: α, β1, δ and ε; however, PC3 cells expressed relatively more PKCε and LNCaP cells expressed relatively more PKCδ (Fig 1 below). Since PKCε is generally growth enhancing and PKCδ is associated with apoptosis, these differences might account for the more aggressive nature of PC3 cells.

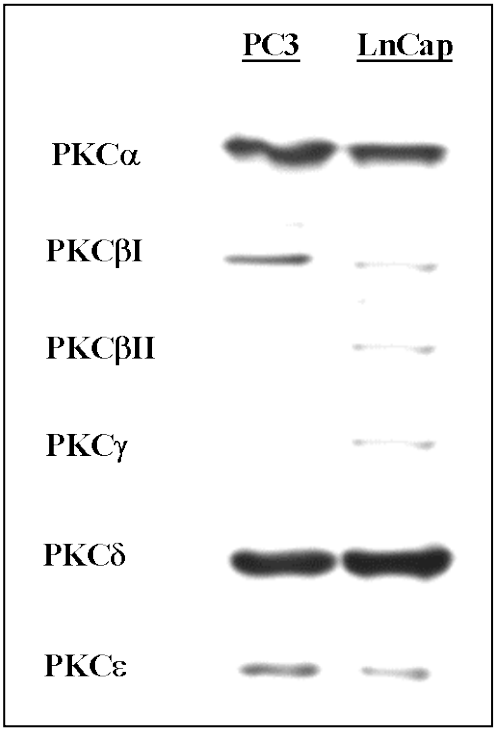


Fig 1. Expression of PKC isotypes in PC3 and LNCaP cells. An equal number of quiescent cells were processed for SDS-PAGE and western blotting using antisera specific for the PKC isotypes indicated.

Fig 2 (below) shows the results for PKC expression in control PC3 cells and for cells stimulated with PMA, which is known to downregulate PKC. Note that PMA downregulated the conventional PKCα and PKCβ1 more effectively than the novel PKCδ and PKCε. This could be important in regards to growth regulation in these cells since PMA is growth stimulatory at low doses (which would not downregulate novel PKCs) and growth inhibitory at high doses (which would downregulate the novel PKCs). In contrast, NT activates PKCs without causing downregulation and thus, it is growth stimulatory at all doses. This led us to hypothesize that growth stimulation by NT and perhaps other inputs might be mediated more by the novel PKCs than by conventional PKCs.

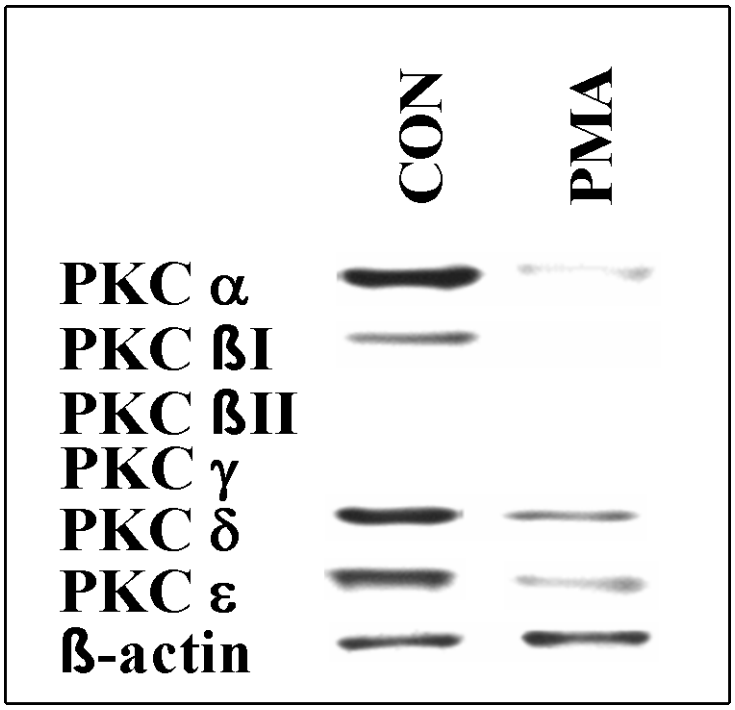


Fig 2. Wesern blot assessing expression of PKC isotypes in control PC3 cells and cells pretreated with PMA. Quiescent cells withdrawn from serum were treated for 24 hrs with 1 μM PMA or vehicle control and cell extracts (40 μg protein) were subjected to SDS-PAGE and western blotting using antisera specific for PKCα, PKCβ1, PKCβ2, PKCγ, PKCδ, and PKCε. β-actin was used as loading control.

Next, we set out to determine the PKC isotypes in PC3 cells that became phosphorylated in response to NT. Using antibodies to the phosphorylated forms of each PKC isotype, we determined the levels of PKC activation for control cells and NT treated cells. Western blotting was performed on cell extracts obtained after 1-30 min treatment. The cells were withdrawn from serum for 24 hrs before the medium was changed and NT was added. Our initial studies indicated that the basal level of activation (constitutive activity) was so high that it was difficult to demonstrate an effect of NT. Subsequent experiments showed that our initial difficulties were due to mechanical effects on the cells. We have

solved this problem by adding NT from a 100 X solution to the quiescent cells, thus avoiding any mechanical disturbance (ie., without changing the medium). Under these conditions, exposure to NT for 5-30 min induced the phosphorylation of PKCα, PKCβ1, PKCδ and PKCε (Fig 3). In addition, we demonstrated that NT caused the phosphorylation of multiple conventional PKC substrates with molecular masses from 30 kDa to 140 kDa (Fig 3). Thus, in PC3 cells, NT activated the conventional PKCs (α, β1) as well as the novel PKCs (δ, ε).

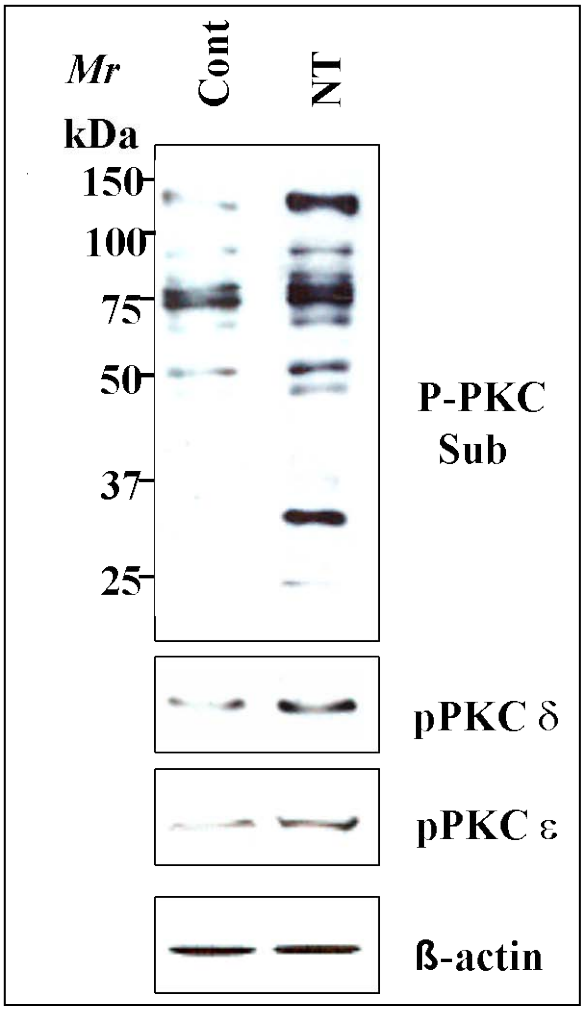


Fig 3. NT stimulates phosphorylation of multiple PKC substrates and activates (phosphorylates) both conventional and novel PKCs in PC3 cells. Quiescent cells, withdrawn from serum for 24 hrs, were stimulated with 10 nM NT for 30 min. Cell extracts were subjected to SDS-PAGE and western blotting. In the upper panel, endogenous PKC substrate phosphorylation was assessed using a phospho-specific antiserum to the motif R/K-X-S-Hyd-R/K. In the lower panels the activation of PKCδ and PKCε was assessed using antisera specific for phosphorylated forms of these PKC isotypes. β-actin was used as the loading control.

B. PKC isotype translocation-

To confirm these results, we then used the translocation assay, which indicates PKC activation by its movement from cytosol to membrane. PC3 cells were treated with NT for 1-30 min as described above and after cell lysis, the membrane and cytosolic fractions were subjected to western blotting using antisera towards the individual PKC isotypes.

Our initial results indicated that the PKCs were often found in the membrane fraction under basal conditions, and thus, it was difficult to show an effect of NT. Again, our studies since then have shown that some of this difficulty was due to mechanical effects on the cells. Stimulating the cells by adding NT without changing the medium reduced the problem and allowed us to demonstrate that NT translocates PKC isotypes in PC3 cells. Translocation studies were performed using a conventional pan PKC antiserum, as well as antisera specific for PKCα, PKCδ and PKCε. As an example, Fig 4 shows results for PKCδ. Although it can be seen that NT stimulated the translocation of PKCδ and this was inhibited by quercetin and resveratrol, the effect was not striking and the results were not as convincing as those obtained with the phosphorylation assays. Therefore, we decided to primarily use the phosphorylation assays to assess PKC activation in response to NT and the various FLAV-like agents.

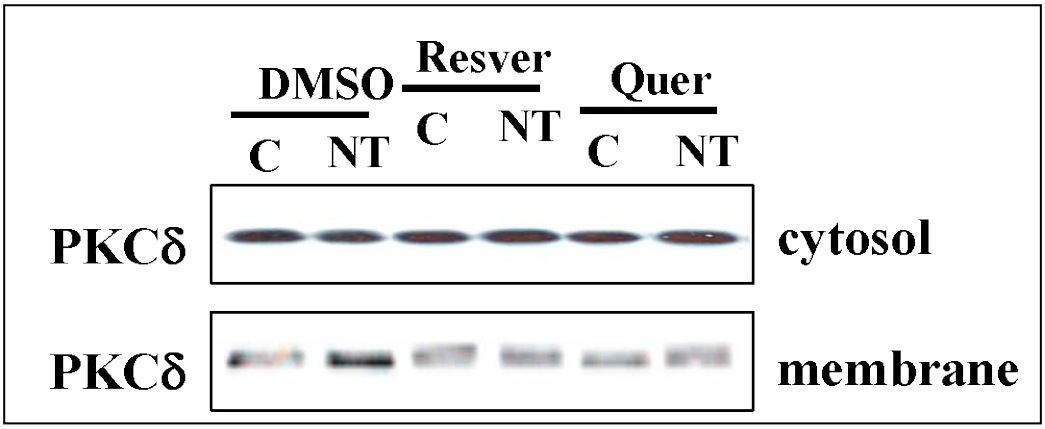


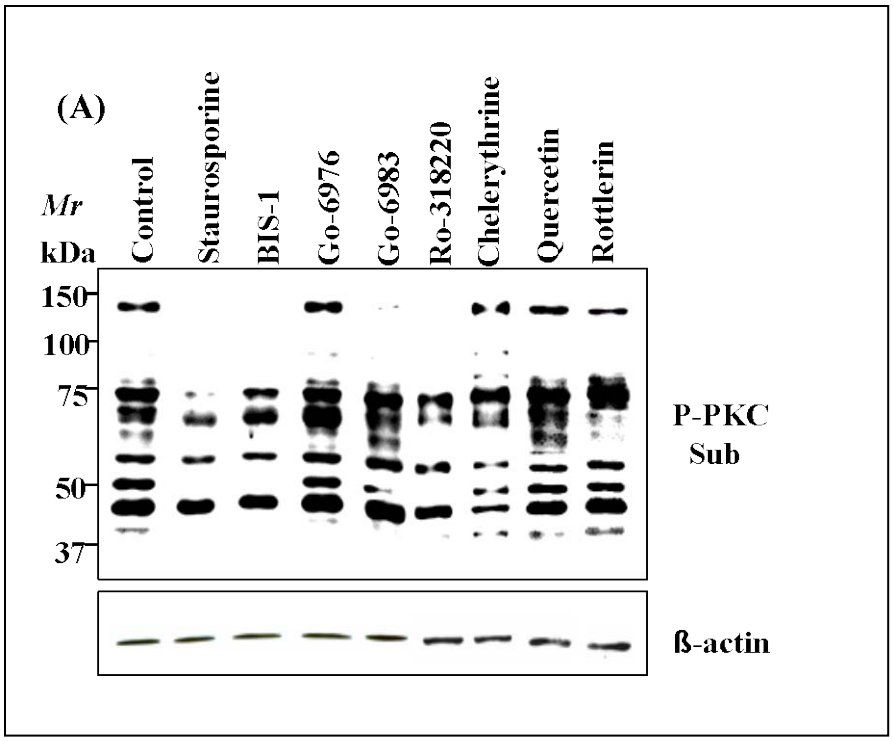
Fig 4. Quiescent cells were treated with 65 μM resveratrol or 35 μM quercetin or vehicle control for 30 min. Then, they were stimulated with 10 nM NT or control for an additional 20 min. Cell homogenates were centrifuged to isolate the cytosolic and membrane fractions, which were subjected to western blotting using an antiserum specific for PKCδ. Note that NT caused the translocation of PKCδ to the membrane fraction.

C. PKC isotype activity-

To assess cellular PKC activity, we choose to measure *in vivo* PKC substrate phosphorylation rather than to employ *ex-vivo* assays that use PKC immunoprecipitates to phosphorylate substrates in a test tube because only the former method could be certain to detect both direct and indirect effects of agents on PKC activity. Thus, the former method would but the later method would not detect any effects on PKC sequestration, substrate availability, cofactor and energy levels that would only exist *in vivo*.

Therefore, to examine the effects of NT and FLAV on cellular PKC activity, we used a western blotting assay to measure *in vivo* PKC substrate phosphorylation. The antiserum (Cell Signaling) was raised towards the phosphorylated PKC consensus motif R/K-X-S-Hyd-R/K. PKC isotype specificity considerations predicted that the assay would primarily detect substrates phosphorylated by conventional PKCs (α, β1, β2, γ) and was likely to respond to novel PKCε substrates and might possibly react (although poorly) with novel PKCδ substrates. Our results indicated that PC3 cells exhibited constitutive PKC activity and that NT caused a time-dependent (peak, 30 min) enhancement of PKC substrate phosphorylation (Fig 3 above).

The constitutive PKC substrate phosphorylation was inhibited differentially by a number of specific PKC inhibitors (Fig 5A and 5C), as was the PMA-induced PKC substrate phosphorylation (Fig 5B and 5D). Interestingly the inhibitory pattern for quercetin differed from those seen for other PKC inhibitors (best viewed in Fig 5C). Note that although the inhibitory effects of quercetin were not remarkable, quercetin appeared to specifically inhibit the phosphorylation of substrates with molecular masses of 60-85 kDa, without much effect on the 50 kDa band (Fig 5C). A similar pattern of inhibition was displayed by the specific PKCδ inhibitor rottlerin (Fig 5C), suggesting that quercetin specifically inhibited this novel PKC. In contrast, the specific inhibitor of conventional PKCs (Go-6976) had little effect on the 60-85 kDa substrates but instead very effectively inhibited the phosphorylation of the 50 kDa band (Fig 5C). These results supported the idea that quercetin was a specific inhibitor of novel PKCs (PKCδ and PKCε), and that this might explain the ability of quercetin to modulate NTR function.



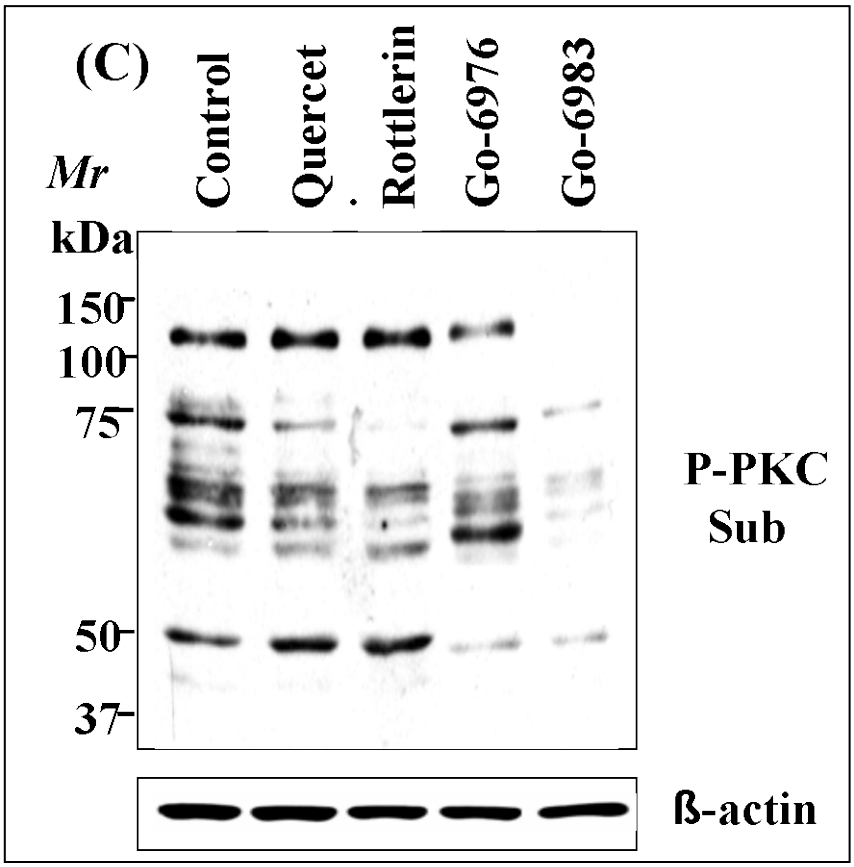
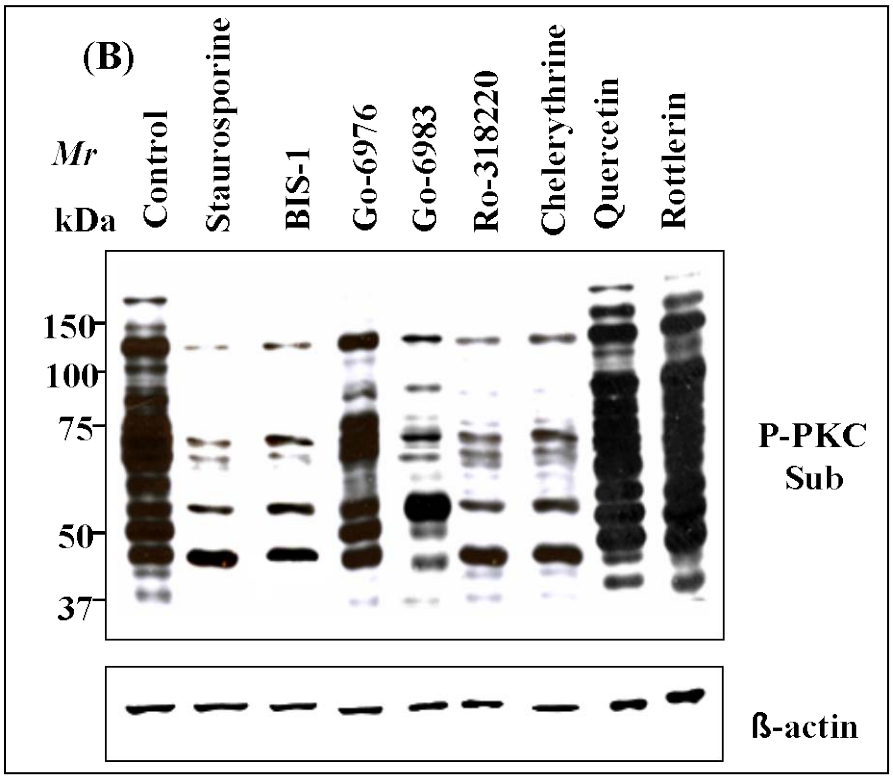


Fig 5A and 5C. Constitutive PKC activity was inhibited by BIS-1 and quercetin. Quiescent cells were incubated with inhibitors shown for 30 min and PKC substrate activity was determined by western blotting. In A, 15 μM quercetin was used. In C, 40 μM quercetin was used. Note that quercetin reduced the intensity of some of the bands (mostly those from 60-85 kDa). Contrasting the pattern for quercetin with that for Go-6976 suggested that quercetin inhibited novel PKCs rather than conventional PKCs.



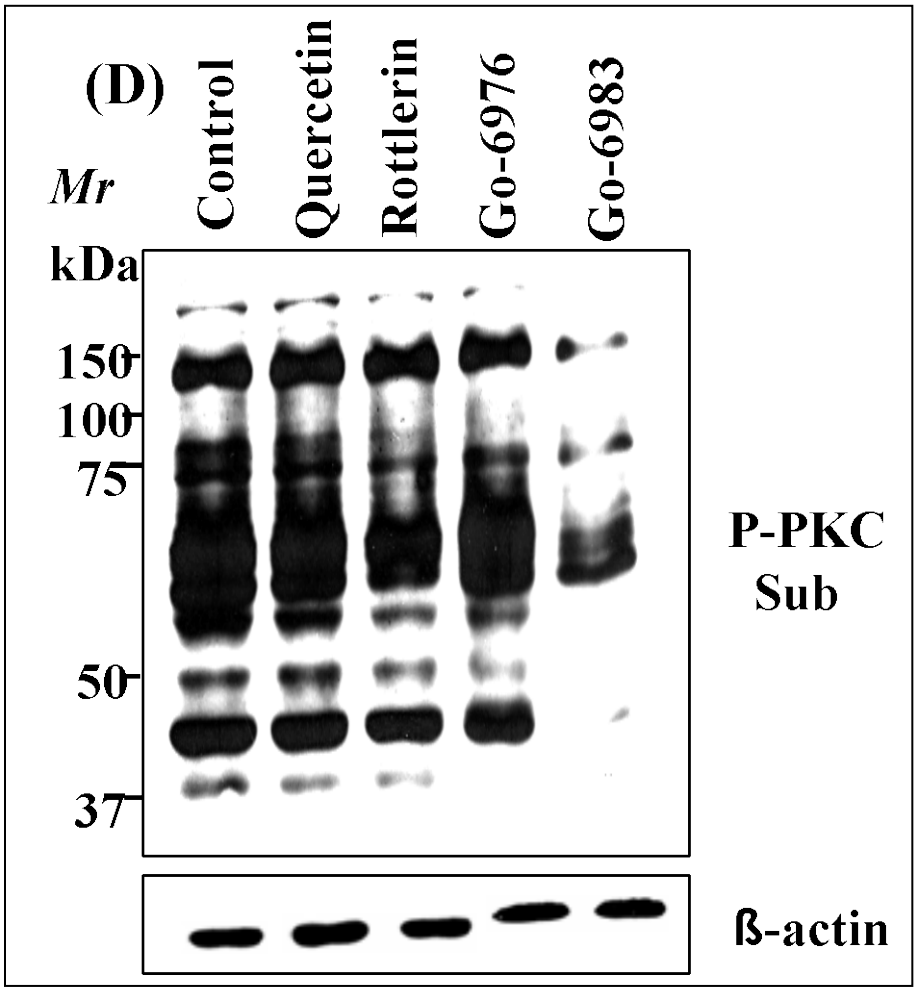


Fig 5B and 5D. PMA-induced PKC substrate activity was reduced by BIS-1 and quercetin. Cells were stimulated with 100 nM PMA for 30 min. In B, 15 μM quercetin and in C, 40 μM quercetin was used.

Task 4. Identify PKC isotypes that are required for NT-induced growth signaling.

A. Antisense Work-

PKC specific antisense oligodeoxynucleotide (ODN) sequences (20 mers) that were specific for PKCα, PKCδ and PKCε were obtained from Invitrogen. PC3 cells in Opti-MEM medium were exposed to lipofectamine (5μg/ml) mixed with the ODNs (1μM) for 3 days. Cell extracts were used to perform western blotting to quantitate changes in the expression of the individual PKC isotypes. The results indicated that PC3 cells were resistant to PKC knockdown by these methods. These negative results led us to investigate the usefulness of RNAi to perform these experiments.

B. RNAi work-

Plasmid DNAs containing shRNA sequences complementary to specific PKC isoforms were obtained from the UMass RNAi Core Facility and Open Biosystems. The sequences included: two silencing sequences for PKCα, two for PKCβ, two for PKCδ and one for PKCε, as well as a non-silencing control. The shRNAs were cloned in pSM2c vector under the control of the CMV promoter for expression in mammalian cells. A puromycin resistance gene was also present to facilitate the selection of clone pools.

Transient transfection was performed using FuGene (Roche) as recommended by the manufacturer. Basically, 5 x 10^4 cells/ml were plated per well of 12-well plates so that 40% confluence was obtained after 24 hrs. These were then transfected using various concentrations of FuGene (0.75-6 μl/ml) and of DNA (0.25-1.0 μg/ml). After 48 hrs, cell extracts were examined by western blotting to assess the effects on PKC expression. FuGene was toxic above 1.5 μl/ml. However, using this amount of FuGene with 0.5 μg DNA gave results consistent with specific knockdown (about 40%) for PKCδ and PKCε). We then performed experiments to test the effects of PKC knockdown on NT binding to the cells. Using our optimal conditions, cells were transfected with the non-silencing control and the silencing constructs, and NT binding was tested after 48 hrs. Basal binding was measured as well as binding in the presence of conventional and novel PKC inhibitors (1-2μM). Although some preliminary findings were obtained that were encouraging (as reported last year), the efficiency of the knockdown with transient transfection was not sufficient to produce reproducible and statistically significant results.

Therefore, we decided to select clone pools based on puromycin resistance. Several clones were obtained that displayed >50% knockdown of PKCδ and PKCε expression (Fig 6). Based on results from

western blotting, one clone from each treatment was selected for further analysis: nonsense (NS clone); PKCδ(-) clone; and PKCε(-) clone.

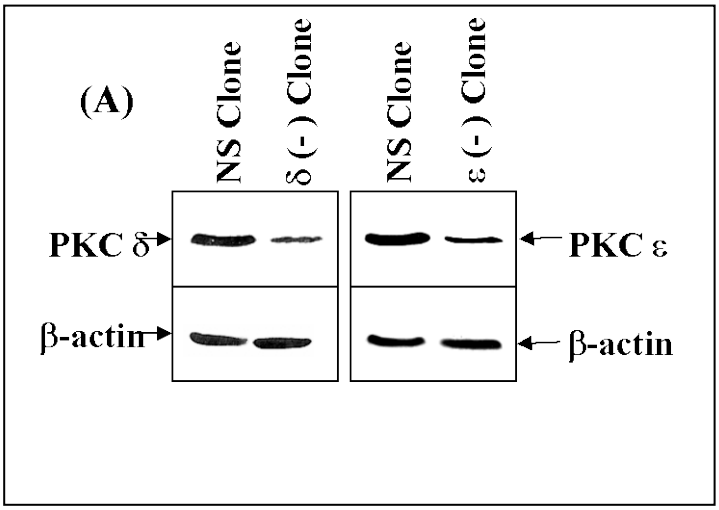


Fig 6. Expression of PKCδ and PKCε in the selected knockdown clones of PC3 cells. An equal number of cells was extracted and 40 ug of protein was subjected SDS-PAGE and western blotting with specific antisera was performed to assess PKC expression. β-actin was used as the loading control.

To determine the importance of PKCδ and PKCε in NTR function, we first measured basal NT binding in the clones when the cells were ≅90% confluent. Basal binding expressed as cpm/μg protein (mean±SEM, n=4) did not differ for the 3 clones (NS clone, 18.0±1.4; PKCδ(-) clone, 15.8±1.2; and PKCε(-) clone, 15.4±1.1). Then we assessed the effects of BIS-1 and rottlerin on cellular NT binding in these clones, hypothesizing that the enhancement of NT binding by these inhibitors might be reduced in clones that lacked the relevant PKC isotype. The results in Table 1A show that the responses in the different clones did not differ significantly. These results indicated that PKCδ knockdown or PKCε knockdown by itself was not sufficient to alter basal NT binding or the effects of BIS-1 and rottlerin on NT binding. One possible explanation was that knockdown of both PKCδ and PKCε (and perhaps PKCα and PKCβI) was necessary to reproduce the effects of these inhibitors on NT binding.

Table 1A

Effect of PKC Inhibitors on NTR Binding in PKC Knockdown and Control Clones of PC3 Cells

Cell Clone	NT Binding (% DMSO control)			
	BIS-1		Rottlerin	
	0.5μM	2.5μM	0.5μM	2.5μM
NS control	155±14	216±20	150±16	181±17
PKCδ (-)	168±13	254±17	145±13	222±18
NS control	140±13	225±19	140±14	236±19
PKCε (-)	167±12	268±21	177±15	244±18

NTR binding to each PC3 cell clone was measured in the presence of BIS-1 or rottlerin at the indicated concentrations as compared to the vehicle (DMSO) control. The cells (80-90% confluent) were preincubated 15 min with the agents in Locke prior to the binding reaction. Control NT binding for each clone was similar when expressed as cpm/μg protein: NS control, 18.0±1.4; PKCδ (-); 15.8±1.2; PKCε (-), 15.5±1.1 (n=4). For each clone, the effects of BIS-1 and rottlerin were expressed as % control NT binding (mean±SEM; n=3 experiments). The results for the different clones were not significantly different.

To further address the importance of PKC isotypes in NTR signaling, we next examined the ability of NT to stimulate inositol phosphate (IP) formation in the various PKC knockdown clones. We found that NT was less effective in elevating IP formation in the PKCδ(-) and the PKCε(-) clones as compared to the NS clone (Fig 7). This was not due to a general effect on PLC activity or the level of phospholipid substrate since the IP response to 3nM BOM was not inhibited in the PKCδ(-) clone (% control response, 95±2; n=6) and was enhanced in the PKCε(-) clone (% control response, 148±13; n=6). These results suggested that PKCδ and PKCε were necessary for NT to maximally stimulate PLC to enhance IP metabolism.

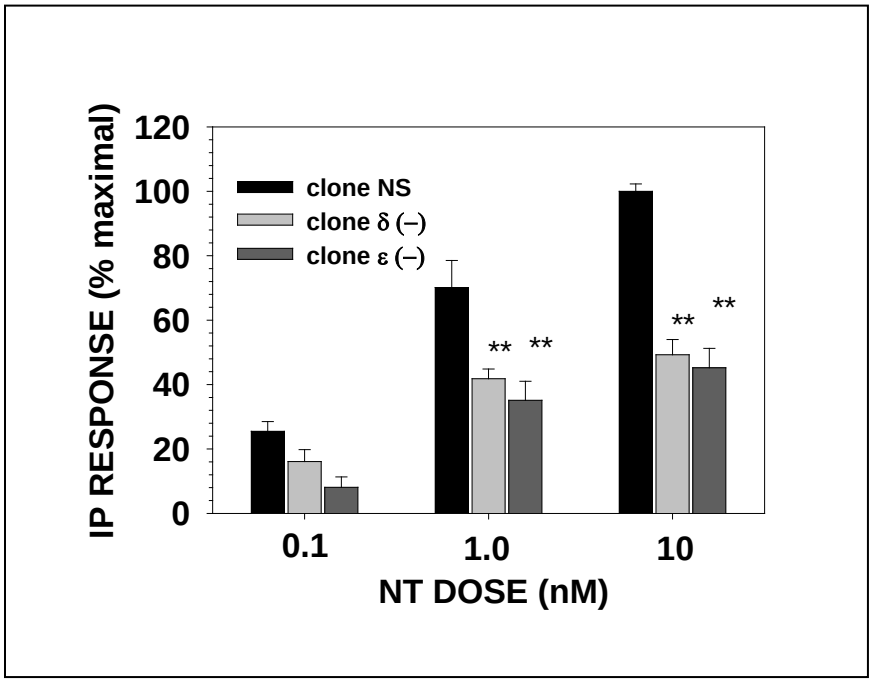


Fig 7. Effect of PKC knockdown on NT-induced IP formation. Equal numbers of cells were stimulated with the indicated doses of NT. IP formation was measured and calculated as % maximal.

These results show that knockdown of either PKCδ or PKCε was sufficient to diminish the IP response to NT. Consistent with the importance of both PKCδ and PKCε in this regulation, treatment with PKC inhibitor BIS-1 further inhibited the NT response and further enhanced the BOM response, even in the PKCδ(-) and PKCε(-) clones (Fig 8). These results were in keeping with the hypothesis that both PKCδ and PKCε activity were required to maintain the ability of NTR to stimulate PLC.

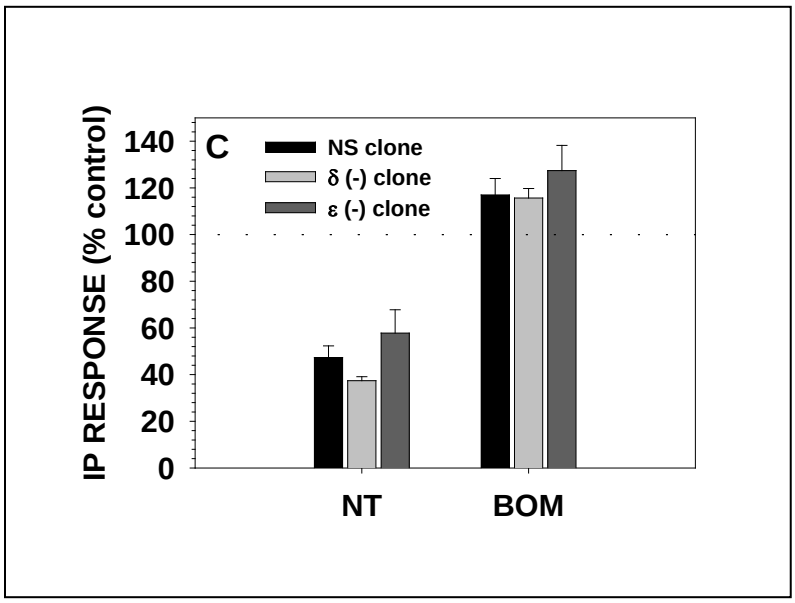


Fig 8. BIS-1 inhibited the effect of NT and enhanced the effect of BOM on IP formation even in the PKCδ(-) and PKCε(-) clones. An equal number of cells, pretreated with 2.5 μM BIS-1 or vehicle control, were stimulated with 10 nM NT or 3 nM BOM. The IP responses were calculated as % control for each individual clone. In the NS clone, IP formation was elevated about 4-fold by NT and about 10-fold by BOM. In the PKCδ(-) and PKCε(-) clones, the control response to NT was about 50% less than in the NS clone, but this response was further inhibited by BIS-1, showing that both PKCδ and PKCε participated in this regulation.

Task 5. Determine effects of FLAV on activation and expression of PKC isotypes.

A- Effects of FLAV on PKC activation by NT.

Next we assessed the effect of FLAV on NT-induced *in vivo* PKC substrate phosphorylation using the western blotting method previously described. NT enhanced PKC substrate phosphorylation, increasing the intensity of bands in the size range of 40-140 kDa (Fig 9). The effects of NT were inhibited by quercetin and BIS-1 (Fig 9). Note that the effects of quercetin again appeared to be selective for PKC substrates in the size range of 60-85 kDa. This led us to hypothesize that these substrates were primarily PKCδ and PKCε substrates. We also showed that the response to NT involved the activation (phosphorylation) of PKCα, PKCβ1, PKCδ and PKCε. Fig 9 shows the activation of PKCδ and PKCε by NT, and also the fact that quercetin and BIS-1 inhibited both the basal and NT-induced phosphorylation of PKCδ and PKCε.

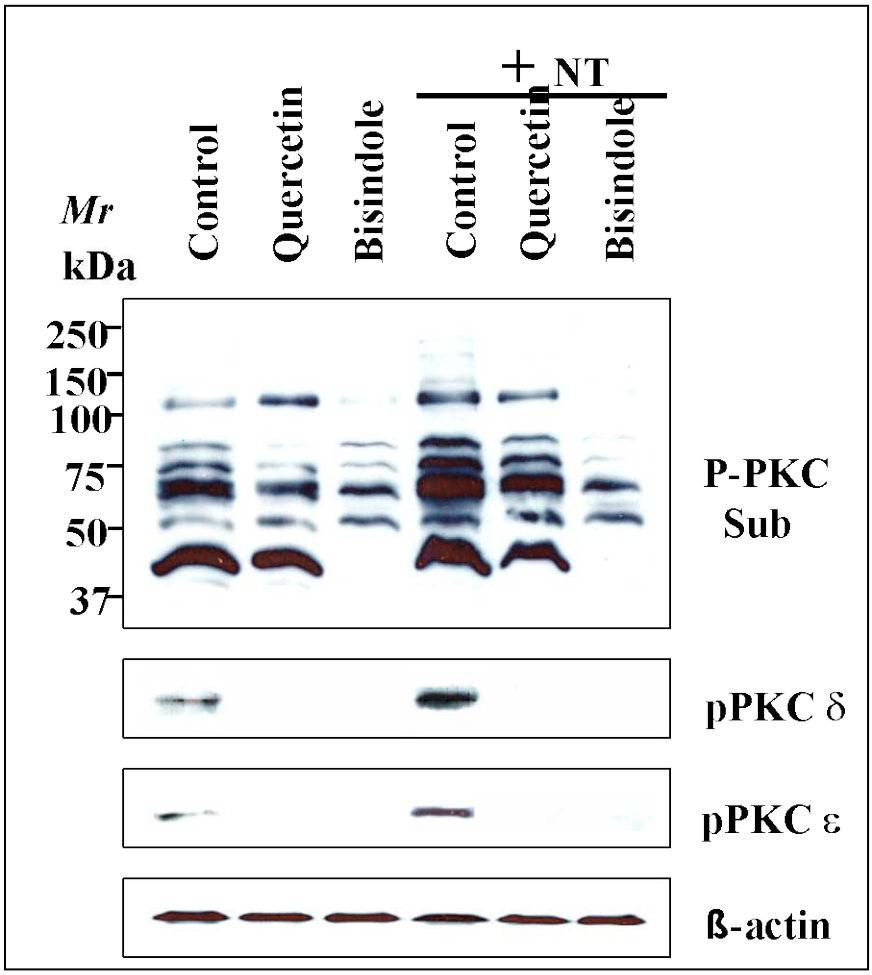


Fig 9. Effect of quercetin and BIS-1 on control and NT-induced PKC substrate phosphorylation and on the activity level (phosphorylation) of PKCδ and PKCε. Quiescent cells were pretreated with 35μM quercetin or 7 μM BIS-1 or vehicle control, and then stimulated with 30 nM NT for 30 min. Cell extracts were processed for western blotting using phospho-specific antisera towards PKC substrates and PKC isotypes. β-actin was used as loading control.

B- Effects of FLAV on PKC Expression in PC3 cells.

Quercetin and BIS-1 induced a dramatic downregulation of novel PKCε and PKCδ, whereas the conventional PKCα was upregulated (Fig 10). Again, this supported our hypothesis that quercetin exerted specific effects on the novel PKCδ and PKCε.

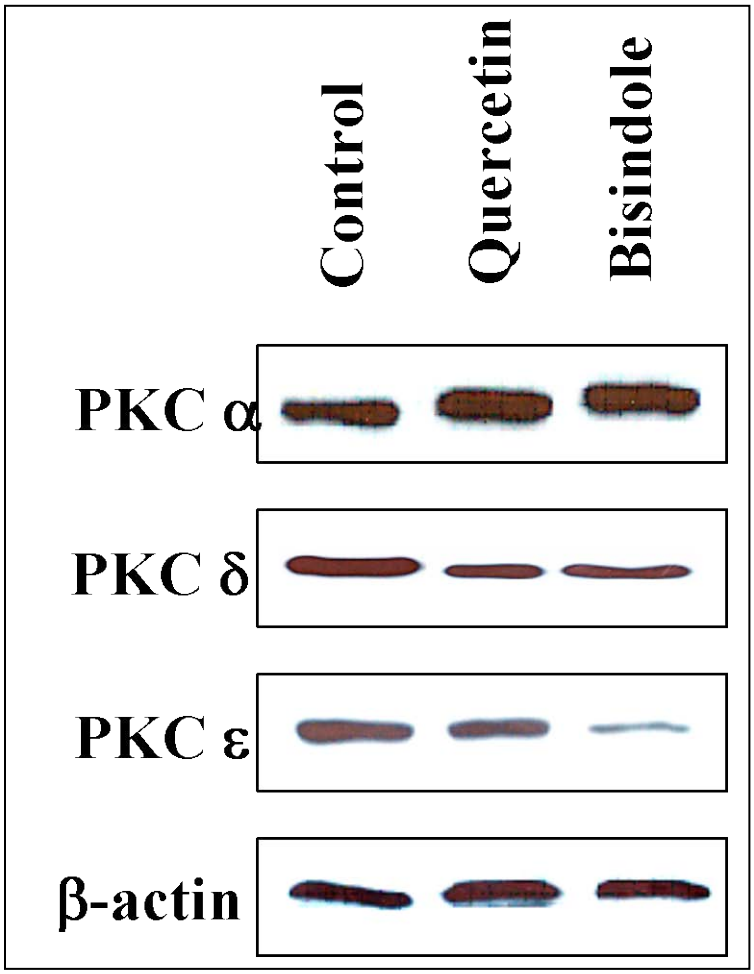


Fig 10. Quiescent cells were incubated with 35 μM quercetin or 7μM BIS-1 or vehicle control for 20 hrs and then cell extracts were subjected to SDS-PAGE and western blotting using antisera specific for PKCα, PKCδ and PKCε. β-actin was used as the loading control.

The time-course of the effect of quercetin and BIS-1 on PKCε expression is shown in Fig 11. Both of these agents downregulated PKCε time-dependently and this downregulation was noticeable after 30 min and very significant after 4 hrs exposure to 7 μM BIS-1 or 100 μM quercetin (high dose), while the low dose of quercetin (35 μM) took a longer time to develop. We hypothesize that the inhibition and downregulation of PKCδ and PKCε by quercetin and BIS-1 relate to their effects on NTR function. Although these agents alter NTR function within 15-30 min, which is well before downregulation was seen, it could be that these PKCs are sequestered as they enter the downregulation pathway and that this is sufficient to inhibit NTR function. In other words, the rapid effects of quercetin and BIS-1 on NTR function could be an early reflection of the sequestration of PKCδ and PKCε (perhaps within vesicles) as they move along the degradative pathway towards lysosomes.

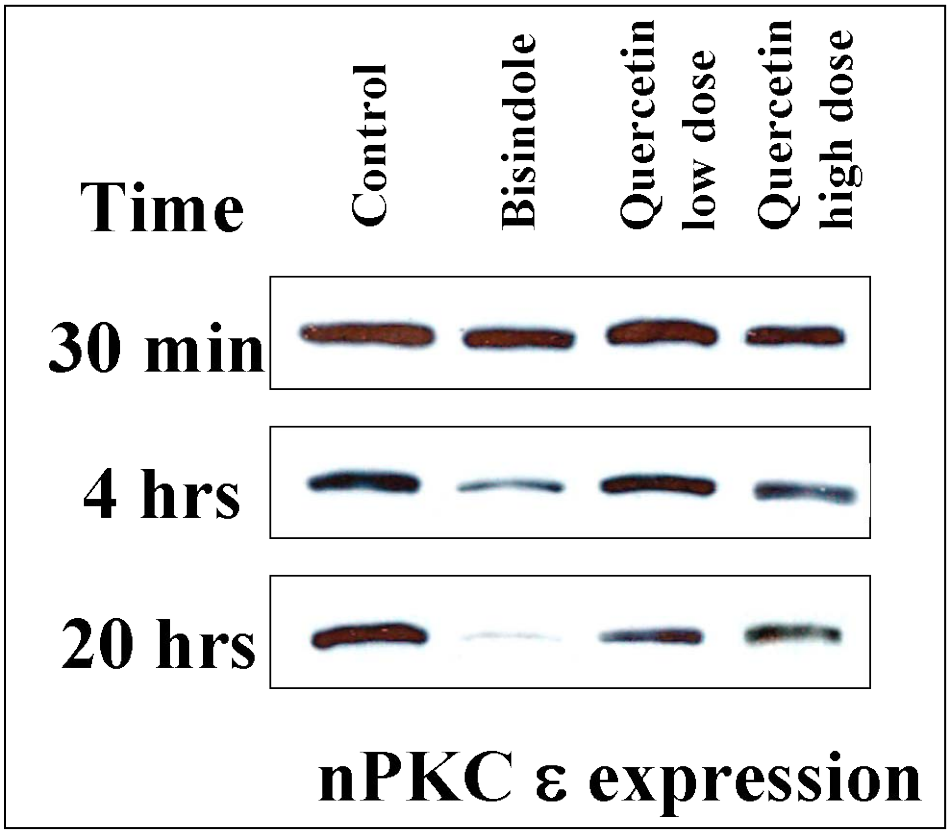


Fig 11. Time course for effects of quercetin and BIS-1 on PKCε expression. Quiescent PC3 cells were incubated with 7 μM BIS-1 or 35 μM quercetin (low dose) or 100 μM quercetin (high dose) or vehicle control for the times indicated. Cell extracts were subjected to western blotting using an antiserum specific for nPKCε. β-actin was used as the loading control.

C. Structure-Function Studies on the Effects of FLAVs on NTR Function.

As noted above, our work shows that FLAVs are not very effective PKC inhibitors *in vivo* using the PC3 cell system, although the literature indicates that they are potent inhibitors using *in vitro* assays. Part of this might be attributed to PKC isotype selectivity, as suggested by the results in Fig 9 where quercetin inhibited the phosphorylation of only a subset of the substrates. Nevertheless, we decided to pursue the hypothesis that PKC was not the only relevant target in regards to the effects of FLAVs on NTR function. Since FLAVs are also known to inhibit receptor tyrosine kinases (RTK), LOX and PI3 kinase, these were other possible targets to explore. To investigate the molecular specificity of FLAV actions, workers have compared the effects of various FLAVs on the activity of PKC, RTK, PI3 kinase and LOX in a number of systems. These studies have shown that the positioning of the hydroxyl groups in the FLAV structure strongly influences the inhibitory effects and that the results differ somewhat for these different enzymes (Ferriola et al, 1989; Agullo et al, 1997; Huang et al, 1999; Nomura et al, 2008; Sadik et al, 2003). These findings led us to characterize the hydroxyl group dependence for the effect of FLAVs on NT binding in PC3 cells, thinking that this information might help us to determine which categories of enzymes are likely to be involved in the effects on NTR.

The chemical structures of the various FLAVs investigated are depicted in Fig 1 and Table 1. The basic structure consists of two benzene rings (rings A & B) linked through a heterocyclic pyran or pyrone (ring C). Ring C is in the middle of rings A and B (Fig 12). Ring B is usually attached at C2 of ring C. The different categories are defined by the presence of various substituents, most important being the oxy group (ketone) at C4 (ring C), the C2-C3 double bond (ring C) and an OH group at C3 (ring C). Note that the C2-C3 double bond greatly increases the aromaticity, since it conjugates the aromatic double bonds in ring A and ring B through the central ketone. Flavones, isoflavones, flavonols, and chalcones have the C2-C3 double bond, whereas this structure is missing in the flavanones and catechins. Flavonols have an OH group at C3, whereas isoflavones have ring B attached at this position. Note that isoflavones will be less aromatic than flavones and flavonols, due to the loss of resonance structures involved in shifting ring B from position 2 to position 3. Ring C is not closed in chalcones, which lack the oxygen (ether) at C1. The C ring is also missing in stilbenes but they do have a central double bond that is analagous to the C2-C3 double bond of the flavones (Fig 12).

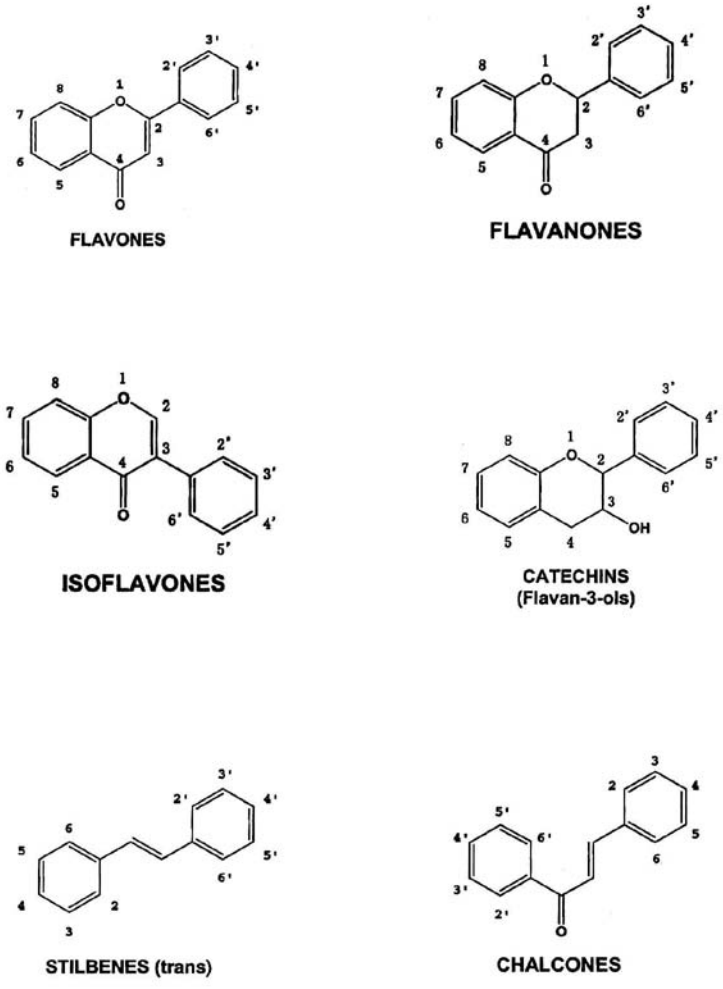


Figure 12- Chemical Structures of FLAVs and Polyphenols

Table 1- EC50 for the Effect of Specific Categories of Polyphenols on NT Receptor Binding

	Ring Position Number							# OH * Groups	EC50 ** (μM)
	3	5	6	7	3'	4'	5'		
FLAVONES									
3-OH flavone	OH							1	58
5-OH flavone		OH						1	60
7-OH flavone				OH				1	62
chrysin		OH		OH				2	35
apigenin		OH		OH		OH		3	24
luteolin		OH		OH	OH	OH		4	23
baicalein		OH	OH	OH				3	55
ISOFLAVONES									
Genistein		OH		OH		OH		3	47
Daidzein				OH		OH		2	>100
FLAVONOLS									
galangin	OH	OH		OH				3	16
fiscetin	OH			OH	OH	OH		4	28
kaempferol	OH	OH		OH		OH		4	16
quercetin	OH	OH		OH	OH	OH		5	28
myricetin	OH	OH		OH	OH	OH	OH	6	45
FLAVANONES									
naringenin		OH		OH		OH		3	>200
taxifolin	OH	OH		OH	OH	OH		5	>200
CATECHINS									
catechin	OH	OH		OH	OH	OH		5	>200
ECGC	galate	OH		OH	OH	OH	OH	5	>100
STILBENES									
cis-stilbene								0	>200
trans-stilbene								0	>100
resveratrol	OH	OH				OH		3	65
diethylstilbestrol	4-OH				OH			2	12
CHALCONES									
Phloretin	4-OH			2'-OH	4'-OH	6'-OH		4	30

* Total number of OH groups present in the molecule.
** EC50 for the change in NT binding to intact PC3 cells at 37 C for 30 min.

Using our standard procedure, we measured the effects of multiple doses of each FLAV on NT binding to intact cells during 30 min at 37° C, and the average EC50 determined in 4 experiments is given in Table 1. Comparing the results for the stilbenes, it can be seen that stilbenes lacking OH groups on the benzene rings are unable to alter NT binding whereas resveratrol and diethylstilbestrol are quite active. Comparing the results for the flavones, it can be seen that the potency for the effect on NT binding increases with the total number of OH groups (peaking at 4). This is also true for the flavonols where potency peaks at 4 total OH groups and then decreases for 5 and 6 total OH groups. This trend is

likely due to the negative effect of increased polarity on penetration into the cells. The essential nature of the C2-C3 double bond is clear from the inactivity of all of the flavanones and catechins. This is best seen by comparing naringenin to apigenin and by comparing taxifolin to quercetin. The importance of the OH group at C3 can be seen by comparing galangin to chrysin and by comparing kampferol to apigenin, each of which shows enhanced potency with addition of the C3 OH. By comparing results for apigenin and phloretin, one concludes that the ether group that closes the middle ring is not essential for the effect on NT binding.

By comparing our results for the effects of FLAVs on NT binding to published results for effects on the enzymes in question, it appears that our results are most similar to those for RTKs. For example, the order of potency for the NT system was nearly the same as that for inhibition of RTK activity (luteolin > quercetin > genistein > myricetin >> taxifolin and catechin). Both responses showed an absolute requirement for the C2-C3 double bond. On the other hand, for the inhibition of PI3 kinase, myricetin was the most potent and galangin was the least potent of the flavonols, which is opposite to our results for the effect on NT binding. Similarly for PKC inhibition, myricetin, quercetin and luteolin were more effective than apigenin and kaempferol, whereas the opposite was obtained for the effect on NT binding. Although the potency order for the effects on NTR was also similar to that for LOX inhibition (Sadik et al, 2003), the fit was less impressive than for RTKs. Also consistent with an involvement of RTKs in the regulation of the NTR by FLAVs was the ineffectiveness of daidzein, which is the standard inactive control for the RTK inhibitor genistein. In total, these results suggested that the effects of FLAVs on NTR function in PC3 cells could involve an inhibition of RTK activity in the cells.

D. Effect of FLAVs on RTK Activity in PC3 Cells

To further test this idea, we next assessed the ability of a number of FLAVs to inhibit RTK initiated activation of the MAP kinase pathway in PC3 cells. Cells were pretreated 30 min with FLAVs and then stimulated for 15 min with 1% human serum plus 10 nM EGF. Cell extracts were processed for western blotting to determine the levels of activated ERK. The results in Fig 13 show that FLAVs inhibited this RTK-mediated response. As was seen previously for the effects on NTR binding (Table 1), luteolin was more effective than quercetin and resveratrol in inhibiting this RTK response.

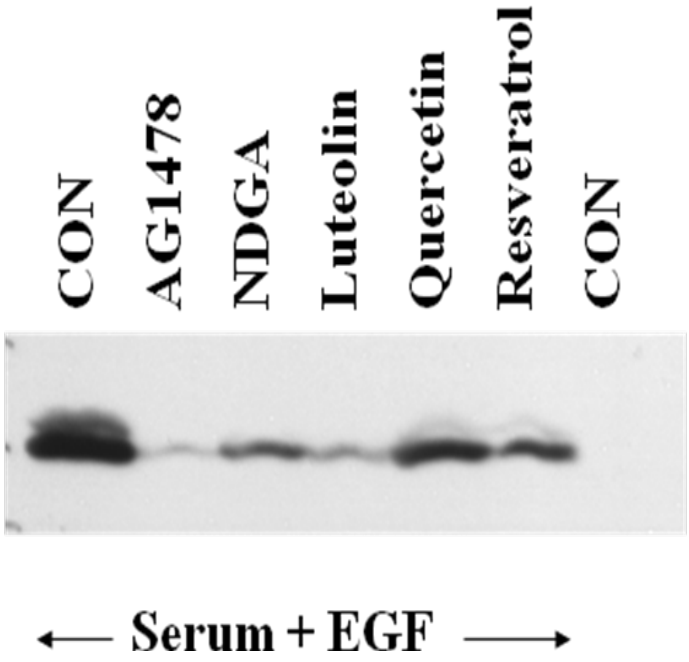


Figure 13 – FLAVs inhibit RTK – mediated MAP kinase activation by serum and EGF. Quiescent cells were pre treated with the agents (30 uM) or the DMSO control for 30 min and then stimulated for 15 min. Cell extracts were subjected to western blotting for phosphorylated ERK.

Since RTK activity is generally opposed by the action of tyrosine phosphatases (Tyr P'ase), our idea that RTKs regulated the NTR could also be tested by targeting Tyr P'ase. These enzymes possess viscinal sulfhydryl groups that are highly sensitive to certain heavy metals that bind to them avidly. The most active and well-studied inhibitors of Tyr P'tase are peroxovanadate (Posneer et al, 1994) and zinc (Brautigan et al, 1981). To prove that these Tyr P'tase inhibitors would enhance RTK activity in PC3 cells, we assessed the effects of peroxovanadate and zinc on MAPK phosphorylation, an indicator of RTK activity in these cells. Fig 14 and Fig 15 show that the expected results were obtained. Peroxovanadate (Fig 14) enhanced the endogenous RTK activity, and this response was blocked by the antioxidant N-acetyl cysteine (NAc) and the viscinal sulfhydryl agent 2,3-dimercapto pyridine (MP). Zinc pyrithione (Fig 15) gave precisely the same effect. Pyrithione was used since this ionophore facilitates the entry of zinc ion into the cells. Again this response was blocked by NAc and MP, which is in keeping with the mechanism involving viscinal sulfhydryl groups.

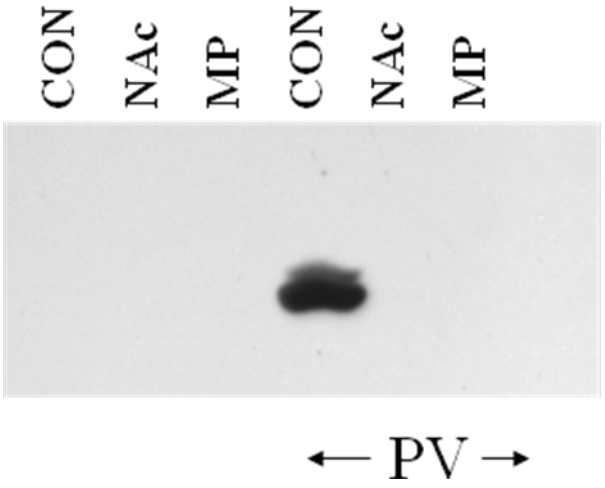


Figure 14 – Peroxovanadate (P'Tase inhibitor) stimulates RTK – mediated activation of MAP kinase, which is inhibited by antioxidant N-acetyl cysteine (NAc) and viscinal SH-agent 2,3 –dimercapto-pyridine (MP).

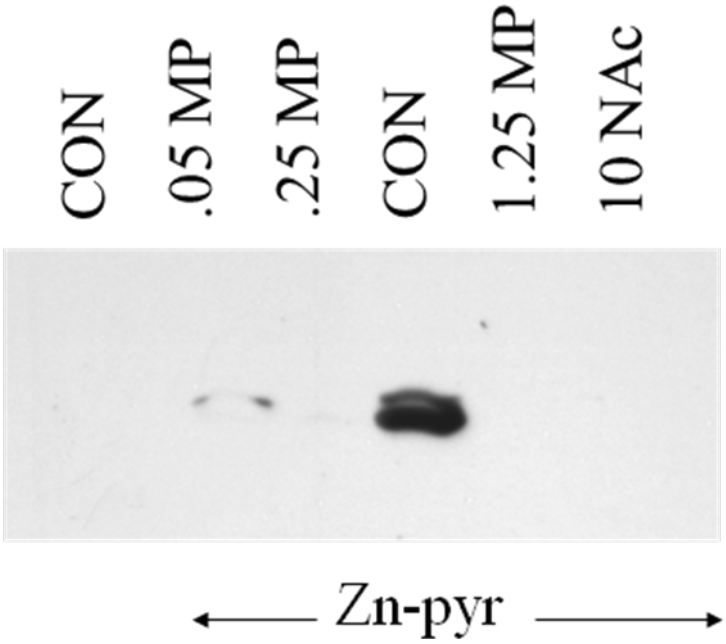


Figure 15 – Zinc - pyrithione (P'Tase inhibitor) stimulates RTK – mediated activation of MAP kinase, which is inhibited by 10 mM NAc and MP at 0.05 – 1.25 mM concentrations.

To test our hypothesis that Tyr P'tase could regulate the NTR, we next assessed the effect of peroxovanadate on NT binding, the expectation being that by inhibiting Tyr P'tase, peroxovanadate would enhance RTK activity, thereby inhibiting NT binding. Other workers have shown for the classical Tyr kinase EGFR that peroxovanadate is far more effective in enhancing kinase activity than vanadate or peroxide. Therefore, we assessed the effects of these 3 agents on NT binding, and the results in Fig 16 show that peroxovanadate was far more potent than vanadate or peroxide in diminishing NT binding.

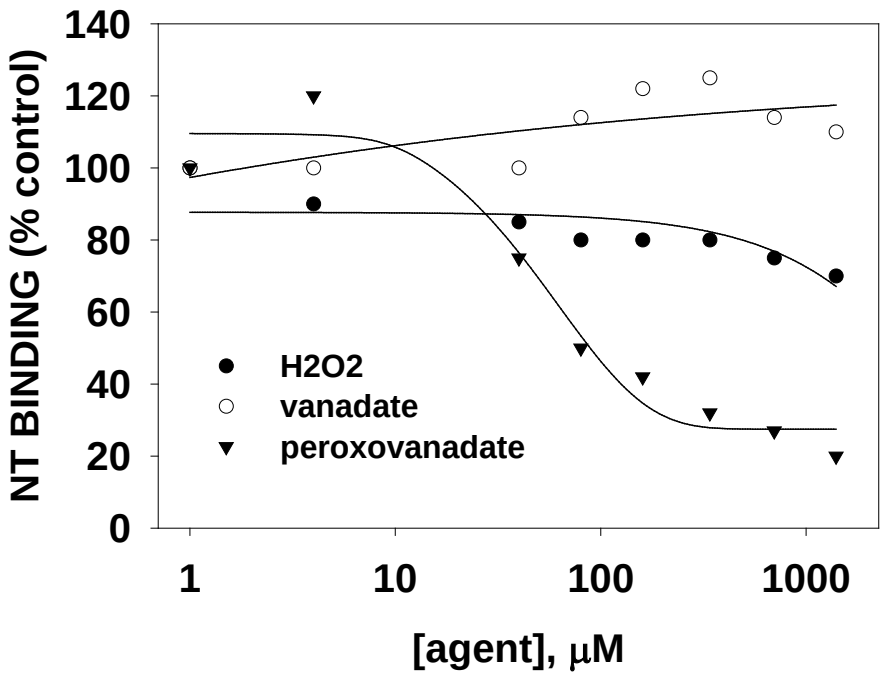


Figure 16 – Effect of peroxovanadate, vanadate and peroxide on NT binding to intact PC3 cells. Cells were pretreated with the agents for 10 min and binding was performed at 37 C for 30 min. Note that peroxovanadate was far more active than the other compounds in inhibiting NT binding. Results are representative of at least 3 experiments.

To further test our idea that Tyr P'tase could regulate NT binding, we examined the effects of zinc ion, the expectation being that zinc would enhance RTK activity, thereby inhibiting NT binding. Fig 17 shows that the expected results were obtained. NT binding to intact PC3 cells was inhibited progressively by the addition of zinc sulfate to the medium, especially at levels just above 10μM, the physiologic extracellular zinc concentration. However, addition of the ionophore pyrithione to facilitate entry of zinc ion into the cells dramatically potentiated the effect. These results indicate that intracellular levels of zinc ion can greatly inhibit NT binding to the cell surface, most likely by altering Tyr P'tase activity.

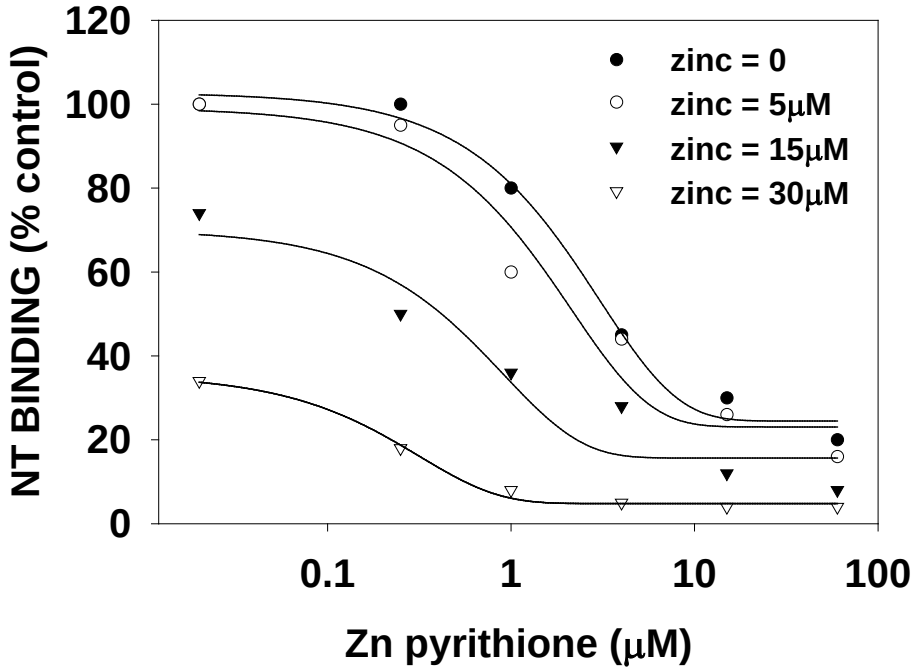


Figure 17 – Effect of zinc ion and zinc pyrithione on NT binding to intact PC3 cells. NT binding was progressively decreased as zinc levels were raised above the physiologic level of 10 μM. The effect of the zinc ionophore pyrithione was to greatly potentiate the inhibition by zinc ion. Results are representative of at least 5 experiments.

To further test the involvement of Tyr P'tase in the regulation of NT binding, we assessed the effects of zinc chelators on NT binding in intact PC3 cells. Zinc chelators would be expected to have the opposite effect as the zinc ionophore since chelators would reduce intracellular zinc levels, thereby increasing Tyr P'tase activity. Fig 18 shows that the expected results were obtained. Each of the zinc chelators tested were found to increase NT binding, and the order of potency was the same as that for their zinc affinity (TPEN > phenanthroline > bipyridyl). These results were in keeping with the idea that the effects of FLAVs on NTR function might be partly attributed to their ability to inhibit RTK activity.

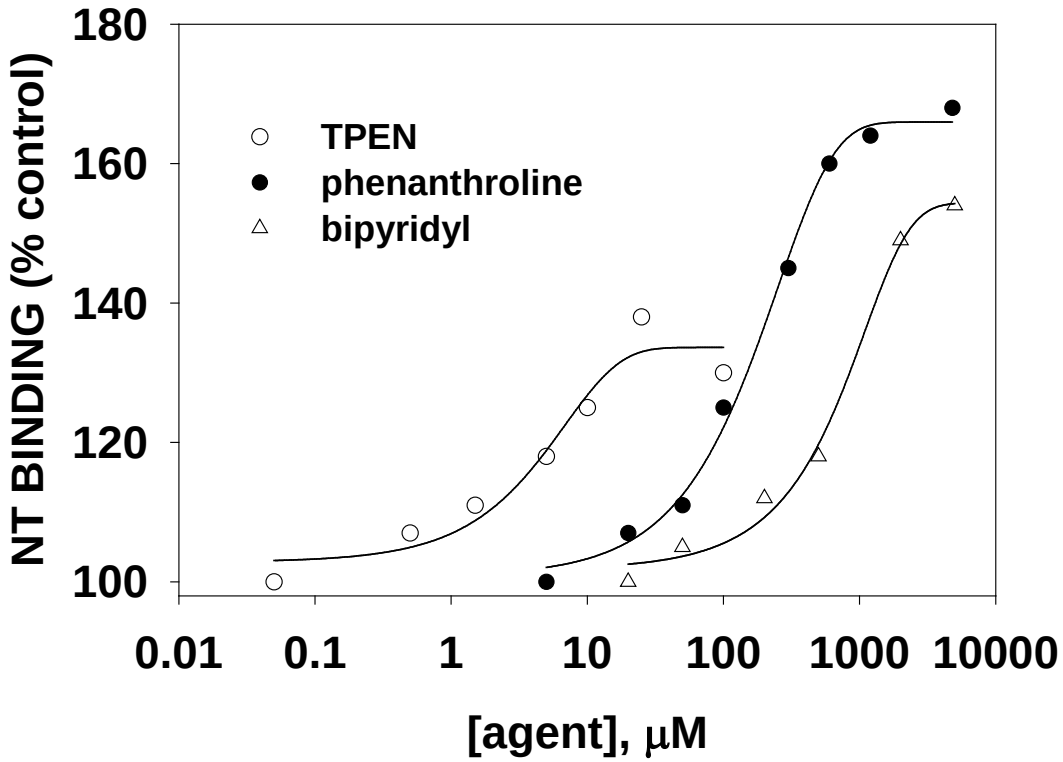


Figure 18 – Effect of zinc chelators TPEN, phenanthroline, and bipyidyl on NT binding to intact PC3 cells. Cells were pretreated for 15 minutes and binding was performed for 30 minutes. The order of potency of the agents to increase NT binding was the same as the order of affinity for zinc. Results are representative of at least 3 experiments.

In total, these results support the notion that tyrosine phosphorylation events can regulate NTR function. However, it is not yet clear if RTKs directly phosphorylate NTR or exert indirect effects that involve phosphorylation of G proteins or other regulators. It is also unclear if this regulation involves a change in receptor affinity (as seen with PKC) or a change in receptor number and internalization.

E. Do the effects of FLAV on NTR correlate to their effects on cancer cell growth?

It is well established that FLAV exert inhibitory effects on cancer cell growth while having much less effect on the growth of normal cells (Haddad et al, 2006). Although the long-term effects (>24 hrs) of these agents in cancer cells have been described, almost nothing is known about the rapid effects that initiate them. For example, quercetin can stimulate the classical mitochondrial apoptotic pathway and also enhance cell killing in response to death receptor activation. However, the mechanisms are not well

understood because the changes that have been studied usually begin after 8-12 hrs or later. NT provides a system where the rapid effects (5-30 min) of FLAV can be studied using assays that yield results within hours. If the effects of FLAV on the NT system correlate to those on cancer cell growth, NT assays might be used as screening tools to facilitate the identification of more potent agents in the FLAV category that might be appropriate for therapeutic uses.

The results in Fig 19 relate the effects of various FLAVs on NTR binding to those on the growth of PC3 cells in our laboratory. Note that the EC50 to alter NTR binding correlates to the IC50 to inhibit cell growth. This suggests that FLAV may target the same mediators in producing these two effects. Our growth data is in fair agreement with that of others (Haddad et al, 2006), showing higher potencies for flavonols (e.g., fiscetin, kaempferol, luteolin, quercetin) than for flavonones. In addition, the similarity shown in the structure-function studies to the effects on RTKs suggests that the ability of FLAVs to inhibit RTKs could be an important input for both NTR and growth. Since the effects on NTR binding occur within 30 min, there could be an early step that is common and which determines cell viability as subsequent steps proceed. At a minimum, this indicates that NT binding could be used to predict the toxicity of new synthetic FLAVs and to shed light on the early steps involved in their anti-cancer effects.

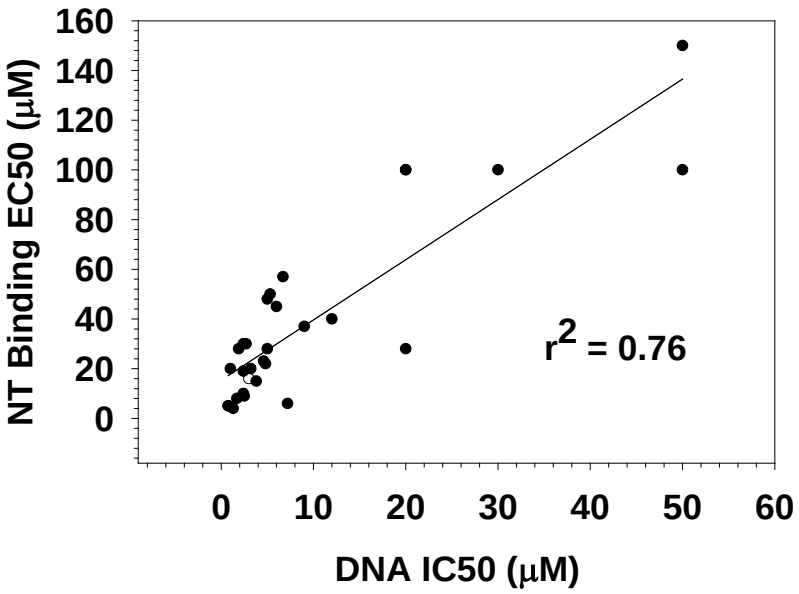


Fig 19. The potency for FLAV to alter NTR binding correlates to the potency to inhibit PC3 cell growth. Dose-response assays were performed to measure the effects of FLAV compounds on NTR binding to intact cells (during 30 min) and the EC50 was determined for each agent. Similarly, dose-response studies were performed using a 2-day assay for cell growth that measured ^{3}H-thymidine incorporation into DNA, and the IC50 was determined for each agent.

Task 6. Determine effects of FLAV on NT signaling modules in caveolae.

Since the apparent upstream effects of FLAVs on NT binding and signaling can be mimicked by β-cyclodextrin which disrupts caveolar signaling modules by adsorbing cholesterol, we hypothesized that FLAVs might disrupt caveolae. Using sucrose gradient ultracentrifugation of membrane fractions from PC3 cells (Pike and Miller, 1998), we demonstrated the localization of NTR in light density membranes that contained caveolin (Fig 20, fraction 4). Pretreatment of the cells with β-cyclodextrin greatly diminished the levels of caveolin, NTR, Gαq and PLCβ in the light membrane fraction (Fig 20, fraction 4)). Thus, β-cyclodextrin appeared to displace NTR from its signaling modules in caveolae, which is consistent with the fact that it alters NT binding and signaling (β-cyclodextrin caused a 2-fold increase in NT binding affinity, while it inhibited NT-induced IP formation. In comparison, quercetin and Bis-1 caused about a 3-fold increase).

To determine if quercetin and Bis-1 shared with β-cyclodextrin the ability to disrupt caveolae, we examined their effects in this system. As compared to β-cyclodextrin, quercetin and Bis-1 had much less effect on the distribution of NTR, caveolin, Gαq and PLCβ in the sucrose gradient (Fig 20). Since β-cyclodextrin has not been reported to inhibit PKC, LOX or RTKs, these results argue that β-cyclodextrin might inhibit NT signaling by disrupting caveolae, whereas quercetin and Bis-1 might inhibit NT signaling by a different mechanism involving PKC, LOX and/or RTKs.

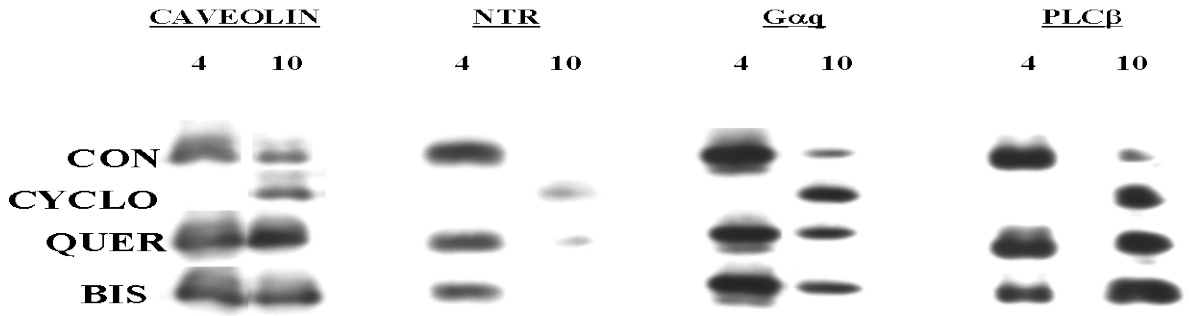


Figure 20 - Effect of β-cyclodextrin, quercetin and BIS-1 on the distribution of NTR, G protein (Gαq) and phospholipase C (PLCβ) in caveolae and heavy membranes. PC3 cells were treated with 5 mM β-cyclodextrin or 50 μM quercetin or 10 μM BIS-1. Sucrose centrifugation was used to separate caveolae from heavy membranes. Western blotting was performed for caveolin, NTR, Gαq and PLCβ in the 10 fractions. Shown are the results for the peak fractions (#4 = caveolae; #10 = dense membranes). For the control, NTR, caveolin, Gαq and PLCβ were primarily in fraction #4. β-cyclodextrin decreased their levels in fraction #4. Quercetin and BIS-1 had less effect.

Since it appeared that FLAVs did not displace NTR from caveolar signaling modules, we hypothesized that they may have altered NTR function in a more subtle way, perhaps by modulating the response to NT itself. Therefore, we investigated the effect of NT on the membrane distribution of NTR. PC3 cells were exposed to 100 nM NT for 30 min and membranes were separated by sucrose density centrifugation. NTR was measured by western blotting each of the 10 fractions. Fig 21 shows the results of a typical experiment. Under control conditions, NTR was primarily present in the light density caveolae (fraction #5). Exposure to NT reduced the level of NTR in caveolae by >60%.

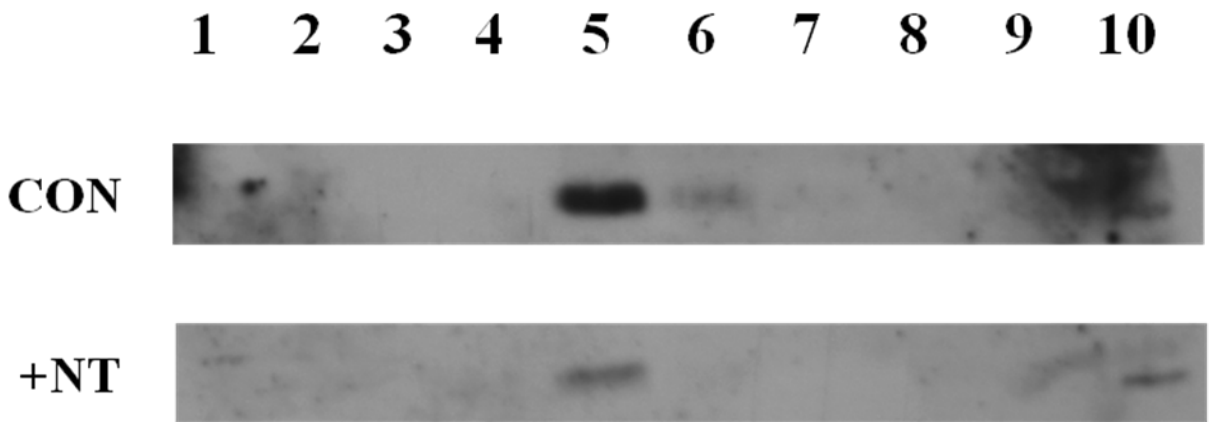


Figure 21 – Western blot showing the levels of NTR in fractions obtained during sucrose density gradient centrifugation of membranes from PC3 cells treated with 100 nM NT or control. Fraction 1 is the top and fraction 10 is the bottom of the gradient. For the control, NTR was primarily located in the caveolae (fraction 5) and it was displaced by treatment with NT.

To further characterize this effect, we next examined the time course for the response to 100 nM NT. Fig 22 shows that 100 nM NT diminished the level of NTR in caveolae within 1 min. The fact that NT can stimulate rapid elevations in cellular calcium that peak within 1 min suggests that this caveolar pool of NTR could represent cell surface receptors that mediate responses to NT. Since many of the responses to NT display a rapid tachyphylaxis, the rapid decrease in the level of NTR could represent a desensitization response.

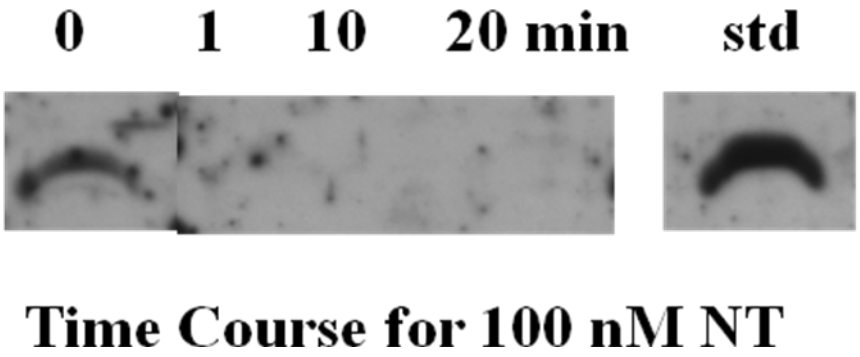


Figure 22 - The time course for the effect 100 nM NT on the level of NTR in caveolae. PC3 cells were treated with 100 nM NT for times indicated in cellular membranes were separated by sucrose density centrifugation. Western blotting was performed to measure NTR in the caveolar fraction.

Our next goal was to compare the dose dependence for this effect on the distribution of NTR to the dose dependence for binding of NT to the receptor. Using our standard procedure, NT binding to intact PC3 cells comes to equilibrium in 30 min and the displacement curve indicates that the IC50 is about 1 nM. Therefore, we used these identical conditions (30 min at 37° C) to determine the dose dependence for the ability of NT to displace NTR from caveolae. Fig 23 shows that increasing doses of NT progressively diminished the level of NTR in the caveolar fraction and that the IC50 was about 1 nM. These results are in keeping with the binding data and suggest that the binding of NT to the receptor initiates the displacement of NTR from the caveolar fraction.

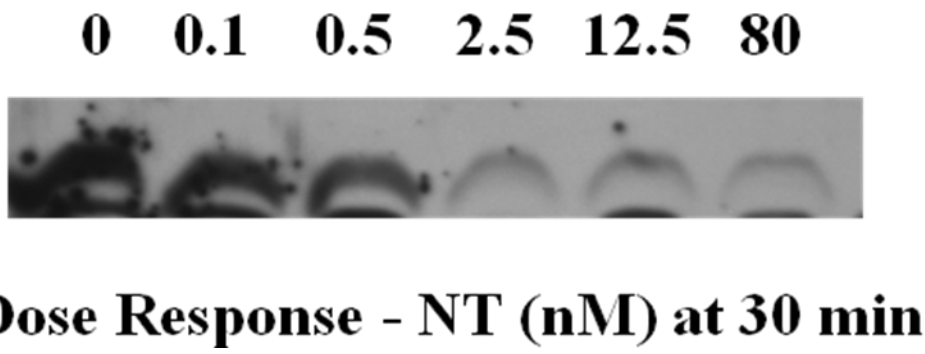


Figure 23 – Dose response for the effect of NT on the level of NT are in caveolae. PC3 cells were treated with doses of NT for 30 minutes. Cellular membranes were separated by density centrifugation and western blotting for NTR was performed on the caveolar fraction. Note that the IC50 for the displacement of NTR was about 1 nM.

Our next goal was to determine if the ability of NT to displace NTR from caveolae was subject to antagonism. SR48692 is a highly specific NTR antagonist that binds to the NT binding site but does not activate the receptor. PMA is a PKC activator that reduces the affinity of the receptor, most likely through phosphorylation events. Cells were pretreated with SR48692 or PMA for 30 min and then stimulated with NT for an additional 30 min. Fig 24 shows that the effect of 3 nM NT to displace NTR from the caveolar fraction was totally inhibited by 1μM SR48692 and diminished by 100 nM PMA. These results are in keeping with the known ability of these compounds to inhibit NT binding and NTR function.

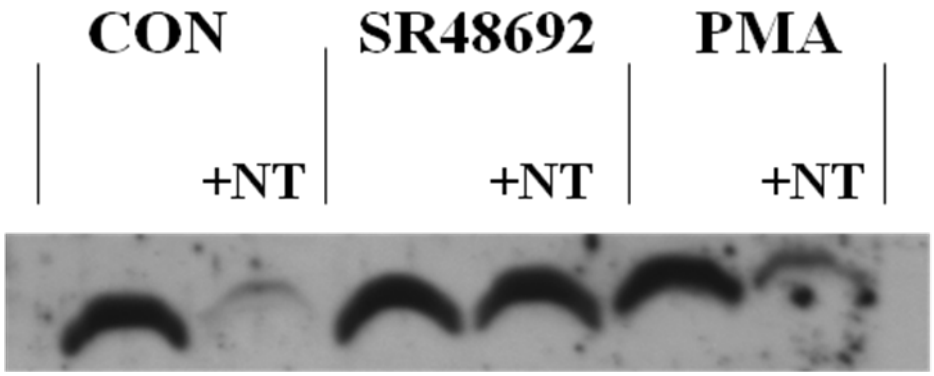


Figure 24– The effect of NT on the distribution of NTR in caveolae was blocked by SR48692 and diminished by PMA. Cells were pretreated with the agents shown and then exposed to 100 nM NT for 30 minutes. Membranes were separated and NTR was measured in the caveolar fraction.

Our final goal was to examine the effects of FLAVs on this response to NT. As before, cells were pretreated with the agents for 30 min and then stimulated with 3 nM NT for an additional 30 min. The results in Fig 25 confirm the effect of NT to displace NTR from the caveolar fraction and the ability of SR48692 to block this effect. Although the effects seen for the agents examined suggest that they antagonized the response to NT, we have not done a sufficient number of these studies to draw a definite conclusion. Difficulties have arisen in regards to the stability of NTR in some of our recent experiments, in that the western blots show variable amounts of lower molecular weight bands. Since the various agents might affect the stability of NTR, we have to be cautious in interpreting the results. We are investigating a number of factors that might input into this problem, such as incubator contamination, failure of the protease inhibitors, temperature of the samples during work-up and non-specific effects of the agents on protease action. Despite these reservations however, our preliminary conclusion from the studies performed to date is that some of these agents can inhibit the ability of NT to displace NTR from the caveolar fraction.

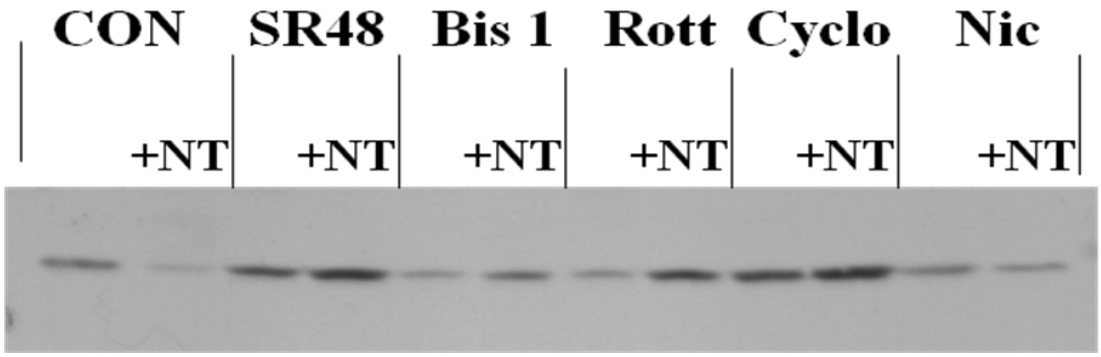


Figure 25 – The inhibitory effect of various agents on the displacement of NTR from caveolae by NT. C ells were pretreated with SR48692, Bis 1, rottlerin, methyl β - cyclodextrin or micardipine for 30 minutes and then with 3 nM NT for an additional 30 minutes. Membranes were separated and NTR levels were measured in the caveolar fraction by western blotting. Note that the agents shown inhibited the effect of NT.

One of the perplexing things about the effects of FLAVs is that they enhance (by 3-fold) the binding of NT to its receptor, whereas they inhibit NTR signaling and the cellular responses to NT. Although this seems counter intuitive, there is precedence for it in that EGFR binding is elevated 5-fold by agents that inhibit its intrinsic tyrosine kinase activity. In the case of EGFR, the tyrosine kinase inhibitor prevents EGFR from phosphorylating its own tyrosine residues, perhaps then preventing receptor desensitization upon binding. The end result is an apparent increase in binding in the face of inhibited responses. Based on our results, one might suggest that FLAVs somehow inhibit PKCs, RTKs and LOXs that regulate NTR function and also participate in its desensitization. The loss of NTR from the caveolar signaling module may be one aspect of this desensitization. Nevertheless, by blocking the receptor signaling and the subsequent desensitization, FLAVs would increase the apparent binding.

C- Effect of FLAVs on cellular ATP levels.

It is conceivable that inhibition of PKC, RTKs and LOX might in some cases involve changes in cellular ATP levels. In our manuscript provided in the Appendix (Carraway et al, 2007) we showed that NTR binding and signaling were sensitive to metabolic inhibitors. Glycolytic and mitochondrial inhibitors, which reduced cellular ATP levels and activated AMP kinase, produced the same effects on NT growth signaling that are seen in the presence of FLAVs. Therefore, we tested the effects of quercetin and BIS-1 on cellular ATP levels. The results in Fig 26 indicate that quercetin and BIS-1, tested across the concentration range that altered NTR signaling, did not affect cellular ATP levels. In contrast, antimycin A (which served as the positive control) dramatically reduced cellular ATP levels across the concentration range that altered NTR binding and signaling.

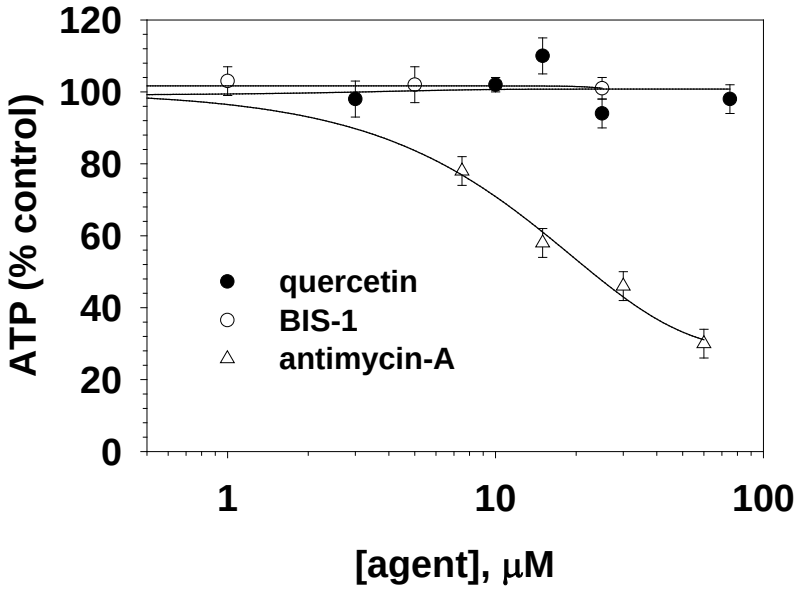


Figure 26 - Quercetin and BIS-1 do not alter cellular ATP levels in PC3 cells. Quiescent cells were incubated with the indicated concentrations of quercetin, BIS-1 or antimycin A for 45 min. Cells were extracted with TCA and cellular levels of ATP were measured using a luciferase based assay. Results are representative of at least 3 experiments.

However, it is possible that FLAVs exert more subtle effects on metabolism that are not reflected as a global decrease in ATP levels. For example, FLAVs might have modest effects on mitochondrial metabolism that are compensated by increased glycolysis. In order to address this idea, we examined the effects of FLAVs in cells deprived of glucose. Quiescent cells were withdrawn from glucose for 1.5 hrs in order to put more demand on metabolic systems. However, this did not compromise their ability to maintain cellular ATP levels under control conditions throughout the experiment. Cells were then treated with FLAVs or control for 45 min in the absence of glucose and cellular ATP levels were measured. Fig 27 shows that FLAVs dose-dependently decreased cellular ATP levels relative to the control. In addition, the potency order (galangin > luteolin > apigenin > quercetin > 5-OH flavone) was similar to the activity order for the effects on NTR. These results suggest that the effect of FLAVs on kinase activity in live cells may be due to metabolic stress. In other words, the metabolic effects of FLAVs could be localized or subtle, such that an effect on total cellular ATP levels can only be seen under stressful conditions. Nevertheless, such local effects on metabolism could limit availability of substrates for kinases such as PKC and RTKs.

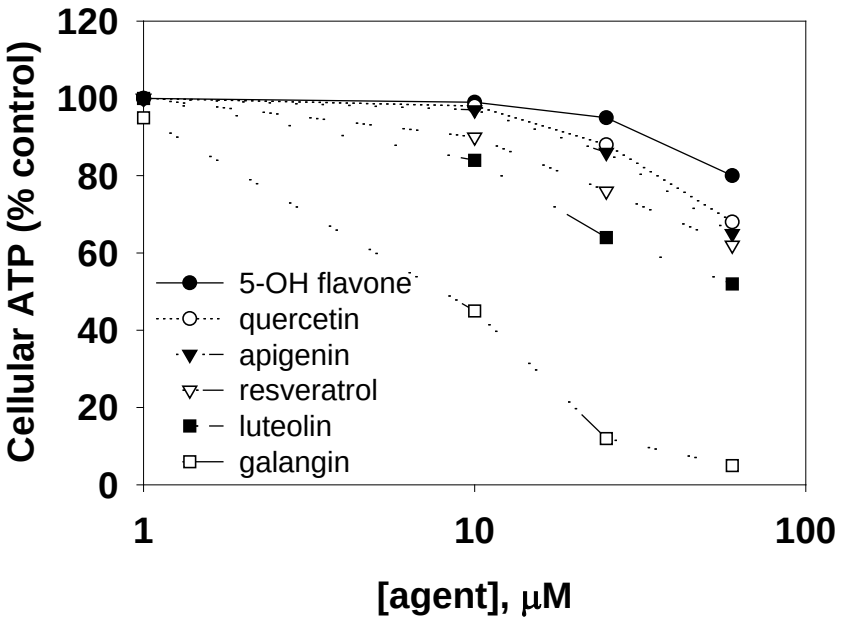


Figure 27- FLAVs dose-responsively diminish cellular ATP levels during treatment of PC3 cells for 45 min in the absence of glucose. Note that removing glucose by itself did not cause a significant decrease in ATP levels (<10%) as compared to cells incubated in 5 mM glucose.

6. KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that lipoxygenase inhibitors mimicked FLAV effects on NTR.
- Demonstrated that metabolic inhibitors reduced ATP and mimicked FLAV effects on NTR.
- Demonstrated that sub-class of PKC inhibitors (BIS-1 like) mimicked FLAV effects on NTR.
- Demonstrated that FLAV inhibited and downregulated novel PKCδ and PKCε.
- Demonstrated that knockdown of either PKCδ and PKCε mimicked FLAV effects on NTR.
- Demonstrated that disruption of caveolae (by removing cholesterol from membranes) mimicked FLAV effects on NTR.
- Demonstrated that FLAV were not metabolic inhibitors (did not reduce cellular ATP).
- Concluded that novel PKCδ and PKCε maintain NTR signaling modules in the working state, and that this maintenance process requires ATP, cholesterol and lipoxygenase activity.
- Determined the EC50 for the effects of 22 FLAVs on NTR binding.

- Related the FLAV effects on NTR binding to those for inhibition of various kinases, showing most similarity to the effects on receptor tyrosine kinases.
- Demonstrated ability of FLAVs to inhibit tyrosine kinase-mediated effect on MAPK activation.
- Demonstrated effect of tyrosine phosphatase inhibitor peroxovanadate on NTR binding.
- Demonstrated effect of tyrosine phosphatase inhibitor peroxovanadate on MAPK activation.
- Demonstrated effect of tyrosine phosphatase inhibitor zinc pyrithione on NTR binding.
- Demonstrated effect of tyrosine phosphatase inhibitor zinc pyrithione on MAPK activation.
- Demonstrated effect of zinc chelators on NTR binding
- Demonstrated that NTR was localized to caveolae in PC3 cells and could be displaced by removing cholesterol from the membrane using β-methyl cyclodextrin.
- Demonstrated that NT displaced NTR from the caveolar fraction and that NT antagonist SR48692 and PKC activator PMA inhibited this effect
- Demonstrated that FLAVs exerted metabolic effects in that they reduced cellular ATP levels in the absence of glucose.

7. REPORTABLE OUTCOMES

A. Hassan S and Carraway RE. Involvement of arachidonic acid metabolism and EGF receptor in neurotensin-induced prostate cancer PC3 cell growth. Regulatory Peptides, 133:105-114, 2006.

B. Carraway RE, Hassan S and Cochrane DE. Regulation of neurotensin receptor function by the arachidonic acid-lipoxygenase pathway in prostate cancer PC3 cells. Prostaglandins, Leukotrienes and Essential Fatty Acids, 74:93-107, 2006.

C. Carraway RE and Plona AM. Involvement of neurotensin in cancer growth: evidence, mechanisms and development of diagnostic tools. Peptides, 27:2445-2460, 2006.

D. Carraway RE and Hassan S. Neurotensin receptor binding and neurotensin-induced growth signaling in prostate cancer PC3 cells are sensitive to metabolic stress. Regulatory Peptides, 141, 140-153, 2007.

E. Carraway RE, Hassan S and Dobner PR. Protein kinase C inhibitors alter neurotensin receptor binding and function in prostate cancer PC3 cells. Regulatory Peptides, 147, 96-109, 2008.

8. CONCLUSIONS

Our results show that the NTR is subject to heterologous regulation by PKC and that there appear to be two mechanisms. Although speculative, our results are consistent with the idea that one mechanism involves negative effects on NTR binding and signaling which seem to be brought about primarily by conventional PKCs (α, β). A second mechanism appears to involve the actions of novel PKCs (δ, ε) which maintain the ability of NTR to stimulate PLC. Since NT can activate PKC upon binding to its receptor, it is possible that NTR is also subject to homologous regulation by PKC. Polyphenols and FLAVs, which are known to inhibit PC growth, can exert multiple effects on NTR function leading to an inhibition of growth signaling. Our studies on quercetin suggest that it could specifically inhibit and downregulate novel PKCs, and this may be the mechanism by which it disrupts NTR signaling. However, FLAVs can also inhibit LOX and RTKs, and our results do not rule out the possible importance of these effects on the regulation of NTR signaling. In addition, FLAVs produced subtle metabolic effects that were exacerbated in the absence of glucose, and these could underlie the effects on NT signaling since NTR function is sensitive to metabolic inhibition.

Other findings reported here indicate that NTR is localized to membrane caveolae where the receptor presumably associates with effectors to induce its effects. Our results clearly indicate that the agonist NT causes a decrease in the level of NTR in these signaling modules, presumably by activating the receptor and initiating desensitization. β-cyclodextrin, which is known to disrupt caveolae by removing cholesterol from cell membranes, displaced NTR from caveolae and produced FLAV-like effects on NT binding and signaling. Although FLAVs did not displace NTR from caveolae, this suggests that FLAVs and the removal of membrane cholesterol may induce similar conformational changes in NTR that cause similar alterations in receptor function. One idea that we are pursuing is that FLAVs alter the conformation of NTR such that NT binds tighter but it cannot activate the receptor and cannot displace the receptor from caveolae. However, our results are too preliminary draw a definitive conclusion at this time.

The relevance of this work to the regulation of PC growth and to the development of useful therapeutics is shown by the fact that the effects of various FLAVs on NTR binding correlate to those on DNA synthesis in cultured PC cells. Thus, the mechanism by which FLAVs alter NTR function may be similar to that by which FLAVs inhibit the growth of PC cells. Our studies point towards effects that involve inhibition of PKC, RTK and/or LOX, which are important in the regulation of PC cell growth.

The effects on these enzymes appear to have a unique ability to feedback on NTR, such that there is a dramatic increase in receptor binding associated with a decrease in signaling..

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10. APPENDIX

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Involvement of arachidonic acid metabolism and EGF receptor in neurotensin-induced prostate cancer PC3 cell growth

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Abstract

Dietary fats, which increase the risk of prostate cancer, stimulate release of intestinal neurotensin (NT), a growth-promoting peptide that enhances the formation of arachidonic acid metabolites in animal blood. This led us to use PC3 cells to examine the involvement of lipoxygenase (LOX) and cyclooxygenase (COX) in the growth effects of NT, including activation of EGF receptor (EGFR) and downstream kinases (ERK, AKT), and stimulation of DNA synthesis. NT and EGF enhanced [^{3}H]-AA release, which was diminished by inhibitors of PLA2 (quinacrine), EGFR (AG1478) and MEK (U0126). NT and EGF phosphorylated EGFR, ERK and AKT, and stimulated DNA synthesis. These effects were diminished by PLA2 inhibitor (quinacrine), general LOX inhibitors (NDGA, ETYA), 5-LOX inhibitors (Rev 5901, AA861), 12-LOX inhibitor (baicalein) and FLAP inhibitor (MK886), while COX inhibitor (indomethacin) was without effect. Cells treated with NT and EGF showed an increase in 5-HETE levels by HPLC. PKC inhibitor (bisindolylmaleimide) blocked the stimulatory effects of NT, EGF and 5-HETE on DNA synthesis. We propose that 5-LOX activity is required for NT to stimulate growth via EGFR and its downstream kinases. The mechanism may involve an effect of 5-HETE on PKC, which is known to facilitate MEK-ERK activation. NT may enhance 5-HETE formation by Ca^{2+}-mediated and ERK-mediated activation of DAG lipase and cPLA2. NT also upregulates cPLA2 and 5-LOX protein expression. Thus, the growth effects of NT and EGF involve a feed-forward system that requires cooperative interactions of the 5-LOX, ERK and AKT pathways.

Keywords: Prostate cancer; 5-lipoxygenase; 5-HETE; Neurotensin; EGF; DNA synthesis

1. Introduction

Epidemiological studies and animal experiments suggest a link between dietary fat intake and the risk of cancer including prostate cancer (PC) [1–3]. This may be due to an elevation of ω-6 fatty acids such as arachidonic acid (AA) since AA can exert important effects on the proliferation of PC cells [4,5]. In addition, the turnover of AA is 10-fold greater in malignant than in benign prostatic tissue [6], suggesting that metabolic pathways involving lipoxygenase (LOX) and/or cyclooxygenase (COX) are activated. Work by Anderson [7] and Ghosh [8] using specific inhibitors have shown that LOX pathways are particularly important in regulating PC cell growth. LOXs are a family of nonheme iron oxygenases named according to site specificity (5-LOX, 12-LOX and 15-LOX), which catalyze the conversion of AA to peroxy-(HpETE) and hydroxy-(HETE) derivatives, and to various leukotrienes [9–11]. PC specimens and cell lines often overexpress LOXs [12–15] and display increased levels of LOX metabolites [8]. Since LOX metabolites can promote PC cell growth [4,16] and LOX inhibitors can induce apoptosis [8,17], it is clear that LOX activity (particularly 5-LOX activity) is key to the growth and survival of PC cells.

Neurotensin (NT), a peptide found in brain, gut and peripheral nerves [18,19], enhances growth of normal intestinal mucosa [20] and stimulates mitogenesis in a variety of cancer cell lines [21–23]. Since NT is released by fat ingestion in animals and humans [24,25], NT might contribute to fat-induced cancer cell growth. PC cells over-express the NT receptor (NTR1) [26], and NT stimulates growth of both androgen-dependent (LNCaP) [27] and androgen-independent (PC3) cells [26]. We have shown that the growth effect of NT in PC3 cells involves protein kinase C (PKC)-dependent,

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EGFR-mediated activation of multiple signaling cascades, including the Raf/MEK/ERK pathway (MAPK) and the PI3K/AKT pathway [28]. Since NTR1 is coupled to $G_{q/11}$ [29], NT typically produces inositol phosphates (IP) by activation of phosphatidylinositol-specific phospholipase C (PLC) and mobilizes intracellular Ca^{2+} [30]. By emptying the Ca^{2+}-store, NT stimulates Ca^{2+}-influx through store-operated Ca^{2+}-channels [31]. Recent work indicates that this can be a strong stimulus for the release of AA from cellular phospholipids and its conversion to bioactive eicosanoids [32]. The fact that NT increases the formation of LOX metabolites in animals [33] suggests that NT might stimulate AA metabolism in PC3 cells by affecting LOX pathways. Since LOX pathways exert important effects on PC cell growth, we hypothesize that LOXs are involved in the growth-enhancing effects of NT.

AA plays a pivotal role in cell signaling [34,35] and proliferation [36], and its release has been shown in response to EGF and other growth stimuli [37–40]. Phospholipase A2 (PLA2) plays an important role in the release of AA from membrane phospholipids [41,42], and diacylglycerol (DAG)-lipase can liberate AA by acting on DAG formed by activated PLC [43]. The fact that PLA2, DAG-lipase and LOXs are Ca^{2+}-sensitive enzymes suggests that Ca^{2+}-signaling by NT could enhance their activity. However, the effects of NT on LOX pathways in cancer cells have not been investigated and the role of AA and its metabolites in NT-induced cancer cell growth is not known.

In the present study, we use PC3 cells to examine the effects of NT on AA release and on the activity of LOX systems; we test LOX inhibitors for effects on NT-induced signaling and cell growth; and we investigate the role of the MAPK pathway in the effects of NT on AA metabolism and cell growth. Our results indicate that the ability of NT and EGF to stimulate growth via the MAPK pathway is highly dependent on AA release and 5-LOX activity. In a reciprocal manner, the ability of NT and EGF to stimulate AA release is dependent on the activity of the MAPK pathway. Thus, NT and EGF appear to initiate a feed-forward cycle involving the 5-LOX and MAPK pathways, which cooperatively stimulate PC3 cell growth.

2. Materials and methods

2.1. Cell culture and reagents

PC3 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured using F12K medium supplemented with 10% fetal bovine serum [26]. Antibodies to cPLA2, phospho-ERK, ERK, phospho-Akt, Akt, phospho-EGFR and EGFR were from Santa Cruz Biotechnology, CA. 5-(*S*)-HETE, 12-(*S*)-HETE, 15-(*S*)-HETE and antibodies to human 5-LOX, 12-LOX and 15-LOX were from Cayman Chemical Company (Ann Arbor, MI). The radiochemicals [methyl-^{3}H]-thymidine and [5,6,8,9,11,12,14,15,^{3}H]-arachidonic acid were from Perkin Elmer Life Science (Boston, MA). Bisindolylmaleimide-I, AG1478, PD153035, MK886 and AACOF3 were from Calbiochem (San Diego, CA). Delta Pak HPLC cartridges were from Waters (Milford, MA) and HPLC grade solvents were from Baker Chemical Company (Phillipsburg, NJ). RHC80267 was from Biomol (Plymouth Meeting, PA). NT, NDGA, ETYA, Rev-5901, AA861 and all other chemicals were from Sigma (St. Louis, MO).

2.2. DNA synthesis assay

The assay was performed as described [28]. In brief, 10^4 cells were plated per well in 24-well plates. After 24 h, cells were serum-starved 48 h, and fresh medium was added with stimuli. After 24 h, [^{3}H]-thymidine (1 μCi/well) was added for 3 h. When used, inhibitors were added 30 min before stimulation. DNA was precipitated with 6% TCA, washed in PBS and ethanol, solubilized in 0.3 N NaOH, 0.1% SDS, and counted by liquid scintillation spectrometry.

2.3. Western blot analysis

Western blot analysis was performed as described [28]. In brief, PC3 cells were grown in 60-mm dishes and serum was withdrawn for 24 h. Cells were washed with hepes-buffered Locke (Locke): 148 mM NaCl; 5.6 mM KCl, 6.3 mM hepes; 2.4 mM $NaHCO_3$; 1.0 mM $CaCl_2$; 0.8 mM $MgCl_2$; 5.6 mM glucose and 0.1% BSA. Cells were exposed to stimuli as indicated, washed in ice-cold PBS with phosphatase inhibitors, placed on ice, scraped into 2 × SDS buffer with phosphatase inhibitors and sonicated. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF (Immobilon P, Millipore). After blocking in 5% non-fat dry milk in TTBS: 20 mM Tris, 0.05% Tween-20, 0.5M NaCl for 1h and washing in TTBS, blots were incubated 18 h at 4 °C with the primary antiserum. After washing, blots were incubated with HRP-linked secondary antibody for 1 h, and ECL was performed according to manufacturer (Santa Cruz). After staining, blots were stripped and reprobed with different antibodies for comparison and for normalization.

2.4. Measurement of AA release

PC3 cells (80% confluent in 24-well plates) were withdrawn from serum for 20 h, labeled by incubation with 1 μCi [^{3}H]-AA/ml for 20 h, and then washed 5× with 0.5% fat-free BSA. Inhibitors were added for 30 min, followed by stimulation with NT and EGF for 30 min at 37 °C. After stimulation, a sample of the medium was centrifuged 12 000 ×*g* for 10 min and radioactivity in the supernatant was determined by liquid scintillation counting. Released radioactivity was expressed as a percentage of total radioactivity in the cells, measured after solubilizing with 0.2 NaOH.

2.5. Measurement of AA metabolites

PC3 cells, grown to 40% confluence in 10-cm dishes and withdrawn from serum for 24 h, were incubated with stimuli for 24 h. After washing in PBS (Dulbecco, Gibco), cells were incubated for 2 h in 4 ml PBS containing 1.5 mM $CaCl_2$, 1.0 mM

$MgCl_2$, 5 mM glucose, 5 mM pyruvate. Reactions were stopped by adding 2 ml CH_3OH plus 0.2 ml 2N HCl and the plates were placed at −20 °C overnight. The medium was removed and cells were gently washed with 2 ml CH_3CN. The combined washes were concentrated (Savant Speed-Vac) to a final volume of 1.4 ml having 30% CH_3OH, centrifuged at 14 000 ×*g* and analyzed by reverse-phase HPLC using Water's equipment, a radial-pak cartridge column (μ-Bondapak C18; 8x100mm) and modified methods [44]. Separation of AA metabolites was achieved by linear gradient elution at 1.5 ml/min from buffer A (42% H_2O, 32% CH_3CN, 26% CH_3OH, 0.04% TFA) to buffer B (2% H_2O, 42% CH_3CN, 56% CH_3OH) while monitoring A_{236} nm (0.05 AUFS). TFA was added to buffer B until its absorbance matched that of buffer A. Peaks were identified by relating retention times to those for authentic standards: 5-HETE, 20.5 min; 12-HETE, 19.5 min; 15-HETE, 18.7 min and quantities were measured by peak integration. Results were normalized to cellular protein (Bio-Rad protein assay) and expressed as ng/mg protein and nmoles/liter incubate.

2.6. Cellular ATP measurement

Approximately 10^4 cells were plated per well in 24-well plates. After 24 h, cells were withdrawn from serum for 48 h and fresh medium was added with stimuli. After 24 h, medium was aspirated, 250 μl 1.2% TCA was added and the plates were placed at −20 °C. After 24 h, the pH was adjusted to 7 using NaOH, and 20 μl was added to each well of a 96-well luminometer plate. The plate was placed in a Beckman LD400 luminometer, which was programmed to add 100 μl of luciferase bioluminescent reagent (Sigma) and to integrate luminescence over 20 s.

2.7. Statistical analysis

All experiments were performed at least three times with similar results. The experimental values obtained were expressed as mean ± SEM with N indicating the number of independent observations. Statistical analysis was performed using Graph-Pad Prism. Comparisons showing $p < 0.05$ were considered statistically significant.

3. Results

3.1. PC3 cells express cPLA2, 5-LOX and 12-LOX

To investigate the involvement of LOX pathways in the effects of NT, we first determined which LOX pathway enzymes were expressed in PC3 cells. Cellular AA is generally liberated from membrane phospholipids by cPLA2, and then peroxidized at specific sites by 5-LOX, 12-LOX and 15-LOX. To determine which of these enzymes is expressed in PC3 cells, we performed western blotting using specific antisera. PC3 cells tested positively for cPLA2, 5-LOX and 12-LOX, giving bands corresponding to the standards (Fig. 1). In contrast, 15-LOX was not detected. These findings indicate the presence of proteins similar to cPLA2, 5-LOX and 12-LOX in PC3 cells.

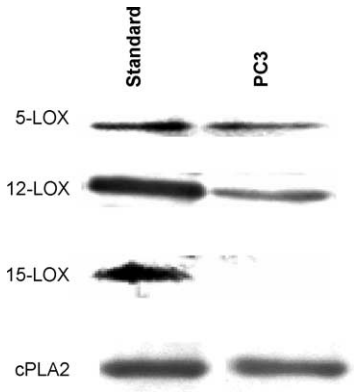

Fig. 1. Western blot assessing expression of 5-LOX, 12-LOX, 15-LOX and cPLA2 in PC3 cells. Cells were extracted in SDS loading buffer containing inhibitors and ≅70 μg of protein was subjected to SDS-PAGE. After transfer, the PVDF membrane was blotted using specific antisera and ECL was performed. Shown is a typical result for 3 experiments. Standard (S) was an extract of human white blood cells for LOXs and an extract of HL60 cells for cPLA2.

3.2. Effect of NT, EGF and HETEs on growth responses in PC3 cells

NT and EGF activate EGFR, ERK and AKT, stimulating DNA synthesis in PC3 cells. Maximal effects are observed using 30 nM NT and 10 nM EGF [28]. To assess the involvement of 5-LOX and 12-LOX in NT-induced and EGF-induced growth, we examined the effects of 5(S)-HETE and 12(S)-HETE, alone and in combination with a maximal dose of NT or EGF. Fig. 2A shows that 100 nM 5(*S*)-HETE and 100 nM 12(*S*)-HETE stimulated DNA synthesis (≅40% increase) as did 30 nM NT (≅60% increase) and 10 nM EGF (≅100% increase). However, their combined effects were less than additive for either HETE plus NT and for either HETE plus EGF (Fig. 2A). This was not due to a ceiling effect since 2% serum increased DNA synthesis by 300% (results not shown). In addition, 5(*S*)-HETE and 12(*S*)-HETE both stimulated ERK phosphorylation, and this effect was less than additive when combined with NT or EGF (Fig. 2B). These results suggest that NT and EGF share a common growth-signaling pathway with 5(*S*)-HETE and 12(*S*)-HETE.

3.3. Effect of NT and EGF on release of [^{3}H]-AA from labeled PC3 cells

The release of AA from membrane phospholipids can be rate-determining for cellular LOX activity [45,46], and this step can be catalyzed by activation of PLA2 [47]. Here, we found that NT and EGF stimulated [^{3}H]-AA release from labeled PC3 cells in a time-dependent and dose-dependent manner. The release of [^{3}H]-AA was significantly enhanced at 15, 30 and 60 min, with an optimal effect at 30 min (results not shown). The maximal effect of NT was to increase basal release by ≅70% and the EC50 was ≅0.3 nM (Fig. 3A). EGF increased basal release by ≅80% and displayed an EC50 of ≅1.2 nM (Fig. 3A). These results show that doses of NT and EGF that are known to stimulate PC3 cell growth also enhance AA release from cellular membranes.

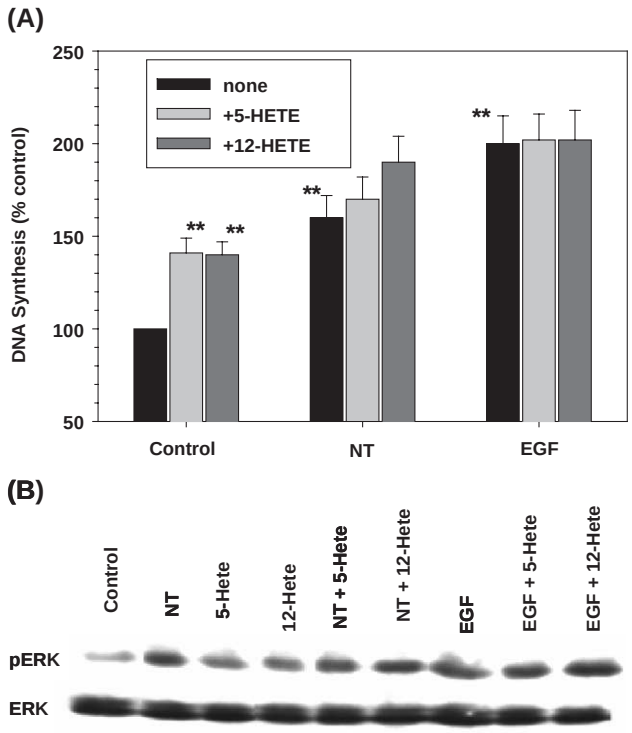

Fig. 2. Non-additivity for the effects of HETEs with those of NT and EGF on DNA synthesis (A) and ERK phosphorylation (B) in PC3 cells. In (A), quiescent cells were treated with NT (30 nM), EGF (10 nM), 5-HETE (100 nM) and 12-HETE (100 nM) alone or in combination for 24 h. The incorporation of [^{3}H]-thymidine into DNA was measured, and the data were expressed as % control. * $p<0.05$; ** $p<0.01$ as compared with control. In (B), quiescent cells were stimulated with NT (30 nM), EGF (10 nM), 5-HETE (500 nM) and 12-HETE (500 nM) alone or in combination for 5 min. Cell lysates were subjected to western blotting using phospho-specific and enzyme-specific antibodies to ERK. Shown is a typical result for 3 experiments.

3.4. Effect of PLA2 and DAG-lipase inhibitors on NT-induced and EGF-induced AA release

AA release can be performed by PLA2 or by the actions of PLC and/or PLD followed by DAG lipase [48]. Pretreatment of PC3 cells with the PLA2 inhibitor (quinacrine) diminished AA release in response to NT and to EGF (Fig. 3B). In contrast, the DAG lipase blocker (RHC-80267) selectively inhibited the response to NT, while having little effect on the response to EGF (Fig. 3B). These results indicate that the AA release response to NT involves activation of PLA2 and DAG lipase, while the response to EGF depends primarily on PLA2.

3.5. Involvement of EGFR and MAPK pathway in NT/EGF-induced AA release

Since cPLA2 can be activated by ERK [49] and since NT and EGF are known to activate ERK by way of EGFR and the MAPK pathway, we assessed the importance of EGFR and the MAPK pathway for the effects of NT and EGF on AA release. Pretreatment of cells with EGFR-specific tyrosine kinase inhibitors (AG1478, PD153035) or with MAPK inhibitor (U0126) dose-dependently diminished [^{3}H]-AA release in response to NT and EGF (Fig. 4). These results indicate that EGFR and the MAPK pathway must be functional for NT and EGF to fully promote AA release.

3.6. NT and EGF elevate 5-HETE levels

Products of AA metabolism in PC3 cells incubated with and without NT and EGF were separated by reverse-phase HPLC. Attention was focused on LOX metabolites using absorbance at 236 nm to quantitate 5-HETE, 12-HETE and 15-HETE identified by retention time. To assess the ability of NT and EGF to upregulate LOX pathways, quiescent cells were stimulated with these peptides for 24 h in RPMI and then fresh medium was added for 2 h to determine the generation of LOX products. Under basal conditions, 5-HETE was the major metabolite (94±17 ng/mg protein) while only minor amounts of 12-HETE and 15-HETE were recovered (<9 ng/mg protein). NT and EGF significantly increased the formation of 5-HETE (30–50% increase) but the effects on

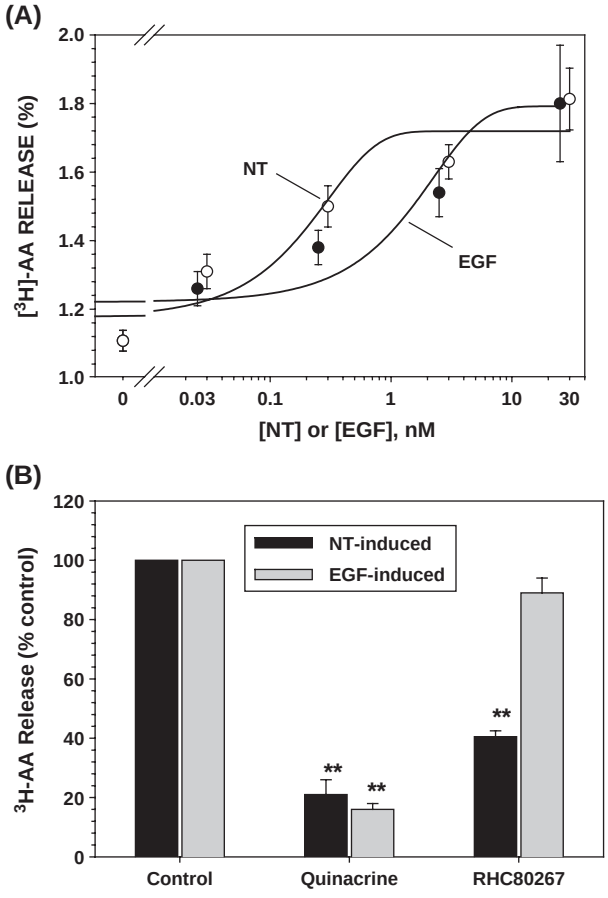

Fig. 3. Dose-response relationships for stimulatory effects of NT and EGF on [^{3}H]-AA release from PC3 cells (A) and inhibition of these responses by agents known to inhibit phospholipase A2 (quinacrine) and DAG lipase (RHC80267) activity (B). In A, cellular phospholipids were labeled with [^{3}H]-AA, the cells were incubated with the indicated concentrations of NT or EGF for 30 min, and the release of [^{3}H]-AA was determined as % of total cellular radioactivity. In B, cells labeled with [^{3}H]-AA were pre-treated for 15 min with each agent (10 μM) or 0.1% the indicated concentrations of agents or 0.1% DMSO (control) prior to being stimulated with 30 nM NT or 25 nM EGF for 30 min. [^{3}H]-AA was determined and data were expressed as % control. The data shown represent 3 experiments. * $p<0.05$; ** $p<0.01$ compared with control.

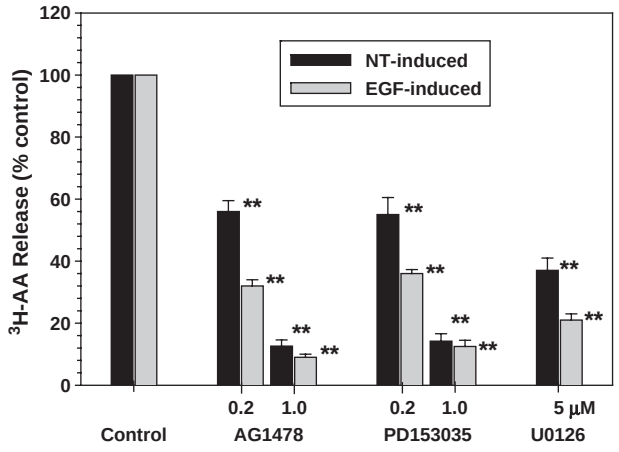

Fig. 4. Effects of tyrosine kinase inhibitors (AG1478, PD153035) and MEK inhibitor (U0126) on [^{3}H]-AA release from PC3 cells in response to NT and EGF. Labeled cells were pre-treated with the indicated concentrations of inhibitors or 0.1% DMSO (control) for 15 min and then stimulated with 30 nM NT or 25 nM EGF for 30 min. The [^{3}H]-AA release was determined and expressed as % control. The data shown represent 3 experiments. * $p<0.05$; ** $p<0.01$ compared with control.

12-HETE and 15-HETE levels were immeasurable (Table 1). The calculated concentration of 5-HETE in the medium (basal, ≅70 nM; stimulated, ≅100 nM) was near to that found to activate ERK (Fig. 2B) and to enhance DNA synthesis in PC3 cells (Fig. 2A).

3.7. NT enhances expression of 5-LOX pathway

Since NT enhanced the formation of 5-HETE, we used western blotting to assess the effect of NT on cPLA2 and 5-LOX protein expression. The results in Fig. 5 show that the levels of cPLA2 and 5-LOX protein were increased in PC3 cells incubated with NT or EGF for 24 h.

3.8. LOX blockers inhibit NT-/EGF-induced activation of ERK and AKT

Various LOX pathway blockers were tested for effects using doses of each agent known to inhibit the enzymes under study and shown in preliminary experiments not to dramatically alter basal activity. PLA2 inhibitor (quinacrine) diminished the activation of MAPK and AKT in response to NT (Fig. 6A) and EGF (Fig. 6B). Similar effects were

Table 1
Effect of NT and EGF on the formation of 5-HETE by PC3 cells

Treatment[a]	Concentration (nM)	5-HETE level[b] (% control)
NT	10 nM	132±9**
	100 nM	128±10*
EGF	10 nM	131±11*
	100 nM	154±20

[a] PC3 cells in 10-cm dishes were treated for 24 h with the indicated stimuli and then incubated for 2 h in 4 ml of buffer.
[b] 5-HETE levels were determined by HPLC, and under control conditions were 94±17 ng/ mg protein (mean±SEM, $n=6$). Given are 5-HETE levels (mean±SEM) determined in 4 or 5 experiments and expressed relative to control (100%). Under all conditions, the levels of 12-HETE and 15-HETE were <9 ng/mg protein.
* $p<0.05$; ** $p<0.01$.

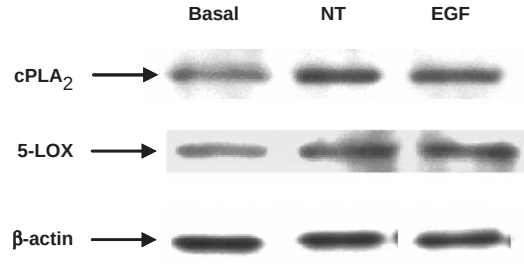

Fig. 5. NT and EGF enhance cPLA2 and 5-LOX protein expression in PC3 cells. Quiescent cells were incubated with 30 nM NT, 25 nM EGF or vehicle (control) for 24 h, and cell extracts were subjected to western blotting with specific antisera. Shown is a typical result for an experiment that was performed 3 times. The relative intensities of the bands for cPLA2 and 5-LOX were elevated about 2-fold when normalized to β-actin.

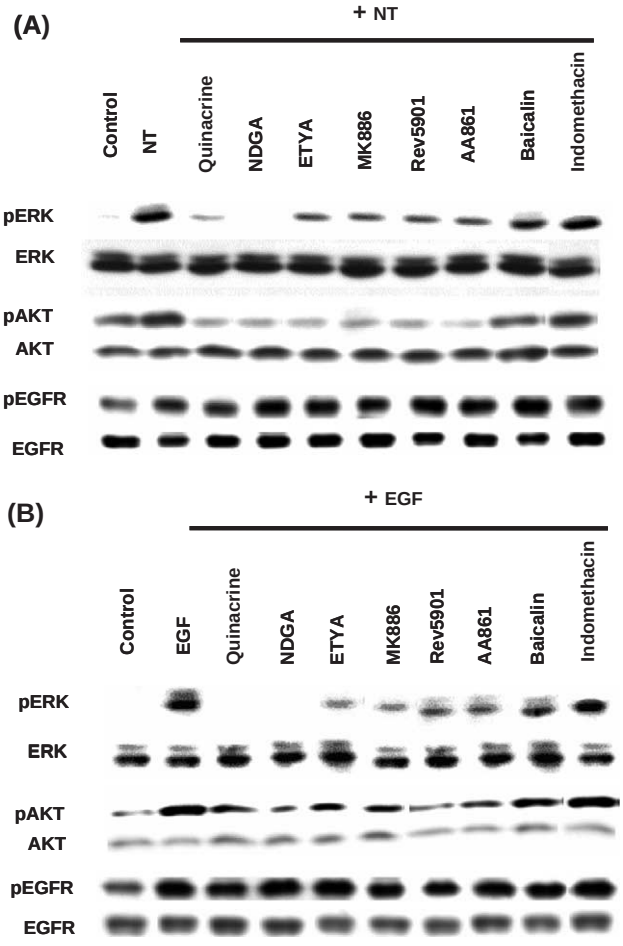

Fig. 6. Effect of various inhibitors of PLA2, LOX and COX on phosphorylation of ERK, AKT and EGFR in response to NT (A) and EGF (B) in PC3 cells. Quiescent cells were preincubated with PLA2 inhibitor (quinacrine, 30 μM) or LOX-inhibitors (NDGA, 30 μM; ETYA, 30 μM) or FLAP inhibitor (MK886, 30 μM), selective 5-LOX inhibitors (Rev5901, 30 μM; AA861, 30 μM), 12-LOX inhibitor (baicalin, 30 μM), COX inhibitor (indomethacin, 50μM), or control (0.3% DMSO) for 15 min and then stimulated with NT (20 nM) or EGF (1 nM) for 3 min. Shown is a typical result for an experiment that was performed 3 times. Note that LOX blockers inhibited the effects of NT and EGF on phosphoryation of ERK and AKT without altering the effects on phosphorylation of EGFR.

displayed by broad specificity LOX inhibitors (NDGA and ETYA), FLAP inhibitor (MK886) and selective 5-LOX inhibitors (Rev 5901 and AA861). The 12-LOX inhibitor (baicalein) was less effective, and the COX inhibitor (indomethacin) had no effects (Fig. 6AB). None of the agents diminished the ability of NT (Fig. 6A) or EGF (Fig. 6B) to phosphorylate EGFR, indicating that these inhibitors act downstream of EGFR to block MAPK activation. These results indicate an involvement of the 5-LOX pathway in the activation of MAPK and AKT by NT and EGF.

3.9. LOX blockers inhibit NT-induced and EGF-induced DNA synthesis

LOX pathway blockers were also tested for an ability to inhibit NT-induced and EGF-induced DNA synthesis. The doses of the agents used here were ≅30-fold less than for the MAPK experiments (above) due to the longer exposure time (24 h vs. 15 min). The results were similar to those for MAPK activation in that inhibitors of PLA2 and 5-LOX, but not the COX inhibitor, diminished responses to NT and EGF (Fig. 7). To confirm the lack of effect by the COX inhibitor, we also tested the ability of PGE2 to stimulate DNA synthesis. Treatment of quiescent PC3 cells for 24 h with PGE2 (10 nM, 100 nM, 1 μM and 10 μM) did not significantly enhance DNA synthesis (3 experiments; $p > 0.05$). These results indicate that the 5-LOX pathway participates in NT-induced and EGF-induced DNA synthesis, while COX pathways are not actively involved.

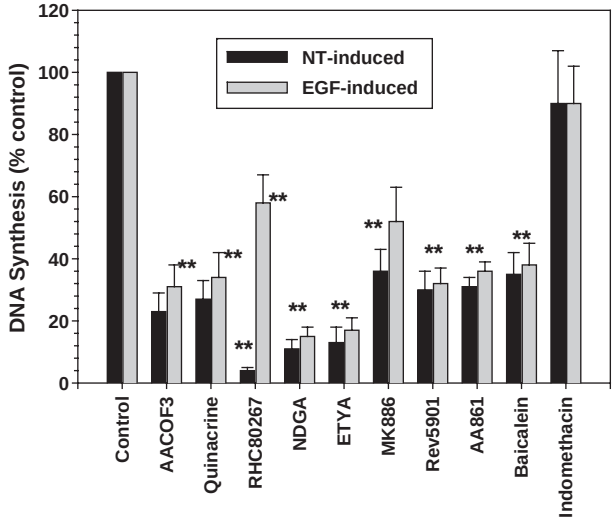

Fig. 7. Effects of inhibitors of PLA2, LOX and COX on DNA synthesis response to NT and EGF in PC3 cells. Quiescent cells were preincubated with PLA2 inhibitors (AACOCF3, 1 μM; quinacrine, 1 μM), DAG-lipase inhibitor (RHC80267, 2 μM), LOX-inhibitors (NDGA, 1 μM; ETYA, 1 μM), FLAP inhibitor (MK886, 1 μM), selective 5-LOX inhibitors (Rev5901, 3 μM; AA861, 2 μM), 12-LOX inhibitor (baicalin 1 μM), COX inhibitor (indomethacin, 5 μM), or control (0.02% DMSO) for 30 min followed by stimulation with NT (10 nM) or EGF (10 nM) for 24 h. The incorporation of [^{3}H]-thymidine into DNA was measured and the data were expressed as % control. The data shown represent at least 3 experiments. * $p < 0.05$; ** $p < 0.01$ as compared with control.

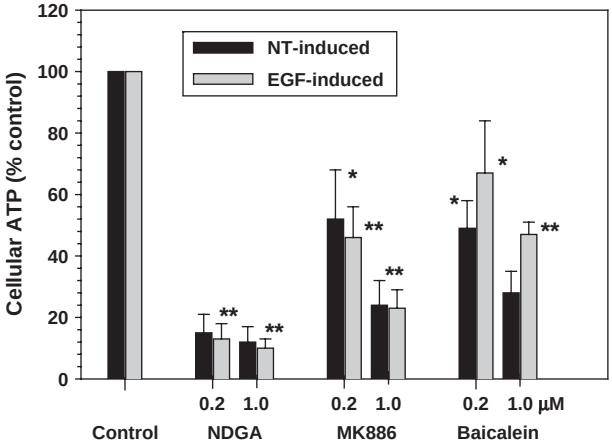

Fig. 8. LOX blockers inhibit NT-induced and EGF-induced elevation in ATP levels in PC3 cells, although they do not affect basal ATP levels. Quiescent cells were preincubated with indicated concentrations of LOX inhibitors for 30 min prior to stimulation with 30 nM NT or 10 nM EGF for 24 h. Cellular ATP levels were determined and expressed as % control. NT and EGF elevated ATP levels by 50–60%. The data shown represent 3 experiments. * $p < 0.05$; ** $p < 0.01$ as compared with control.

3.10. LOX blockers inhibit NT-induced and EGF-induced ATP levels

Cellular ATP was used as a measure of proliferation and cytotoxicity [50]. Initial experiments indicated that an 18-h exposure of cells to NT (30 nM) and EGF (10 nM) increased cellular levels of ATP by 52 ± 5% and 57 ± 5%, respectively (3 experiments; $p < 0.01$ versus control). When cells were incubated with NDGA, MK886 or baicalein (0.2–1.0 μM), basal levels of ATP were unchanged (results not shown), indicating that these agents are not cytotoxic. Nevertheless, these agents dose-responsively inhibited the ability of NT and EGF to elevate ATP levels (Fig. 8). Baicalein was less effective than NDGA and MK886. These results indicate that these LOX blockers inhibit the growth effects of NT and EGF without inducing cytotoxicity.

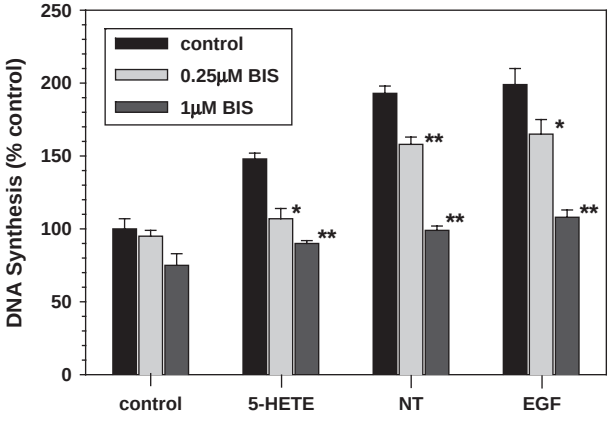

Fig. 9. PKC inhibitor bisindolylmaleimide-I (BIS) dose-responsively inhibits the DNA synthesis response to 5-HETE, NT and EGF in PC3 cells. Quiescent cells were pretreated with indicated concentrations of BIS for 30 min prior to measuring the effects of 5-HETE (100 nM), NT (30 nM) and EGF (10 nM) on DNA synthesis. The data shown represent 3 experiments. * $p < 0.05$; ** $p < 0.01$ as compared with control.

3.11. Growth effects of 5-HETE, NT and EGF are PKC-dependent

Prior work has shown that the growth effects of NT are PKC-dependent. [28] To better understand the role of PKC, we have now tested the effects of PKC inhibitor bisindolylmaleimide-I on growth responses to 5-HETE, NT and EGF. Our results in Fig. 9 show that bisindolylmaleimide-I dose-responsively inhibited 5-HETE-induced, NT-induced and EGF-induced DNA synthesis.

4. Discussion

The results of this study indicate that the ability of NT and EGF to stimulate PC3 cell growth via the MAPK/PI3K pathways requires AA release and the activity of 5-LOX. Consistent with this, NT and EGF (at concentrations known to activate MAPK/PI3K and stimulate DNA synthesis) were found to induce AA release and 5-HETE formation. The amount of 5-HETE formed was in the range needed to mimic the growth effects of these peptides, and the effects of 5-HETE were non-additive with those of NT and EGF. Furthermore, inhibitors of PLA2 and DAG-lipase (shown to block NT-induced and EGF-induced AA release), as well as a battery of 5-LOX inhibitors, diminished the growth effects of NT and EGF. In a reciprocal manner, the ability of NT and EGF to stimulate AA release was found to depend on the activity of the MAPK pathway. Thus, our results suggest that NT and EGF initiate a feed-forward cycle involving the 5-LOX and MAPK pathways, which stimulate PC3 cell growth in a cooperative manner (Fig. 10).

In order to address the hypothesis that the growth-enhancing effects of NT and EGF involve the release of AA and its subsequent metabolism, we employed a number of approaches. Initially, we demonstrated that the release of [^{3}H]-AA incorporated into cellular membranes was enhanced by doses of NT and EGF that stimulated PC3 cell growth. Since the release of AA often involves activation of PLA2 [46,51], we assessed the effects of PLA2 inhibitors (quinacrine and AACOCF3) on AA release, MAPK activation and DNA synthesis in response to NT or EGF. Quinacrine inhibited each of these responses. AACOCF3 inhibited AA release and DNA synthesis but it interfered in the MAPK assay, precluding an assessment of its effects. Since DAG lipase can also promote AA release in some systems [43], we tested the effects of DAG lipase inhibitor RHC-80267. Our finding that RHC-80267 selectively attenuated the effects of NT without altering the responses to EGF is consistent with the fact that NT can activate PLC, presumably releasing the substrate DAG [48]. Thus, NT appears to promote AA release via effects on PLA2 and DAG lipase, whereas the primary effect of EGF is on PLA2. These results are in keeping with the known sensitivity of cPLA2 activity to elevations in [Ca^{2+}] and to phosphorylation by ERK and PKC [52,53]. Recent work has also linked Ca^{2+} entry through store-operated Ca^{2+} channels directly to stimulation of AA release [32]. Overall, these results suggest that AA release in response to NT and EGF participates in the activation of MAPK and the stimulation of DNA synthesis by these peptides.

Released AA can be metabolized by LOX and COX to yield a host of biologically active eicosanoids including HETEs, leukotrienes, prostaglandins and others [54]. In order to

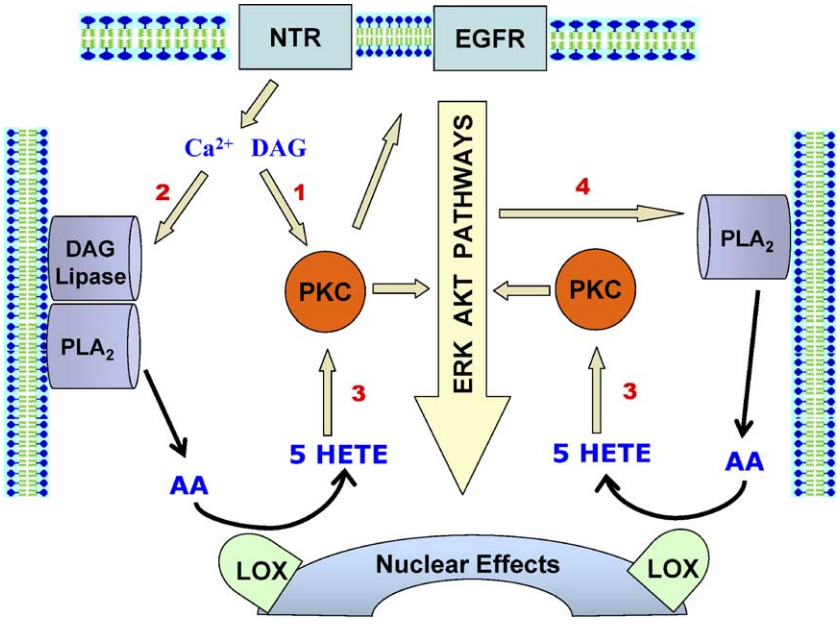

Fig. 10. Diagram of NT-induced and EGF-induced growth signaling in PC3 cells. NT stimulates PLC-mediated increases in cellular levels of Ca^{2+} and DAG. This leads (via path 1) to activation of PKC, EGFR and the ERK/AKT pathways, and (via path 2) to the activation of PLA2 and DAG lipase, enhancing LOX-mediated conversion of AA to 5-HETE. These changes set up a feed-forward loop since 5-HETE further activates PKC (path 3), and the ERK/AKT pathways further activate PLA2 (path 4). Thus, the 5-LOX pathway cooperatively interacts with the ERK/AKT pathways to produce growth enhancement in response to NT and EGF.

investigate the roles of these enzymes in NT-induced and EGF-induced growth, we examined the ability of specific enzyme inhibitors to alter responses to NT and EGF. Whereas COX inhibitor indomethacin was without effect, six structurally and mechanistically diverse LOX inhibitors diminished the effects of NT and EGF on AA release, MAPK activation and DNA synthesis. Since some of the LOX blockers used here (NDGA, ETYA, Rev5901, AA861, baicalein) are antioxidants that inhibit LOX activity by reducing Fe^{+3} to Fe^{+2} [55], they could conceivably act on other targets as reducing agents. However, MK886, which is thought to act by binding to the 5-LOX activating protein FLAP (25), produced similar effects in our hands. Furthermore, the doses of the various agents shown here to effectively diminish the growth effects of NT and EGF were in the range of those shown by others to inhibit 5-LOX in blood cells [11,56]: MK886 (0.1–1 μM), NDGA (0.3–2 μM), AA861 (0.1–2 μM) and ETYA (6–50 μM). Based on our results, it seems likely that inhibition of 5-LOX activity underlies the ability of these agents to disrupt the growth effects of NT and EGF.

Ghosh and Myers were the first to demonstrate the importance of 5-LOX in prostate cancer cell growth [4] and to assess (using radioimmunoassay) the ability of PC3 cells to generate 5-HETE from AA [8]. The present study is the first to employ HPLC to measure the production of 5-HETE, 12-HETE and 15-HETE by PC3 cells in response to NT and EGF. In general, our results confirm their conclusions and extend them by defining a scheme for regulation by growth factors (Fig. 10). One important advance is that we have succeeded in showing that the levels of 5-HETE produced by PC3 cells in the absence and presence of NT and EGF (basal, ≅70 nM; stimulated, ≅100 nM) lie on the dose–response curve for the enhancement of DNA synthesis by 5-HETE. Another advance is that we have correlated PC3 cell growth to the expression of cPLA2 and 5-LOX, and to the production of 5-HETE. Thus, exposure of PC3 cells to NT and EGF not only induced the expression of these enzymes and elevated the levels of 5-HETE but also stimulated DNA synthesis to an extent that corresponded to the 5-HETE level.

Other workers have also shown that 5-HETE activates ERK and AKT in PC3 cells, stimulates cell growth and reverses the growth-arrested state induced by LOX inhibitors MK886 and AA861 [16]. Finding that pertussis toxin inhibits some of the growth effects of 5-HETE, this group proposed that PC3 cells express a novel G protein-coupled receptor that mediates the actions of 5-HETE. Thus, in our model (Fig. 10), it is possible that a cell-surface receptor for 5-HETE is interposed between 5-HETE and the activation of PKC. However, arguing for a direct mechanism is the fact that 12(*S*)-HETE has been shown to directly activate PKCϵ [57]. A third possibility is that HETE becomes esterified to membrane phospholipids and facilitates the translocation of PKC to membranes. Evidence supporting such a mechanism has been gathered using human tracheal epithelial cells [58] and human corneal epithelial cells [59].

Another feed-forward growth regulating system involving eicosanoids has recently been described in PC3 cells [5]. Exogenous AA was shown to upregulate cPLA2 and COX-2 expression and to stimulate a growth response that correlated to the production of PGE2. Suggesting that this pathway is positively regulated by downstream PGE2 was the finding that COX inhibitor fluriprofen diminished all of the effects of AA. Although we did not measure PGE2 levels in our experiments, the effects of NT and EGF on ERK/AKT phosporylation and DNA synthesis were not diminished by COX inhibitor indomethacin. Furthermore, we were unable to stimulate DNA synthesis by exposing PC3 cells to PGE2 over a broad concentration range (0.01–10 μM) for 24 h. Thus, it appears that COX activity is not required and PGE2 is not actively involved in the growth effects of NT and EGF under the conditions of our experiments.

Cross-talk amongst signaling pathways is an important mechanism by which cells integrate environmental stimuli [60]. NT and EGF are potential regulators of cellular growth and over-expression of their receptors in cancer is thought to contribute to the malignant state [61–63]. In a prior study [28], we found that NT stimulates PC3 cells to release HB-EGF, which presumably transactivates EGFR and leads to MAPK activation and cell proliferation. Since EGF is known to induce AA release in a number of cell systems [38,64,65], we focused attention in the present study on AA metabolism. In general, NT and EGF produced similar effects that were equally sensitive to inhibitors of EGFR tyrosine–kinase, 5-LOX and PKC, suggesting that the effects of NT on 5-HETE formation, MAP kinase activation and DNA synthesis are closely tied to the activation of EGFR.

In conclusion, our findings suggest that NT activates the MAPK/PI3K pathways and stimulates DNA synthesis by way of PKC and EGFR (Fig. 10); however, operation of this signaling mechanism requires 5-LOX activity. This regulation may involve an effect of 5-HETE on PKC activity, which is known to facilitate MEK activation. Our results suggest that NT enhances 5-LOX activity by a)—Ca^{2+}-mediated activation of cPLA2 and DAG-lipase; b)—MAPK-dependent activation of cPLA2; and c)—upregulation of cPLA2 and 5-LOX expression. Since enzymes in the MAPK/PI3K pathways can activate cPLA2, liberating AA and enhancing 5-LOX activity, this appears to be a feed-forward system.

Acknowledgements

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Regulation of neurotensin receptor function by the arachidonic acid–lipoxygenase pathway in prostate cancer PC3 cells

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Abstract

Neurotensin (NT) elevates leukotriene levels in animals and stimulates 5-HETE formation in prostate cancer PC3 cells. PC3 cell growth is stimulated by NT and inhibited by lipoxygenase (LOX) blockers. This led us to test LOX blockers (NDGA, MK886, ETYA, Rev5901, AA861 and others) for effects on NT binding and signaling. LOX blockers dramatically enhanced ^{125}I-neurotensin binding to NT receptor NTR1 in PC3 cells, whereas they inhibited NT-induced inositol phosphate formation. These effects were indirect (binding to isolated membranes was unaffected), receptor-specific (binding to β_2-adrenergic, $V1_a$-vasopressin, EGF and bombesin receptor was unaffected) and pathway-specific (cyclooxygenase inhibitors were inactive). NT receptor affinity was increased but receptor number and % internalization were unchanged. Also supporting the involvement of arachidonic acid metabolism in NTR1 regulation was the finding that inhibitors of PLA2 and DAG lipase enhanced NT binding. These findings suggest that NTR1 is regulated by specific feedback mechanism(s) involving lipid peroxidation and/or LOX-dependent processes.

1. Introduction

Neurotensin (NT), a regulatory peptide found in brain, intestine and peripheral nerves [1], stimulates growth of prostate cancer cells, including the human PC3 cell line that expresses the high affinity, G protein-coupled NT receptor NTR1 [2]. Because NTR1 is often coupled to $G_{q/11}$, NT typically stimulates phosphatidylinositol-specific phospholipase C (PLC)-mediated inositol phosphate (IP) formation [3] and the release of intracellular Ca^{2+} [4]. One pathway that can be activated by Ca^{2+} involves the release of arachidonic acid (AA) from cellular phospholipids [5,6] and its conversion to bioactive eicosanoids [7]. The fact that NT can enhance the formation of leukotrienes, presumably from endogenous AA, in some systems [8] suggests that NT might stimulate AA metabolism in PC3 cells. Support for this possibility derives from the finding that NT increases the release of ^{3}H-AA from PC3 cells and its conversion into HETE-like products [9].

Studies with prostate cancer specimens and cell lines suggest important roles for AA metabolites in regulating cancer cell growth [10]. The turnover of AA is 10-fold greater in malignant than in benign prostatic tissue, [11] suggesting that AA metabolism is increased in malignancy via activation of the lipoxygenase (LOX) and/or cyclooxygenase (COX) pathways. Using specific inhibitors, Anderson [12] concluded that LOX pathways are particularly important in regulating DNA synthesis in PC3 cells. Ghosh and Myers [13] supported this idea, showing that the 5-LOX product 5-HETE promoted cell growth. Furthermore, 5-LOX inhibitors MK886 and AA-861 blocked PC3 cell growth induced by AA, and the resulting DNA degradation was reversed by 5-HETE [14]. The possibility that AA metabolites are involved in NT-induced cell growth is suggested by the following observations: (a) PC3 cells express 5-LOX and 12-LOX [15]; (b) NT stimulates PC3 cells to release AA;

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and (c) LOX blockers, but not COX blockers, inhibit NT-induced PC3 cell proliferation [9].

In an effort to understand the mechanism(s) involved in NT-induced responses, we performed the present study investigating the effects of LOX- and COX-directed agents on NT binding and signaling in PC3 cells. Here we have shown that LOX blockers but not COX blockers greatly enhanced the binding of NT to its cellular receptor NTR1, while they inhibited NT-induced IP formation. This phenomenon, which occurred in five different cancer cell lines, appeared to be receptor-specific, pathway-specific and to involve an indirect effect on NTR1. AA metabolism was implicated in this regulation by the fact that inhibitors of PLA2 and DAG lipase enhanced NT binding, and subsequent treatment with AA reversed these effects. Since our prior work has shown that similar effects on NTR1 function occur in response to specific antioxidants and flavoprotein inhibitors [16] and since LOX can contribute to the cellular generation of reactive oxygen species (ROS), [17] we measured the effects of LOX blockers and AA on cellular ROS formation, as well as the effects of ROS on NT binding. Our findings are compatible with the idea that LOX products (oxidized lipids or other ROS) or LOX-dependent processes cause feedback effects that influence NTR1 function.

2. Materials and methods

2.1. Materials

[^{125}I]-sodium iodide (2000 Ci/mmol) and [1,2–3H(N)]-myo-inositol (60 mCi/mmol) were obtained from Perkin Elmer Life Science (Boston, MA). MK886, LY171883, LY294002 and SB203580 were from Calbiochem (San Diego, CA). Rev-5901, AA861, retinoic acid, LY83583, SQ22536 were from Biomol (Plymouth Meeting, PA). NT, L-NMMA, L-NAME, NDGA, ETYA, DTT, gossypol and all other chemicals were from Sigma (St. Louis, MO). 5(S)-HETE, 12(S)-HETE, 5(S)-HpETE, 12(S)-HpETE, 15(S)-HpETE and AA were obtained from Cayman Chemical (Ann Arbor, MI). DCFH-DA was from Molecular Probes (Eugene, OR). SR48692 was provided by Sanofi-Synthelabo (Toulouse, France). [4-azido-Phe6]-NT was synthesized by us as described [3].

2.2. Tissue culture

PC3, PC3M, DU145, and HT29 cells, obtained from American Type Culture Collection (Manassas, VA), were maintained using F12K medium (PC3, PC3M) and DMEM medium (DU145, HT29), supplemented with 10% fetal bovine serum [2]. PC3, PC3 M (its highly metastatic clone), and DU145 [18] are androgen-independent cell-lines derived from human prostatic adenocarcinomas. HT29 is a cell-line obtained from a human colon cancer [19]. LNCaP^{CS}, an androgen-independent clone derived from human prostate cancer LNCaP cells by multiple passages in charcoal-stripped serum [20], was a generous gift from Shuk-mei Ho, Department of Environmental Health, University of Cincinnati Medical School. LNCaP^{CS} were maintained in phenol red-free RPMI medium as described. MatLy-Lu cells, an androgen-independent cell-line derived from a rat prostatic adenocarcinoma [21], was obtained from Manny Menon, Urology Institute, Henry Ford Health System, Detroit. Cells were routinely grown to 85–95% confluence in 24-well culture plates to study cell binding and IP formation, in 10-cm culture dishes to perform receptor crosslinking studies, and in 600 ml culture flasks to prepare cell membranes. For IP studies, cells were labeled with ^{3}H-inositol using medium 199 (Difco) which has a low inositol content.

2.3. Binding to cultured cells

HPLC-purified monoiodinated NT (^{125}I-NT) at 2000 Ci/mmol was prepared and binding was performed as described by us [3]. In brief, cells in 24-well plates were washed with hepes-buffered Locke-BSA (Locke): 148 mM NaCl; 5.6 mM KCl, 6.3 mM hepes; 2.4 mM $NaHCO_3$; 1.0 mM $CaCl_2$; 0.8 mM $MgCl_2$; 5.6 mM glucose; 0.1% BSA; pH 7.4. Stock solutions (10 mM) of each agent in dimethyl sulfoxide (DMSO) were prepared just before use and were diluted in Locke to give $\leqslant$1% DMSO. Equilibrium binding at 37 °C was performed for 30 min using 10^5 cpm/ml ^{125}I-NT in 1.0 ml Locke. The reaction was stopped on ice, the medium was aspirated and the cells were washed three times in ice-cold saline. Total cellular binding was assessed by measuring radioactivity (Packard γ-counter) and protein (Bio-Rad assay) in cells extracted in 0.6 ml 0.2 M NaOH. Specific binding, displaceable by 1 μM NT (20,000-fold excess), was 95% of total binding. Binding displacement curves were constructed and binding parameters were determined by Scatchard analysis and by using the Cheng–Prusoff equation {Ki = IC50/1 + [L]/Kd}, where Kd and [L] are the dissociation constant and the ligand concentration, respectively. Cell surface binding and internalization of ^{125}I-NT were assessed by washing cells at room temperature for 2 min with 0.6 ml of 0.2 M acetic acid, 0.5 M NaCl [3].

The assay conditions and the binding parameters for the ligands used to measure receptor binding for bombesin, vasopressin, β2-adrenergic and EGF in PC3 cells were described by us [3]. In brief, equilibrium binding was performed in a manner identical to that for NT using HPLC-purified ^{125}I-labeled ligands (>1000 Ci/mmol; 10^5 cpm/ml) incubated with PC3 cells in 24-well plates for 30 min at 37 °C. The reaction was

stopped as described for NT and specific binding was defined as that displaceable by 1 μM unlabeled ligand (20,000-fold excess).

2.4. Binding to cell membranes

PC3 cell membranes were prepared [2] and the assay was carried out as described by us [17]. In brief, binding of ^{125}I-NT (10^5 cpm) to membranes (20–40 μg total protein) was performed at room temperature for 60 min in 10 mM Tris–HCl (pH 7.5), containing 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1% BSA and protease inhibitors. Reactions were stopped on ice. Membranes, collected on GF-B glass fiber filters, were washed with ice-cold 10 mM Tris buffer using a Brandel cell harvester. Filters were counted using the γ-counter. Specific binding was calculated as that displaceable by 1 μM NT (20,000-fold excess).

2.5. Crosslinking of ^{125}I-[4-azido-Phe6]-NT to NTR1

This method has been described previously by us [3]. Briefly, [4-azido-Phe6]-NT was iodinated and purified by HPLC. PC3 cells in 10-cm dishes were incubated with 0.3×10^6 cpm/ml ^{125}I-[4-azido-Phe6]-NT in 8 ml Locke 30 min at 37 °C in the presence of the agents indicated ± unlabeled NT (1 μM). Cells were placed on ice, irradiated at 254 nm with a handheld UV light for 5 min at 3 cm, washed in PBS and lysed in 10 mM Hepes (pH 7.4), 1 mM EDTA, 0.5 mM *o*-phenanthroline, 0.5 mM PMSF, 0.5 mM TPCK. After centrifugation, membranes were solubilized in 250 μl 50 mM Tris buffer (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.5% NP-40, 5% glycerol at 4 °C for 2 h. Solubilized NTR1, diluted 3-fold in buffer without detergent, was immunoprecipitated using rabbit antiserum (Ab-NTR1) towards the C-terminus of human NTR1 (1:100). During western blotting, Ab-NTR1 detected two bands in PC3 cell extracts, the parent 50 kDa protein and a breakdown product of 33 kDa [3]. After 18 h at 4 °C, protein A-agarose (10 mg, Sigma) was added for 6 h, the agarose was washed in PBS and associated radioactivity was measured using the γ-counter. The vehicle control (0.05% DMSO) typically gave ≅5000 cpm specifically bound to the immunoprecipitated NTR1. The results for each agent were expressed as % control.

2.6. IP formation

Formation of [^{3}H]-IP was measured as described previously by us [3]. Briefly, PC3 cells in 24-well plates were incubated 48 h with myo-[^{3}H]-inositol (2.5 μCi/ml) in medium 199, 5% fetal calf serum. After washing in Locke, cells were preincubated at 37 °C with test agents or vehicle control (DMSO) in Locke, 15 mM LiCl. After 10 min, reactions were started by adding NT or vehicle (Locke). After 30 min, medium was aspirated, ice-cold 0.1 M formic acid in methanol (1 ml) was added and plates were placed at −20 °C overnight. IP was adsorbed to AG-1 × 8 (formate form, Bio-Rad), which was washed five times in 5 mM myo-inositol and eluted in 1.5 M ammonium formate, 0.1 M formic acid. Scintillation counting was performed in Ecoscint (National Diagnostics).

2.7. Assay of ROS formation

Intracellular ROS formation was measured by fluorescence [22] using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), a dye that permeates cells where it is trapped as the de-esterified free acid, which can then react with ROS to form fluorescent 2′,7′-dichlorofluorescein (DCF). PC3 cells grown to 80% confluence in 24-well plates were washed in Locke, loaded with 10 μM DCFH-DA for 30 min, and then incubated with the indicated reagents for 20 min at 37° C. Reactions were stopped by aspiration and cells were trypsinized, collected on ice, and analyzed on a FACSCalibur™ (Becton Dickinson Biosciences, San Jose, CA) at the UMMS Flow Cytometry Core Facility using Cell Quest and TM software. Fluorescence intensity was measured for 10,000 cells in each sample with excitation (485 nm) and emission (530 nm). Spontaneous fluorescence of cells was <1% that of DCFH-DA treated cells. Results were obtained as histogram plots of cell number versus fluorescence intensity, and the mean fluorescence for each sample within an experiment was expressed as % control. The results of testing each agent in multiple experiments were then summarized as mean ± SEM.

2.8. Statistics

Data were calculated as mean ± SEM for n independent observations. Statistically significant differences were assessed using the Student's *t*-test for single comparisons and using ANOVA (Graph Pad Software, San Diego, CA) with the Tukey–Kramer test for multiple comparisons. Statistical significance was defined As $P < 0.05$.

3. Results

3.1. LOX blockers enhanced NT binding

Specific binding of ^{125}I-NT (10^5 cpm/ml) to PC3 cells, measured at equilibrium, was ≅16.8 ± 0.81 cpm/μg protein ($n = 12$), which corresponded to ≅3000 cpm/well. NDGA, a broad specificity LOX inhibitor, dose-dependently increased the apparent rate of and the steady state level of NT binding to PC3 cells (Fig. 1A).

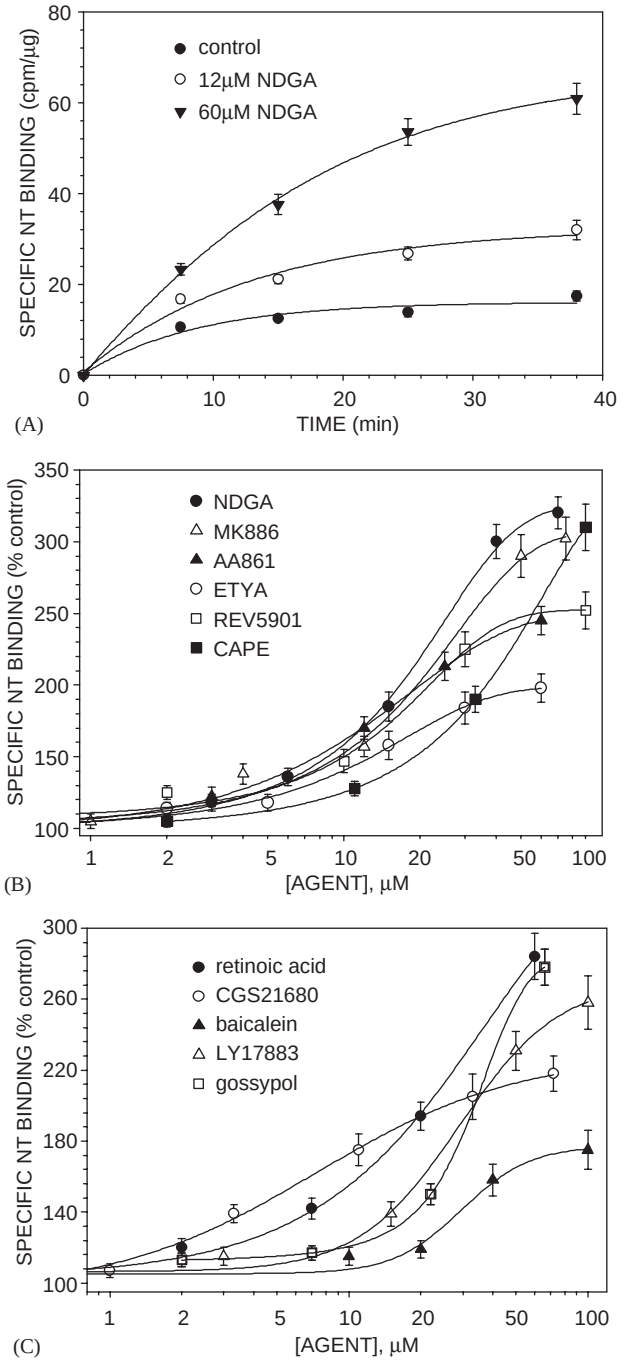


Fig. 1. LOX blockers increased the rate of (A) and the level of (B, C) specific NT binding to PC3 cells. (A) Cells were incubated with indicated concentrations of NDGA or vehicle at 37 °C. After 10 min, ^{125}I-NT (10^5 cpm; 50 pM) was added. Incubation continued for times indicated and specific binding was measured. Non-specific binding was <5% of total binding in all cases (data not shown). The data (mean ± SEM) were from a typical experiment that was repeated. (B and C) Log dose–response plots for effects of LOX blockers on specific NT binding. Cells were pretreated 10 min with agents indicated, ^{125}I-NT (10^5 cpm; 50 pM) was added and specific NT binding was measured at equilibrium (30 min). The minimum dose that significantly ($P<0.05$) increased specific NT binding above control was 2 μM (Rev-5901, retinoic acid), 3 μM (AA861, NDGA, CGS21680), 4 μM (MK886), 5 μM (ETYA), 7 μM (gossypol, LY 171883), 10 μM (CAPE), 20 μM (baicalein). The data (mean ± SEM) were pooled from at least 4 experiments.

Specific binding was enhanced as much as 3-fold, without a change in non-specific binding (data not shown).

Similar effects were displayed by eleven structurally diverse LOX blockers known to act by a number of different mechanisms. These included five non-selective LOX inhibitors (NDGA, ETYA, CAPE, gossypol, retinoic acid), two 5-LOX inhibitors (AA-861, Rev-5901), one 12-LOX inhibitor (baicalein), one FLAP inhibitor (MK886), one indirect LOX inhibitor (CGS-21680) and one LTD4 receptor antagonist (LY171883). NT binding was increased dose-dependently up to 3.2-fold by NDGA, 3.0-fold by MK886, 2.8-fold by CAPE, 2.7-fold by retinoic acid, 2.6-fold by LY171883, 2.6-fold by gossypol, 2.4-fold by Rev-5901, 2.3-fold by AA861, 2.2-fold by ETYA, 2.2-fold by CGS-21680 and 1.8-fold by baicalein (Figs. 1B and 1C). Comparing the magnitude of the responses to these agents suggested that the primary effect on NT binding was likely to involve 5-LOX; however, contributions by 12-LOX and/or 15-LOX were also possible.

3.2. LOX blockers inhibited NT-induced IP formation

Although LOX-directed agents enhanced NT receptor binding, they reduced the ability of NT to stimulate PLC. NT increased IP formation in PC3 cells ≅4-fold, giving an EC50 ≅ 1 nM (Fig. 2A) in agreement with the Kd measured for NTR1 [3]. LOX pathway blockers dose-responsively inhibited NT-induced IP formation (Fig. 2B). The efficacy of NT was reduced, not its potency (Fig. 2A), suggesting that LOX-directed agents shifted NTR1 to a non-functional state.

For each agent (Table 1), the IC50 value for inhibiting NT-induced IP formation was related to the EC50 value for enhancing NT binding, and there was a strong statistical correlation (Fig. 2C). This indicated that these two assays displayed similar chemical sensitivities to the tested agents or that the enhanced binding led to the inhibition of IP formation or visa versa.

3.3. LOX blockers enhanced labeling of NTR1

When ^{125}I-(4-azido-Phe^6)-NT (2.4×10^6 cpm) was bound and crosslinked to PC3 cells in 10-cm dishes under control conditions, and NTR1 in the solubilized membrane fraction was immunoprecipitated, 4674 ± 357 cpm (mean ± SEM; $n=3$) was specifically associated with NTR1. The radioactivity specifically associated with NTR1 was enhanced by 2.6 ± 0.3-fold and 2.7 ± 0.3-fold (mean ± SEM; $n=3$) when the incubation was performed in the presence of 50 μM NDGA and 50 μM MK886, respectively. For both NDGA and MK886, the fold increase in NTR1-labeling was similar to the increase in NT binding observed at

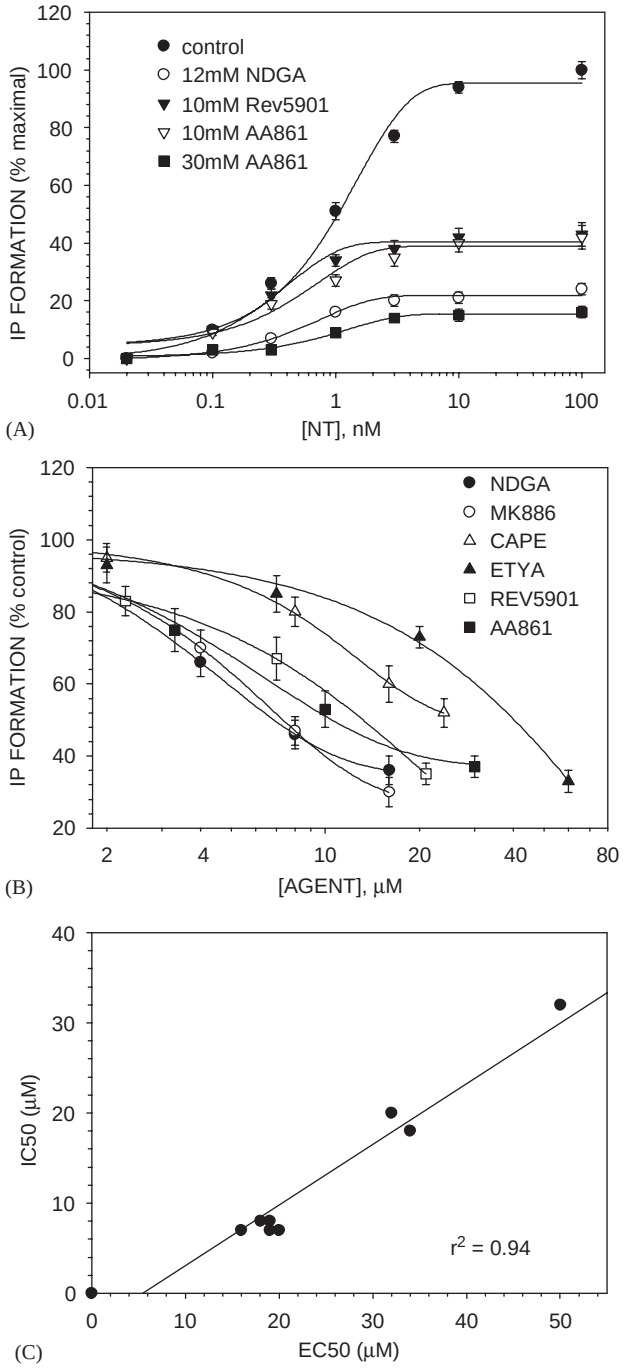


Fig. 2. NT-induced IP formation was inhibited by LOX blockers. (A) Log dose–response plots for the effect of NT in the presence and absence of LOX blockers. NT increased IP formation ≅5-fold, with EC50 ≅1.0 nM. The efficacy of NT, but not its potency, was reduced by LOX blockers. The data (mean ± SEM) were from a typical experiment that was repeated. (B) Log dose–response plots showing that IP formation in response to a maximal dose of NT (30 nM) was inhibited by LOX blockers. The minimum dose that significantly ($P < 0.05$) decreased the response to NT was 4 μM (NDGA, MK886, AA861), 7 μM (Rev5901), 8 μM (CAPE) and 20 μM (ETYA). The data (mean ± SEM) were pooled from 3 experiments. (C) Plot showing that the ability of LOX blockers to inhibit NT-induced IP formation (IC50) correlated to the ability to enhance NT binding (EC50). The values shown in the plot were determined from studies such as those in Figs. 1B, C and 2B, and are presented in Table 1 for the agents used. The plot yielded a correlation coefficient $r^2 = 0.94$.

the dose used (Fig. 1B). These results show that LOX blockers increased the association of ^{125}I-labeled NT ligands with NTR1, the primary NT receptor in PC3 cells.

3.4. LOX blockers did not act directly on NTR1

Binding of ^{125}I-NT to isolated PC3 cell membranes in vitro was not dramatically increased by NDGA, MK886, Rev-5901, AA861 and retinoic acid (Table 2), suggesting that that LOX-directed agents did not act directly on NTR1. The relatively small increases seen for 75 μM NDGA (32%) and for 75 μM retinoic acid (42%) might be attributed to non-specific effects on sulfhydryl groups required for NT binding [23]. Although a key participant in the reaction could have been lost during membrane isolation, it seemed more likely that there was a requirement for cellular metabolism or architecture. Thus, the increase in NT binding in intact cells most likely reflected an indirect effect involving LOX and/or other systems sensitive to these agents.

3.5. Receptor specificity

To determine if these effects were specific to NTR1, we tested LOX blockers for effects on PC3 cell binding of ligands for other receptors using assays validated by us [3]. Table 3 shows that NDGA, MK886, AA861 and Rev-5901 did not have dramatic effects on ligand binding to bombesin, V_{1a}-vasopressin, β2-adrenergic and EGF receptors. There was only a small increase (19–35%) in bombesin receptor binding (all agents) and a 65% increase in EGF binding (only MK-886). Some of these agents are structurally similar to epinephrine, which may explain the small decrease in β2-adrenergic receptor binding. These results show that the robust elevation in cell binding caused by LOX blockers was specific to NTR1 but smaller elevations also occurred for bombesin and EGF receptors.

3.6. Cell specificity

To determine if the effects of LOX blockers were specific to PC3 cells, we assessed the effect of NDGA on NT binding in a number of other cancer cell lines, including prostate carcinomas (PC3M, DU145, LNCaP^{CS}, MatLyLu) and a colon carcinoma (HT29). NT receptor expression, determined as specific cpm bound/μg protein (mean; $n = 3$ experiments), varied according to the cell line: PC3 (19 cpm/μg); HT29 (8 cpm/μg); MatLyLu (6 cpm/μg); PC3M (4 cpm/μg); LNCaP^{CS} (3 cpm/μg); and DU145 (2 cpm/μg). For each cell line, NDGA elevated specific NT binding without an effect on non-specific binding. As compared to the vehicle control (0.04% DMSO), specific binding in the presence of 40 μM NDGA was (mean ± SEM; $n = 3$

Table 1
Activity of LOX blockers on NT Binding and NT-induced IP formation

Inhibitor type	Agent	NT binding[a]		IP formation[b]	5-LOX activity[c]
		Efficacy (% increase)	EC50 (μM)	IC50 (μM)	IC50 (μM)
Non-selective LOX inhibitor	NDGA	230	16	7	0.3–2
	ETYA	100	50	32	6–50
	CAPE	220	34	18	
	Retinoic acid	180	20	7	
	Gossypol	170	32	20	
5-LOX inhibitor	Rev5901	160	19	8	10
	AA861	155	19	7	0.1–2
FLAP inhibitor	MK886	210	18	8	0.1–1
12-LOX inhibitor	Baicalein	75	>100	>100	
Blocker of leukotriene formation	CGS21680	228	22	nd	0.01–1
LTD4 receptor antagonist	LY171883	157	34	nd	

[a]The ability of each agent to enhance NT binding was tested in PC3 cells. The maximal increase observed was ≅200%. Therefore, EC50 was defined as the [agent] that increased NT binding by 100%. Data are from 3 or more experiments.
[b]The ability of each agent to inhibit IP formation in response to a maximal dose of NT (30 nM) was tested in PC3 cells. The maximal inhibition observed was ≅80%. Therefore, IC50 was defined as [agent] that decreased IP formation by 40%. Data are from at least 3 experiments.
[c]IC50 is given as the range of reported values for inhibition of 5-LOX activity measured in various blood cell systems ([7,51–53], and references therein).

Table 2
Effects of LOX blockers on NT-binding to PC3 cell membranes

Agent	Specific NT-binding (% control) at dose of agent[a]		
	10 μM	25 μM	75 μM
NDGA	110±5	121±8	132±10*
MK886	99±3	107±4	97±4
Rev-5901	101±4	113±5	110±5
AA-861	107±4	109±4	110±5
Retinoic acid	102±3	115±4*	142±8**

[a]PC3 cell membranes were preincubated for 10 min with agents or control, and NT binding was performed at room temperature for 60 min. Specific binding was measured in 4 experiments and expressed as % control (mean±SEM).
*Result was significantly different from control ($P<0.05$).
**Result was significantly different from control ($P<0.01$).

experiments): PC3 (240±19%); HT29 (201±10%); MatLyLu (226±16%); PC3M (276±12%); LNCaP^{CS} (193±10%); and DU145 (200±18%). These results indicate that the NDGA-induced increase in NT binding was shown in a number of cancer cell lines and was of similar magnitude.

3.7. Specificity for LOX pathway

NT binding was not dramatically altered by inhibitors of COX (indomethacin, phenbutazone), nitric oxide synthase (L-NAME, L-NMMA), guanylyl cyclase (LY83583), adenylyl cyclase (SQ22536), MAP-kinase (PD98059) and PI3-kinase (LY294002) at concentrations that inhibit these enzymes (Table 4). NT binding was also not much affected by general reducing agents (e.g., sodium borohydride, DTT) and antioxidants (e.g., trolox, ascorbic acid, riboflavin), results reported previously [16]. Thus, the robust increase in NT binding to PC3 cells appeared to be specific to the LOX pathway and was not due to a non-specific change in cellular redox state.

3.8. Cell-surface binding versus internalization

Acid washing of PC3 cells after the binding reaction indicated that 73±2% of the bound ^{125}I-NT was internalized (mean±SEM; $n=8$). The surface-bound ^{125}I-NT and the internalized ^{125}I-NT were both dose-dependently enhanced by NDGA (Fig. 3A) and MK886 (Fig. 3B). Internalization, as percentage of total binding, was unchanged by these agents (range: 68–73%; values given in Fig. 3 legend). NDGA and MK886 also enhanced ^{125}I-NT binding when internalization was fully inhibited by 10 μM phenylarsine oxide (results not shown). The internalization rate at 37 °C for cell-surface ^{125}I-NT, previously bound to cells at 4 °C, was also unaffected by 50 μM NDGA [uptake rate (%/min): control, 9.5±0.8; NDGA, 10.2±0.9; $n=8$]. These results indicate that LOX blockers increased cellular NT binding by enhancing the interaction of NT with NTR1, rather than by enhancing internalization of the NT-NTR1 complex.

Table 3
Effects of LOX blockers on PC3 cell binding of ligands specific for bombesin-, vasopressin-, β2-adrenergic- and EGF-receptors

Ligand	Agent	Specific binding (% control) at dose of agent[e]	
		12 μM	60 μM
^{125}I-[Nle^{14}]-bombesin[a]	NDGA	110±3	127±4 **
	MK886	106±5	125±7*
	AA-861	112±4	135±6 **
	Rev-5901	107±3	119±3 **
^{125}I-HOLVA[b]	NDGA	84±4**	50±4 **
	MK886	93±5	67±3 **
	AA-861	87±3*	57±3 **
	Rev-5901	68±5 **	42±3 **
^{125}I-Pindolol[c]	NDGA	86±4*	66±9 **
	MK886	105±3	102±6
	AA-861	107±5	102±6
	Rev-5901	97±2	76±3 **
^{125}I-EGF[d]	NDGA	114±4	112±6
	MK886	141±7**	165±11**
	AA-861	112±5	100±3
	Rev-5901	101±3	92±5

[a]This ligand for bombesin receptor gave 95% specific binding (Bmax = 1016±64 fmol/mg).
[b]This ligand for V_{1a}-vasopressin receptor gave 77% specific binding (Bmax = 156±12 fmol/mg). None of these agents resembles HOLVA structurally.
[c]This ligand for β2-adrenergic receptor gave 66% specific binding (Bmax = 86±6 fmol/mg). Since NDGA and Rev-5901 resemble epinephrine structurally, the decrease in binding is most likely due to direct competition with the ligand (% crossreaction, ≅0.0005).
[d]This ligand for EGF-receptor gave 95% specific binding (Bmax = 151±11 fmol/mg).
[e]Specific binding of each ^{125}I-ligand (10^5 cpm) was measured to PC3 cells using conditions identical to those for NT binding: 24-well plates with 1.0 ml Locke at 37 °C for 30 min. Binding was expressed as % control (mean±SEM) for at least 3 independent experiments.
*Result was significantly different from control ($P<0.05$).
**Result was significantly different from control ($P<0.01$).

3.9. NTR1 affinity versus NTR1 number

LOX blockers enhanced NT binding and increased the steepness of the NT displacement curve. When NT displacement data were expressed as % maximal binding, LOX blockers shifted the curves to the left by a factor of ≅3 (Figs. 4A and B). The average Ki for NT was decreased from 1.0±0.09 nM (control) to 0.28±0.03 nM (50 μM NDGA; $P<0.01$) and 0.35±0.04 nM (50 μM MK886; $P<0.01$) in 3 experiments (Table 5). Scatchard analyses indicated that NDGA and MK886 increased the affinity of NTR1 for NT without changing the receptor number (Figs. 4C and D; Table 5).

In contrast to the results for the agonist NT, the binding displacement Ki for the antagonist SR48692 was not shifted significantly by NDGA and MK886 (Table 5). Overall, these results indicate that LOX blockers shifted NTR1 to a state with an increased affinity for agonist NT and an unchanged affinity for antagonist SR48692. The fact that NT can stimulate LOX activity in PC3 cells [15] suggested that LOX-dependent processes might feedback to alter NTR1 function. Thus, we hypothesized that a constitutive LOX activity in PC3 cells maintained NTR1 in a low affinity state and that this process was disrupted by LOX blockers.

3.10. NT binding depended on availability of AA

The activity of LOX pathways is often dependent on the availability of AA within cells, which is largely determined by its liberation from membrane precursors by the enzymes PLA2 and DAG lipase [24]. To test our idea that cellular NT binding was regulated by a constitutive LOX activity, we measured NT binding in cells treated with inhibitors of PLA2 and DAG lipase. The results in Fig. 5A show that the PLA2 inhibitor quinacrine and the DAG lipase inhibitor RHC-80267 dose-responsively enhanced NT binding. To further test this idea, an add-back experiment was performed in which the ability of AA to reverse the effect of RHC80267 was examined. The results in Fig. 5B show that AA had no effect on basal NT binding but it reversed the elevating effect of RHC-80267 at doses (IC50≅2 μM) thought to be physiologic and non-toxic [25]. These results, indicating that NT binding depended on the availability of AA as well as the activity of LOX, suggested that LOX-dependent AA metabolism was involved in the regulation of NT binding.

3.11. Identification of relevant LOX products

In an attempt to identify LOX products that were involved in the regulation of NT binding, we performed an add-back experiment in which the ability of various LOX products to reverse the effect of NDGA was examined. Since AA is a key substrate for LOX and its availability was shown to be important, we focused on hydroxy- and hydroperoxy-derivatives of AA. The results in Fig. 5C show that exposure of cells to 4 μM 5(S)-HETE, 12(S)-HETE, 5(S)-HpETE, 12(S)-HpETE or 15(S)-HpETE did not alter basal NT binding and did not reverse the effect of 25 μM NDGA, a less than maximal dose. These results indicate that if NDGA increased NT binding by inhibiting LOX, then either (a) more than 4 μM of these LOX products were required to reverse the effect; or (b) some condition such as a general oxidative tone was needed for LOX products to be effective; or (c) other LOX products were involved.

Table 4
Effects of Various Enzyme Inhibitors on NT Binding in PC3 Cells

Agent[a]	Enzyme	Specific NT-binding (% control) at dose of agent[b]			
		6 μM	20 μM	60 μM	200 μM
Indomethacin	Cyclooxygenase	109±4	95±6	113±7	
Phenylbutazone	Cyclooxygenase	113±4	100±4	89±9	
L-NAME	NO synthase		101±3	103±4	95±6
L-NMMA	NO synthase		103±5	107±3	105±7
LY83583	Guanylyl cyclase	106±4	96±5	95±6	
SQ22536	Adenylyl cyclase	98±3	108±4	105±4	
PD98059	MAPK-kinase	113±7	121±7*	131±9*	
U0126	MAPK-kinase	118±6	108±6	120±9	
LY294002	PI3-kinase	115±9	118±6*	132±8*	
SB203580	p38 MAP kinase	108±8	116±7	125±9	

[a]L-NAME and L-NMMA were dissolved in water and all other agents were in DMSO at 10 mM. Agents were diluted into Locke just before use.
[b]PC3 cells were preincubated with agent or control vehicle for 10 min prior to the NT binding reaction. NT binding was performed as in Fig. 1B using ^{125}I-NT (10^5 cpm, 50 pM) in 1.0 ml Locke at 37 °C for 30 min. Specific NT binding was calculated as % control (mean±SEM) for at least 3 independent experiments.
*Result was significantly different from control ($P<0.05$).

3.12. LOX blockers inhibited basal ROS production

Since LOX can contribute to the formation of ROS in some systems [26,27], we hypothesized that LOX blockers might affect NT binding by altering cellular ROS levels. To determine the effects of LOX blockers on ROS formation, we loaded PC3 cells with DCFH-DA and measured its oxidation by cellular H_2O_2 to the fluorescent derivative DCF using flow cytometry. As shown in Fig. 6A, treatment with 400 μM H_2O_2 (the positive control) shifted the fluorescence histogram to the right. In 3 experiments, the mean cellular ROS level was increased linearly by 20–2000 μM H_2O_2 (Fig. 6B). In contrast, treatment of cells with NDGA dose-dependently shifted the fluorescence histogram to the left (Fig. 6A), indicating that cellular ROS formation was inhibited. Comparing the ability of various LOX blockers to decrease ROS production (Fig. 6C), we found that the order of efficacy was NDGA > CAPE ≅ Rev5901 > AA861 > MK886. These results indicate that LOX blockers either inhibited cellular ROS formation or scavenged ROS. The efficacy order, however, differed from that shown in Table 1 for the effect on NT binding (NDGA > MK886 > Rev5901 ≅ AA861 > CAPE).

The disparity was largely due to the low efficacy of MK886 as an inhibitor of ROS formation. Since MK886 not only blocks LOX activity but also inhibits mitochondrial activity in PC3 cells [28] and since mitochondrial inhibitors generally increase ROS formation [29], we tested the effect of mitochondrial complex I inhibitor rotenone. Treatment of PC3 cells with rotenone was found to dose-responsively increase ROS formation (doses of 6–60μM rotenone gave ROS increases of 16–42% in 3 experiments). These results suggest that the ability of MK886 to diminish ROS levels via inhibition of LOX may have been blunted by an opposing effect on mitochondrial respiration. In total, these results are consistent with the idea that LOX blockers could conceivably alter NT binding by inhibiting LOX-dependent ROS formation.

3.13. LOX blockers inhibited ROS response to AA

The fact that LOX blockers diminished basal ROS levels led us to test the effect of adding LOX substrate AA to the cells. As illustrated in Fig. 7A, treatment of PC3 cells with AA dose-dependently increased cellular ROS levels, producing significant effects at doses as low as 2 μM (Fig. 7B). To determine if ROS production in response to AA was LOX-dependent, we tested the ability of LOX blockers to inhibit AA-induced ROS formation. The results (Fig. 7C) show that LOX blockers inhibited the effect of 10 μM AA, and displayed an order of efficacy (NDGA > CAPE > Rev5901 > AA861 > MK886) similar to that for inhibition of basal ROS production (Fig. 6C). These results suggest that basal and AA-induced ROS production in PC3 cells were at least partly LOX-dependent.

3.14. H_2O_2 reversed the effect of NDGA on NT binding

Hypothesizing that LOX blockers altered NT binding by inhibiting LOX-dependent ROS formation, we tested the ability of H_2O_2 (which we assumed might generate LOX products or mimic their actions) to reverse the effect of NDGA. The elevation in NT binding caused by 25 μM NDGA was dose-dependently diminished by subsequent treatment with H_2O_2; however, there was also a decrease in basal NT binding. The effects of H_2O_2

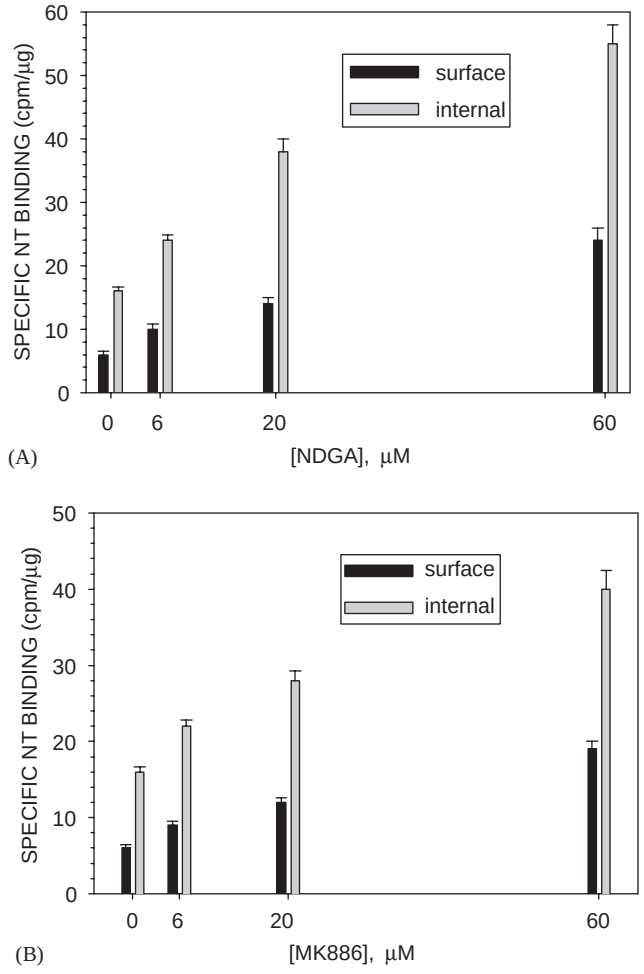


Fig. 3. NDGA (A) and MK886 (B) enhanced cell-surface binding of ^{125}I-NT, without altering the percentage of ^{125}I-NT internalized by PC3 cells. Cells were pretreated 10 min with indicated concentrations of NDGA, MK886 or vehicle control. After NT binding was performed at 37 °C for 30 min, the amounts of ^{125}I-NT bound specifically to the cell surface and internalized were determined by acid washing. The data (mean ± SEM) were pooled from 3 experiments. In A, internalization as a percentage of total binding was (mean ± SEM): 73 ± 2, 71 ± 2, 73 ± 3 and 70 ± 5 at the 0, 6, 20 and 60 μM doses. In (B) internalization was 73 ± 2, 71 ± 2, 70 ± 2 and 68 ± 4 at the 0, 6, 20 and 60 μM doses.

might reflect (a) an ability to produce or mimic LOX products; (b) an ability to decrease NT binding by a process unrelated to LOX; or (c) an ability to react with and inactivate NDGA. Nevertheless, these results are compatible with the idea that NTR1 is held in a low NT binding state by constitutive ROS production via the LOX pathway, and that LOX blockers elevate NT binding by inhibiting ROS production.

4. Discussion

Here, we report for the first time that LOX blockers have major effects on NTR1 function in a number of prostate and colon cancer cell lines. These studies show that a wide variety of LOX blockers can enhance NT binding to PC3 cells while they inhibit NT-induced IP formation. Since our earlier work indicates that NT can activate LOX pathways [8,15], the present findings are compatible with the assumption that LOX products (oxidized lipids or other ROS) or LOX-dependent processes can produce feedback effects that inhibit NTR1 function. AA metabolism was implicated in this regulation since NT binding depended on the availability of AA. The inhibitor of AA formation RHC-80267 caused a striking increase in NT binding that was fully reversed by subsequent addition of near physiologic levels (1–10 μM) of AA. Although a number of LOX-derived AA metabolites were unable (when tested at a concentration of 4 μM) to reverse the NDGA-induced elevation in NT binding, it is possible that higher doses were required or that a peroxidizing tone was needed for them to be effective. H_2O_2 (>1 mM), which may have generated a variety of oxidized lipids or mimicked their effects, was shown to restore NT binding to control levels. Since PC3 cells generated H_2O_2 when incubated with AA and since LOX blockers inhibited this process, it is possible that LOX blockers enhanced NT binding by disrupting LOX-dependent production of ROS (e.g., H_2O_2 or oxidized lipids) that normally occurs in a constitutive manner in PC3 cells (Fig. 8).

Similar effects as shown here on NTR1 function were induced by dihydropyridine Ca^{2+}-channel blockers [3], polyphenolic antioxidants and flavoprotein inhibitors [16]. Given the antioxidant nature of these compounds and the sensitivity of LOX to antioxidants [7], it is possible that all of these agents act by inhibiting LOX and/or other redox-sensitive enzymes. In some systems, lipid peroxidation is inhibited by dihydropyridines [30] and polyphenols [31]. We found that the efficacy for a series of dihydropyridines to alter NTR1 function correlated to superoxide reactivity [16]. Since maximal LOX activity in blood cells requires a peroxidizing tone [32], LOX activity in PC3 cells might depend on the cellular ROS level. The mitochondrial electron transport chain [33] and flavoprotein oxidases [34] are critical sources of superoxide and H_2O_2. While LOX produces lipid peroxides, various other forms of ROS are elaborated by NAD(P)H oxidase, xanthine oxidase, nitric oxide synthase and cytochrome P450 [35] These enzymes constitute a network of systems that contribute to the ROS pool [17]. The results presented here and in our earlier studies point towards an involvement of ROS in regulating NTR1 signaling. Since there is growing support for the participation of ROS as second messengers in receptor signaling [36], one might speculate that the effects observed here represent some form of feedback regulation.

The various LOX blockers shown to alter NTR1 function (Table 1) include non-selective LOX inhibitors

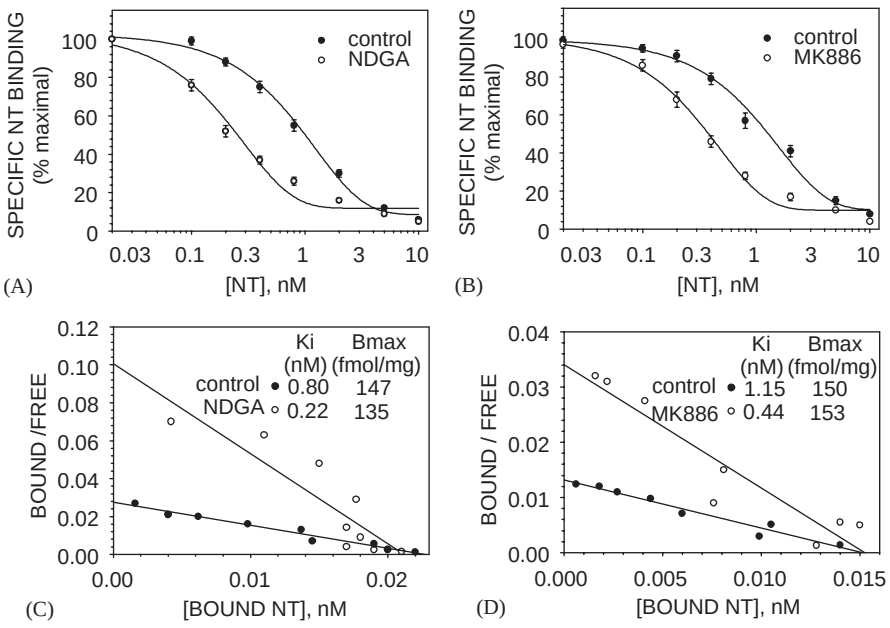


Fig. 4. Binding displacement curves (A, B) and Scatchard plots (C, D) for ^{125}I-NT binding to PC3 cells in the presence and absence of LOX blockers (NDGA and MK886). Specific binding of ^{125}I-NT (15.6 cpm/μg protein) measured at equilibrium was increased 2.9-fold by 50 μM NDGA and 2.6-fold by 50 μM MK886. (A, B) Log dose–response plots show displacement of specific ^{125}I-NT binding by NT, in which binding was expressed as percentage of control. The agents shifted the curves to the left. Results are from a typical experiment repeated twice. (C) Scatchard plots for a typical experiment showing that NDGA increased NTR1 affinity (apparent Ki: control, 0.80 nM; NDGA, 0.22 nM) without increasing receptor number (Bmax: control, 147 fmol/μg NDGA, 135 fmol/μg). (D) Scatchard plots for a typical experiment showing that MK886 increased NTR1 affinity (apparent affinity: control, 1.15 nM; MK886, 0.44 nM) without much effect on receptor number (Bmax: control, 150 fmol/μg; MK886, 153 fmol/μg).

Table 5
Effects of LOX Blockers on NT Binding Parameters in PC3 Cells

Agent[a]	Bmax[b] (fmol/mg)	Ki for NT[b] (nM)	Ki for SR48692[c] (nM)
Control	145±9	1.0±0.09	12±0.9
NDGA	128±10	0.28±0.03**	16±0.6
MK886	143±12	0.35±0.04**	10±0.6

[a]PC3 cells were pretreated 10 min with 50 μM concentrations of each agent or vehicle control. ^{125}I-NT (10^5 cpm, 50 pM) was added and specific binding was measured for 30 min at 37 °C.

[b]Scatchard analyses were performed using 12 concentrations of NT and results for Bmax and Ki (mean±SEM) were obtained from at least 3 experiments for each agent.

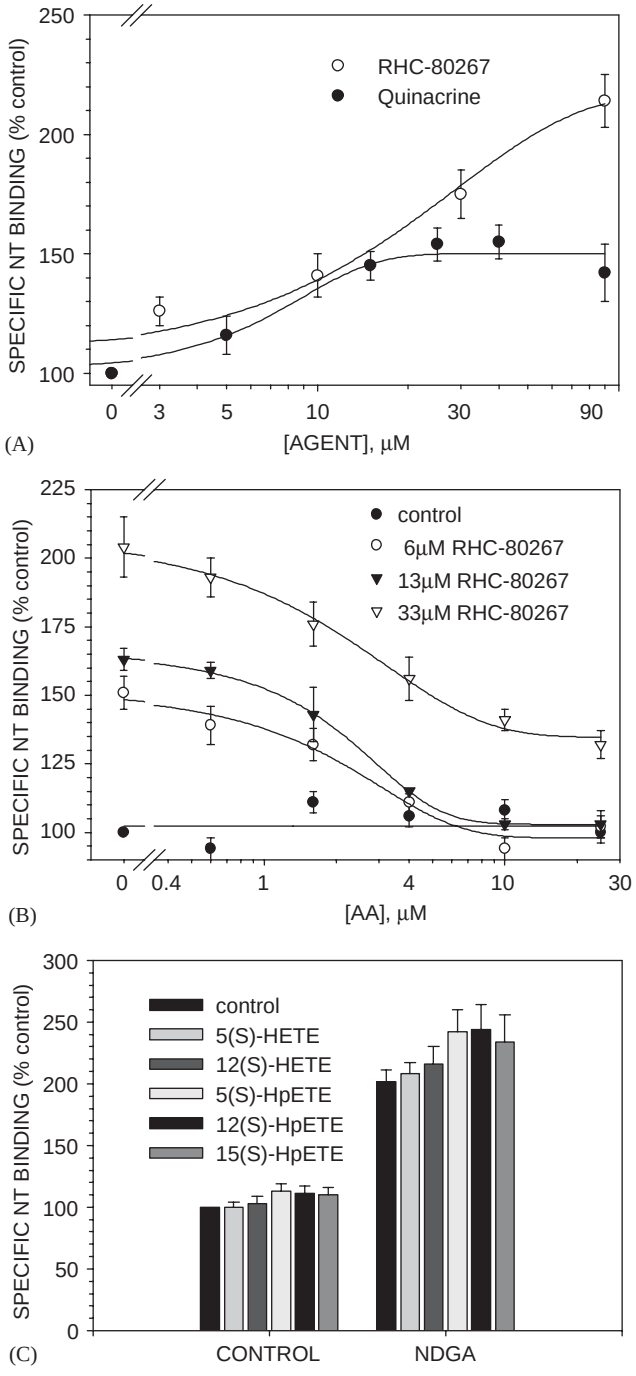
[c]Binding displacement curves using 12 concentrations of SR48692 were used to calculate Ki (mean±SEM) from at least 3 experiments.

**Result was significantly different from control ($P<0.01$).

(NDGA, ETYA, CAPE, retinoic acid, gossypol), 5-LOX inhibitors (AA861, Rev 5901, MK886), 12-LOX inhibitor (baicalein), LTD4 receptor antagonist (LY171883) and adenosine A_{2a} receptor agonist (CGS21680). Some of these agents (NDGA, ETYA, AA861, Rev5901, CAPE, retinoic acid, gossypol, baicalein) inhibit LOX activity by reducing Fe^{3+} to Fe^{2+} [7], so they might reduce other targets or act as ROS scavengers. Arguing against a general cellular redox effect, however, is the fact that common antioxidants (sodium borohydride, ascorbic acid, *N*-acetyl cysteine and vitamins A, B1, B2, C and E) which might be expected to affect protein sulfhydryl groups and cellular glutathione levels, had little effect on NT binding. Instead, only a select group of antioxidants (dihydropyridines and polyphenols) produced the effects seen here for LOX inhibitors [16]. Although dihydropyridines and polyphenols might also inhibit LOX, we cannot rule out the possible involvement of other redox-sensitive systems. On the other hand, several of the LOX blockers tested here (Table 1) are not known to be redox-reactive. Thus, MK886 is thought to act by binding to the 5-LOX activating protein FLAP [37]; CGS21680 is an adenosine A_{2a} receptor agonist that suppresses 5-LOX activity by a cyclic AMP-dependent mechanism [38]; and LY171883 is an LTD4 receptor antagonist. [39] Taken together, our findings indicate that a variety of agents that inhibit LOX by different mechanisms alter NTR1 function, suggesting that LOX (primarily 5-LOX) activity can influence NTR1.

Eleven LOX blockers displayed dose-dependent effects on NTR1 function. Although significant effects on NT binding and NT-induced IP formation occurred for some of these agents at doses near to those for inhibition of LOX in other systems, the doses for half

maximal effects were generally ≅10-fold greater for altering NTR1 function in PC3 cells than for inhibiting 5-LOX in blood cells (Table 1). This disparity could be due to differences in the test systems and incubation times but might also reflect differences in general metabolism [40] and drug metabolism [41] in normal versus cancer cells. Nevertheless, the most efficacious agents that altered NTR1 function in PC3 cells (NDGA, MK886, AA861, Rev5901) were precisely those generally recognized as the most reliable and potent inhibitors of 5-LOX in blood cells [7].



The effects of LOX blockers on NTR1 function were drug-specific and receptor-specific. Whereas LOX blockers dramatically enhanced NT binding, agents known to inhibit pathways involving COX, cAMP, cGMP and MAPK had little effect. Likewise, in contrast to the results for ^{125}I-NT, cellular binding of iodinated ligands for bombesin, vasopressin, β2-adrenergic and EGF receptors were not much affected by LOX blockers. Since LOX blockers did not exert direct effects on NTR1, the effects in intact cells are likely to involve changes in metabolic process(es). The enhancement of NT binding occurred rapidly (within 10 min), involved an increase in receptor affinity without a change in receptor number, and was not due to a change in the rate of receptor internalization. Given that LOX blockers have antioxidative effects, one might propose that LOX blockers elevate NT binding by maintaining sulfhydryl groups that participate in the binding reaction [23]. However, the fact that the cell-permeable reducing agent DTT did not prevent the effects of NDGA (Carraway, unpublished data) nor the effects of dihydropyridines and polyphenols [16] argues against this possibility. Although the mechanism by which LOX blockers alter NTR1 function is not known, a reasonable hypothesis is that these effects are secondary to changes in lipid peroxidation and/or ROS-dependent process(es).

For each LOX blocker, the enhancement of NT binding was associated with a parallel inhibition of NT-induced IP formation, and the drug potencies in these two assays were correlated (Fig. 2C). LOX blockers could exert two separate effects (one to increase NT binding and another to inhibit IP formation), each having the same drug dependence. Alternatively, they could exert one effect (e.g., blocking IP formation) that produces feedback effects on NT binding. Another

Fig. 5. NT binding depended on the availability of AA. (A) Log dose–response plots for the effect of PLA2 inhibitor quinacrine and DAG lipase inhibitor RHC-80267 on specific NT binding. PC3 cells were pretreated with agents indicated for 10 min, ^{125}I-NT (10^5 cpm; 50 pM) was added and specific NT binding was measured at equilibrium (30 min). The data (mean ± SEM) were pooled from 4 experiments. (B) Log dose–response plots showing the ability of AA to reverse the increase in specific NT binding induced by RHC-80267. PC3 cells were pretreated 10 min with 6 μM RHC-80267, 13 μM RHC-80267, 33 μM RHC-80267 or vehicle control (DMSO). Then, AA was added to give the final concentrations indicated and after 2 min, ^{125}I-NT (10^5 cpm; 50 pM) was added and specific NT binding was measured. The data (mean ± SEM) were pooled from 3 experiments. (C) Graph showing effects of indicated LOX products on basal NT binding and the NDGA-induced elevation in NT binding. PC3 cells were pretreated 10 min with 25 μM NDGA and then the indicated LOX products were added (final concentration, 4 μM). After 2 min, ^{125}I-NT (10^5 cpm; 50 pM) was added and specific NT binding was measured. The data (mean ± SEM) from 3 experiments were expressed relative to vehicle control (0.4% ethanol).

possibility is that these agents shift NTR1 to a high affinity state that is unable to regulate IP formation. Precedence for the latter behavior is provided by the effects of tyrosine kinase inhibitors on EGFR, [42] which shift EGFR to a dimer state exhibiting an enhanced ability to bind EGF but a diminished ability to autophosphorylate [43]. Although dimerization has been shown for some G protein-coupled receptors, [44] it has not been described for NTR1 in PC3 cells. However, work in colon cancer HT29 cells and in transfected CHO cells indicates that NTR1 can heterodimerize with NTR3 [45]. These authors found that the ability of NTR1 to mediate IP formation and MAP kinase phosphorylation was altered by cotransfection with NTR3. The EC50 (IP formation) was shifted from 0.3 nM (NTR1 alone) to 1 nM (NTR1 plus NTR3). These results resemble the shift observed here for the NT binding affinity in the presence ($Ki \cong 0.3$ nM) and absence ($Ki \cong 1.0$ nM) of NDGA and MK886 (Table 5). Thus, it might be worthwhile to investigate the effects of LOX blockers on the interaction of NTR1 with NTR3 in PC3 cells.

Since NT is a growth-promoting peptide that is released by fat ingestion in animals and humans [46,47] and since NTR1 is overexpressed in many cancers [48], NT could contribute to fat-induced cancer cell growth [49]. NT stimulates PC3 cell growth by a NTR1-mediated activation of EGF receptor, MAP kinase and PI3 kinase [50], and the ability of NT to stimulate DNA synthesis by activating these pathways is dependent on the availability of AA and is inhibited by LOX blockers [15]. Thus, the present findings, which shed light on possible feedback effects of LOX systems on NTR1 function, give us a better understanding of the operation of this growth pathway and provide fresh ideas for the development of new therapies for prostate cancer.

In conclusion, this study shows that mechanistically diverse LOX blockers enhanced NT binding and inhibited NT-induced IP formation in PC3 cells (and in a number of other cancer cell-lines) via indirect effects that were pathway- and ligand-specific. NTR1 binding affinity was increased without a change in receptor number or internalization. The enhancement in NT binding was associated with a parallel inhibition of NT-induced IP formation, indicating that both responses were similarly sensitive to these agents. The involvement of AA metabolism in the regulation of NTR1 was

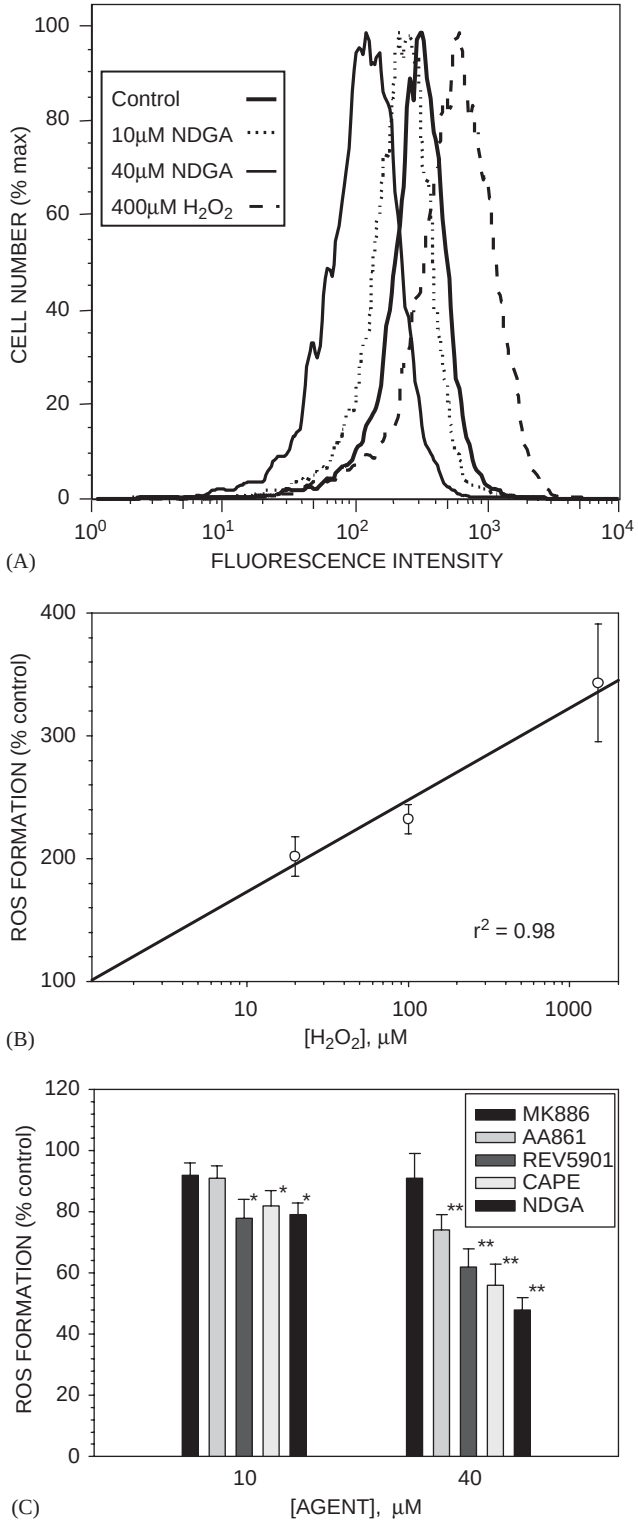
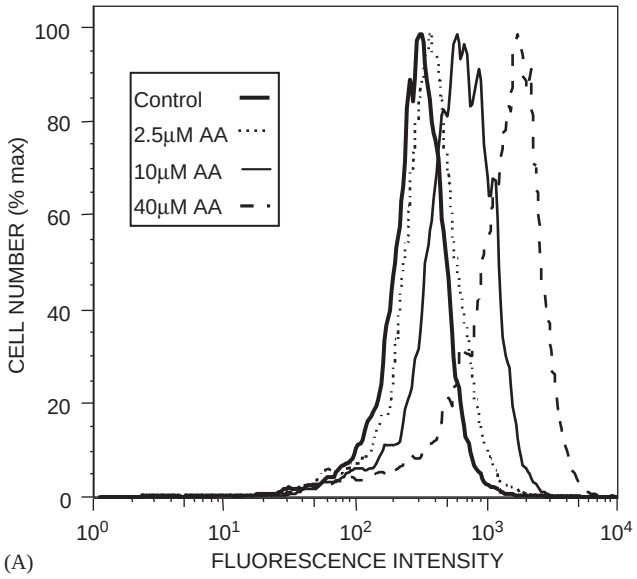
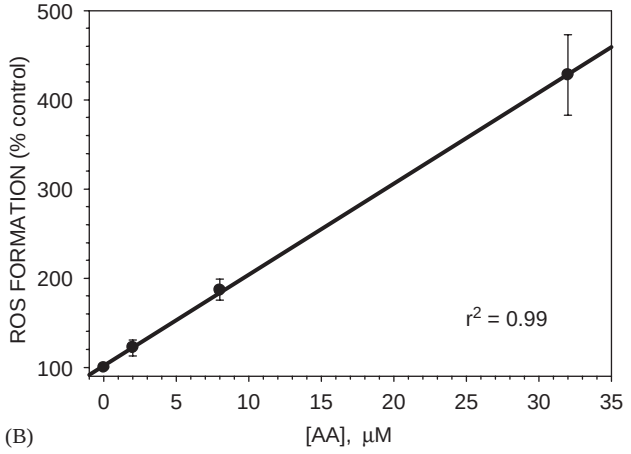


Fig. 6. Effect of H_2O_2, LOX blockers and rotenone on cellular ROS levels measured by flow cytometry. PC3 cells were labeled with DCFH-DA, then incubated with the indicated agents or vehicle (DMSO) for 20 min at 37 °C and harvested for FACS analysis. (A) Representative histograms showing fluorescence intensity versus cell number for control cells and for cells treated with 400 μM H_2O_2, or 10 μM NDGA or 40 μM NDGA. The mean fluorescence intensity was used to quantitate the responses. (B) Dose–response plot for the effect of H_2O_2 on the mean fluorescence intensity measured in 3 experiments (mean ± SEM). (C) Dose–response plots for the effects of various LOX blockers on the mean fluorescence intensity measured in 4 experiments (mean ± SEM).

further supported by the finding that inhibitors of PLA2 and DAG lipase enhanced NT binding. Since NT can activate LOX pathways, we propose that LOX blockers alter NTR1 function by disrupting a specific feedback mechanism that involves lipid peroxidation and/or other LOX-dependent processes.





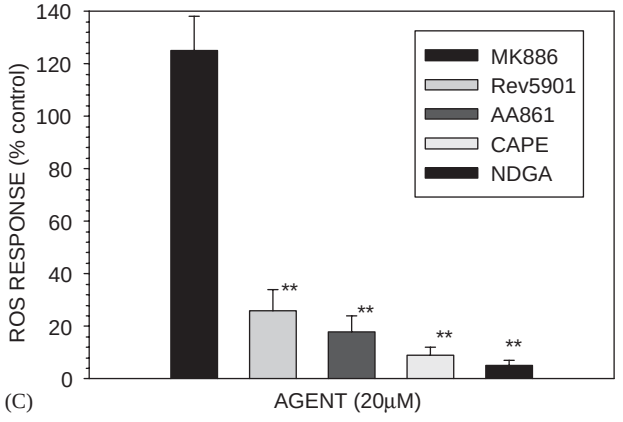


Fig. 7. Effect of AA on cellular ROS levels in the presence and absence of LOX blockers. PC3 cells were labeled with DCFH-DA, then incubated with the indicated agents or vehicle (DMSO) for 20 min at 37 °C and harvested for FACS analysis. (A) Representative histograms showing fluorescence intensity versus cell number for control cells and for cells treated with 2 μM AA, or 10 μM AA or 40 μM AA. The mean fluorescence intensity was used to quantitate the responses. (B) Dose–response plot for the effect of AA on the mean fluorescence intensity measured in 6 experiments (mean ± SEM). (C) Graph comparing the effects of indicated LOX blockers (20 μM) on the ROS response to 10 μM AA expressed as % control (mean ± SEM; $n = 3$ experiments). In the absence of agents, 10 μM AA increased ROS formation by 106 ± 14% above the vehicle control (0.2% DMSO). In the presence of each agent, the response was calculated as the incremental percentage above the appropriate agent control. $^{*}P < 0.05$; $^{**}P < 0.01$.

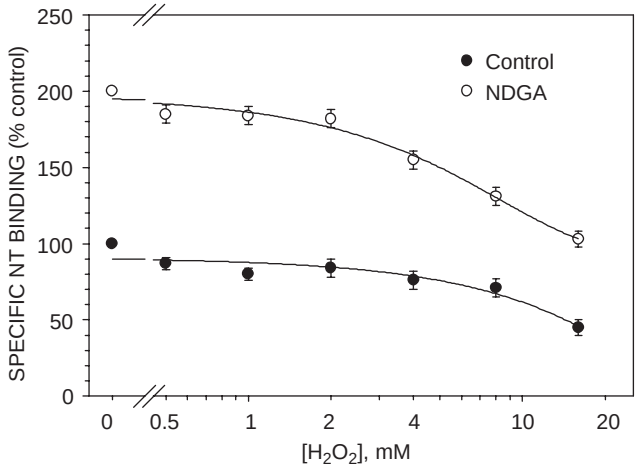


Fig. 8. H_2O_2 dose-responsively reversed the NDGA-induced elevation in NT binding in PC3 cells. After PC3 cells were incubated with 25 μM NDGA or vehicle for 10 min, H_2O_2 was added at the indicated doses and specific NT binding was measured at equilibrium (30 min). The data were expressed as a percentage of the binding measured in cells treated with the vehicle control (0.25% DMSO) and represent 3 experiments (mean ± SEM).

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Review

Involvement of neurotensin in cancer growth: Evidence, mechanisms and development of diagnostic tools

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ABSTRACT

Focusing on the literature of the past 15 years, we evaluate the evidence that neurotensin and neurotensin receptors participate in cancer growth and we describe possible mechanisms. In addition, we review the progress achieved in the use of neurotensin analogs to image tumors in animals and humans. These exciting advances encourage us to pursue further research and stimulate us to consider novel ideas regarding the multiple inputs to cancer growth that neurotensin might influence.

Abbreviations:
AA, arachidonic acid
AC, adenylyl cyclase
Akt, protein kinase B
AR, androgen receptor
Bcl-2, B cell lymphoma/leukemia 2
DAG, diacylglycerol
EGF, epidermal growth factor
EGFR, EGF receptor
ERK, extracellular signal-regulated kinase
FAK, focal adhesion kinase
GPCR, G protein-coupled receptor
Hb-EGF, heparin binding EGF
IP, inositol phosphate
JNK, cJun N-terminal kinase
LCLC, large cell lung carcinoma
LOX, lipoxygenase
MAP kinase, mitogen-activated protein kinase

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Mek, mitogen-activated protein kinase kinase
NMN, neuromedin-N
NT, neurotensin
NTS1, NT receptor subtype 1
NTS3, NT receptor subtype 3
PGE2, prostaglandin E2
PI3K, phosphatidylinositol-3-kinase
PKC, protein kinase C
PKD, protein kinase D (PKCμ)
PLC, phospholipase C
PMA, phorbol-12,13-myristic acid
PSA, prostate specific antigen
PTX, pertussus toxin
RIA, radioimmunoassay
ROS, reactive oxygen species
SCLC, small cell lung carcinoma

Contents

1. Introduction

Among the diverse biologic effects displayed by neurotensin (NT) is an ability to promote the growth of gastroenteropancreatic tissues *in vivo* [84] and the proliferation of dispersed cells in culture [38]. This not only indicates a role for NT in the regulation of normal cell turnover, but it suggests the possibility that NT could contribute to the growth of cancers that express NT receptors. Indeed, tumor cells can both secrete NT and express NT receptors, suggesting that autocrine, paracrine and endocrine regulation by NT is possible. Furthermore, giving NT to animals increases the incidence of and the size of chemically induced tumors [61], whereas giving NT antagonist inhibits the growth of engrafted tumors. These observations suggest that the NT signaling pathway may provide a useful target for the development of diagnostic agents and antitumor drugs. The purpose of this review is to examine the evidence that NT contributes significantly to cancer cell growth and to summarize our knowledge of potential mechanisms. An effort is also made to review our progress in the design and use of NT analogs to image tumors in animals and humans. Finally, to stimulate some controversy and to encourage the reader to consider alternative ideas, we present a novel hypothesis that attempts to incorporate growth regulation by NT into a broad view of its pharmacology and physiology. Due to space limitations, we

have focused our attention on work published within the past 15 years and we apologize for not citing all of the earlier studies.

2. Relevance of NT production and NT receptor expression in cancer

2.1. *Hormonal role for NT in cancer*

The epidemiological association of cancer incidence with high fat intake around the world suggests the possibility that NT, released as a hormone during the digestion of fat, might play a role. Although NT secretion in response to the ingestion of a single meal or to the perfusion of the intestine with fat has been documented, the long-term effects of diet on the synthesis and release of NT are not known. Interestingly, intestinal release of NT is promoted by unsaturated (oleic, linoleic) but not by saturated (stearic) fatty acids [2], and cancer growth is also selectively stimulated by unsaturated fatty acids [42,71]. It could be worthwhile to determine if this relationship extends to the omega-6 (growth stimulatory) and omega-3 (growth inhibitory) fatty acids. Although it is known that fatty acids can exert direct effects on cancer cell growth, the involvement of NT has not been fully investigated.

Pichon et al. reported that levels of NT but not CCK (measured by immunoassay) in fasted sera from patients with pancreatic cancer were significantly elevated in cases involving metastatic and stage IV disease [62]. Whether the increased blood level of NT reflected an increased release by the intestine or by pancreatic tumors is not known but either way, it might possibly impact the disease process. This group found that NT levels fell with progression of the disease for both prostate and pancreatic cancer, showing the possible importance of nutritional status and/or the effects of therapy. In contrast, Meggiato et al. found that plasma levels of NT were normal in pancreatic cancer but elevated in cases where pancreatitis was diagnosed [55]. They argued that the pancreatic insufficiency associated with pancreatitis may have led to enhanced intestinal NT release. In the cancer patients, NT levels appeared to correlate to albumin levels. This could be interesting since fatty acids which release NT are bound to albumin, or it could be of no significance since albumin contains NT-like sequences that crossreact in immunoassays [14].

Thus, it is possible that NT contributes as a hormone to the increased risk of cancer associated with a high fat diet but further investigation is needed to determine the sources of secreted NT in cancer patients as well as the effects of nutritional status and therapy on the circulating levels of NT.

2.2. *Autocrine or paracrine role for NT in cancer*

2.2.1. *NT precursor processing and peptide secretion in cancer*

NT/N gene transcription is developmentally regulated, such that episodes of NT expression occur in some fetal tissues where NT is absent in the adult. Evers [29] has pointed out that the transient appearance of NT in liver, pancreas and colon suggests that these tissues contain ancestral stem cells, which can undergo programmed differentiation. Cancers derived from these tissues often express NT (e.g., 25% of colon cancers), possibly reflecting a reversion to the fetal expression pattern. Dong et al. found that DNA methylation plays a role in NT/N gene silencing in the human colon cancer KM20 and that demethylation at a critical AP-1 promoter site results in NT/N induction [25]. Activation of the Ras pathway can stimulate NT/N expression in the Caco-2 intestinal cell line, suggesting that this could provide a mechanism for inappropriate production of NT by cancer cells or associated tissues [30]. The neuroendocrine phenotype often associated with high-grade small cell lung carcinoma (SCLC) and large cell lung tumors may be related to a resistance to chemotherapy and an enhanced ability to metastasize. Using the Affimetrix GeneChip system to investigate the expression of 12,600 genes, de Lange et al. found that the NT/N gene was one of 73 genes upregulated in a highly metastatic large cell lung tumor cell line H460-M as compared to the less metastatic control [23]. Although they did not measure NT production, the fact that prohormone convertase PC1 was also upregulated suggests that precursor processing to form NT was possible. Whether these results simply reflect the neuroendocrine phenotype or they indicate a role for NT in the metastatic process warrants further investigation.

Using RIA and HPLC to characterize NT-related peptides in thirteen colon cancer cell lines, Rovere et al. found that although all of them expressed proNT/N activity, only six processed the precursor to produce NT-related or neuromedin N (NMN)-related peptides [67,68]. Careful analysis indicated that the major products were large precursor fragments having the NT or NMN sequence located at the C-terminus. NT was a minor product and NMN was not detected. The processing pattern differed from that generally seen in brain and gut, where prohormone convertases PC1 and PC2 produce mostly NT, NMN (brain) and extended NMN (gut). Consistent with this difference, colon cancer cell lines did not express PC1 or PC2 but instead, PC5 was present and was shown to reproduce the processing pattern. Although it was suggested that N-terminally extended forms of NT and NMN might activate NT receptors and exert autocrine growth effects on cancer cells, no evidence for this exciting idea has been reported.

Feurle et al. provided some of the earliest evidence that NT can be secreted by human tumors [31]. The majority of cells in a human pancreatic adenoma stained immunohistochemically for NT and displayed typical N-cell granules. Chromatography of tumor extracts indicated the presence of authentic NT, which was also grossly elevated in the patient's plasma. After surgical removal of the tumor, NT levels returned to normal and the esophageal reflux experienced by the patient subsided. In this remarkable case, the evidence suggested that NT secreted by the tumor not only elevated NT blood levels but also produced a syndrome.

Evers' group has studied the secretion of NT from BON cells, a human carcinoid tumor cell line [49]. Initial work using region-specific RIAs towards proNT/N showed that BON cells display the intestinal pattern of precursor processing, primarily characterized by a large molecular (125 amino acid)

form of NMN [15]. The major products found in BON cells (large NMN, NMN and NT) also appeared in the culture media. In studies to probe the secretory mechanism, Li et al. established that PMA and bombesin, both of which activated PKCα and PKCδ, enhanced NT release [49]. In keeping with the importance of these PKC isoforms were experiments testing the effects of specific PKC inhibitors and of specific PKC isoform overexpression on NT release. These results suggest critical role(s) for PKC signaling in the regulation of NT secretion that should be taken into account when interpreting the effects of PKC-directed agents on cancer cell growth in systems where NT might exert autocrine effects.

Moody's group provided much of the early evidence supporting an autocrine mechanism of growth control by NT [58]. They demonstrated NT production and secretion by small cell lung cancer (SCLC) cell lines, which also expressed the high affinity NT receptor NTS1 and displayed a growth response to NT. In a follow-up study by Davis et al., a number of SCLC cell lines (NCI-N417, NCI-H345, NCI-N592) were found to convert exogenous NT into the fragments NT^{1-8} and NT^{9-13}, reflecting the presence of metallopeptidase 3.4.24.15 in these cells [22]. This raised the issue as to whether NT stability could be a point of control.

Using prostate cancer (LNCaP) cells grown in serum-free defined media, Sehgal et al. showed that immunoreactive NT accumulated in the cells and cell-conditioned media only in the absence of androgen [73]. Furthermore, exogenous NT induced a growth response only in cells that were androgen deprived. Further study suggested that in the presence of androgen, metalloprotease(s) were induced that could degrade NT. The authors concluded that in the absence of androgen, prostate cancer cells develop an autocrine growth mechanism involving NT. Since the expression of NTS1 mRNA was androgen-independent, they reasoned that this autocrine pathway was regulated by modulation of NT levels. In keeping with the notion that androgen withdrawal could enhance NT stability, Pichon et al. have reported that plasma levels of NT are elevated in disease-free prostate cancer patients who received hormone therapy [62]. Moody et al. have also assessed the ability of prostate cancer cells to produce NT and to degrade added NT [60]. LNCaP (androgen-dependent) cells were shown to contain and secrete NT. However, exogenous NT was degraded primarily to NT^{1-11}, consistent with the presence of neutral endopeptidase 3.4.24.11 in these cells. This reaction was inhibited in the absence of androgen and in the presence of metalloprotease inhibitor phosphoramidon. In contrast, androgen-independent cells (PC3, DU145) did not contain or secrete NT and did not degrade exogenous NT. These studies clearly show that peptidases are present in LNCaP cells, but their role in degrading endogenous NT is not yet proven. Firstly, intact NT was recovered from the cells, not fragments of NT. In addition, the results obtained might also be explained if NT production is enhanced by androgen-deprivation as Sehgal et al. have in fact reported [73].

Although these studies argue strongly that androgen withdrawal from prostate cancer cells could induce an autocrine growth mechanism via a change in NT stability, recent work by Dal Farra et al. has added a degree of complexity to this picture [19]. Their results suggest that androgen withdrawal from LNCaP cells permits the NT growth effect by inducing the expression of another NT receptor NTS3. Whereas NTS1 mRNA expression did not change after 8 days in the absence of serum, NTS3 mRNA levels and NTS3 binding activity increased ≅10-fold. Thus, there is evidence supporting the importance of NT stability as well as NT receptor expression in the regulation of NT-induced cancer growth. More studies are warranted to identify other factors that might influence the operation of autocrine/paracrine systems involving NT (e.g., nutrients, hormones and drugs) and to determine whether NT production or NT receptor expression is used as a critical point of control.

2.2.2. *NT receptor expression in cancer*

A variety of primary human tumors have been examined by Reubi et al. for NT receptor content using *in vitro* receptor autoradiography on tissue sections [65]. Binding displacement curves generated for positive tissues were consistent with the presence of NTS1. The incidence of NT receptor expression was: Ewing's sarcoma (65%), meningioma (52%), astrocytoma (43%), thyroid cancer (29%), small cell lung cancer (25%), breast cancer (5%), colon cancer (4%), hepatic (0%), ovarian (0%) and prostate cancer (0%). Reubi's group also used an *ex vivo* scintigraphic technique to show the presence of NT receptor in 75% of pancreatic adenocarcinomas [64]. Interestingly, the reported incidence of NT receptor expression in immortalized cell lines derived from some human cancers (e.g., colon, 45%; Maoret et al. [51]) is higher than reported here for primary tumors. Discrepant results for cell lines and primary tumors could reflect differences induced by cell culturing and cell selection; however, instability of the NT ligand to tissue peptidases may also be a contributing factor. One wonders if results obtained by Western blotting or by measuring NT receptor mRNA might be more reliable. The results obtained by Ehlers et al. [26] and Elek et al. [28] using RT-PCR, argue that the incidence of NTS1 expression in primary human cancers of the pancreas, colon, prostate and ovary could be higher than indicated by the binding assay results above. Binding assays can give false negatives as exemplified by results obtained with prostate LNCaP cells, which express NTS1 by Western blot and respond to NT in functional assays (ERK phosphorylation) but do not bind ^{125}I-NT (Hassan, unpublished results). Thus, in drawing conclusions about the incidence of NT receptor expression in human tumors, one should consider results obtained using several methods including RT-PCR, Western blotting and functional assays.

Dal Farra et al. have done an in depth analysis of NT receptor expression in prostate, colon and pancreatic cancer cell lines, measuring NT receptor mRNA expression and NT binding [19]. In many of the cell lines examined, NTS1 appears not to be expressed, whereas NTS3 is present in all cell lines. Since NT exerts growth effects in some cells which were found to lack NTS1 (most notably LNCaP cells) and since NTS3-transfected CHO cells exhibit a growth response to NT (EC50, ≅200 nM), the authors argue that NTS3 could be an important mediator of NT-induced cancer cell growth. Particularly convincing is their finding that NTS3 mRNA expression and NT binding to soluble receptor increased 10-fold during LNCaP cell differentiation, which is known to be essential to demonstrate the NT growth effects. However, at odds with their results is the report by Elek et al. showing that androgen-deprivation upregulates

NTS1 mRNA expression in LNCaP cells [28]. Although these studies are very exciting, many questions remain unanswered: how does one reconcile the discrepancy between the EC50 for NT-induced growth in LNCaP cells (<1 nM) and that in NTS3-transfected CHO cells (≅200 nM) if not by the presence of NTS1? Do CHO cells transfected with NTS1 exhibit a growth response to NT in the absence of NTS3? Since NTS3 is not G protein-coupled and has a limited association (<10%) with the plasma membrane, how can it mediate the effects of NT which appears to be confined to the extracellular space? A reasonable idea considered by Martin et al. is that NTS3, which they have shown to heterodimerize with NTS1, modulates and/or facilitates the effects of NTS1 [52]. This study is discussed at more length in Section 3.3.

Results published by Souaze et al. argue convincingly that the upregulation of NTS1 in colon cancer is largely attributable to activation of the Wnt/APC signaling pathway [78]. Alterations in Wnt/APC occur often at the early premalignant stage, and are known to be critical to the progression of colon cancer. This group observed activation of the NTS1 gene by agents that caused β-catenin accumulation, as well as a decline of endogenous NTS1 when the tumor suppressor gene APC was restored to cancer cells. Given that chronic inflammation may initiate carcinogenesis, and that NT has important effects in models of colonic inflammation [89], the upregulation of NTS1 by the Wnt/APC pathway could be a key event in tumor progression.

3. Growth effects of NT agonists and antagonists on cancer cells *in vitro*

3.1. *Lung cancer*

Moody's group [21] was one of the first to document the ability of NT (0.5–10 nM) to stimulate clonal growth of SCLC cells (lines: NCI-H345, NCI-N417). Probing the mechanism of signal transduction, Staley et al. [79] found that NT elevated $[Ca^{2+}]_i$ levels (EC50, ≅1 nM) in the NIH-H345 cell line. The response to 0.1 nM NT was shown to decay over 6 min while the response to 10 nM NT decayed within 2 min. Since the decline was not due to depletion of the intracellular Ca^{2+} pool, the authors suggested that desensitization and/or internalization of NT receptor occurred in response to higher levels of NT. Supporting this idea was their finding that pretreatment of cells with 10 nM NT induced a tachyphylactic effect.

Further work by Moody et al. [59] was directed towards characterizing NT receptor in these cells and testing the effects of NT receptor antagonists on cell growth. SCLC cells (NCI-H209) displayed specific binding of ^{125}I-NT and ^{3}H-SR48692 in a manner suggesting that their binding sites overlapped partially. In keeping with this, the elevation of $[Ca^{2+}]_i$ and the increase in c-fos mRNA caused by NT (10 nM) were inhibited by pretreatments with SR48692 (5–10 μM). In addition, the inhibition of SCLC proliferation caused by SR48692 (1 μM) was reversed by NT (10 nM). Based on the effects of NT antagonists, the growth-promoting effect of NT appeared to be mediated by NTS1. Since these cells are known to produce NT, the inhibition of SCLC growth by SR48692 suggests that NT can function as an autocrine growth factor in some lung cancers.

Pursuing a completely different path, Tallet et al. hypothesized that NT might induce Tyr-phosphorylation as part of its growth effect [83]. NT (1–100 nM) was found to stimulate rapid (peak response, 5 min), concentration-dependent Tyr-phosphorlyation and Tyr-kinase activity in SCLC cells (lines: NCI-H69, NCI-H345, NCI-H510). The major Tyr-phosphorylation substrate was identified as p125 focal adhesion kinase (FAK) a cytoplasmic Tyr-kinase activated by phosphorylation on Tyr residues. The effects of NT were inhibited by 25 μM tyrphostin, which also inhibited the growth response to NT. Since the phorbol ester PMA and calcium ionophore A23187 did not induce Tyr-phosphorylation, it was concluded that the effects of NT on Tyr-phosphorylation occurred independently of PKC activation and mobilization of internal calcium.

Important mechanistic studies by Leyton et al. have shown that large cell lung cancer NCI-H1299 cells bind NT (IC50, ≅3 nM) and respond to NT (1–100 nM) with a transient (1–5 min) activation of FAK, followed by a doubling in colony formation [48]. Since NT binding and NT-induced FAK activation and cell proliferation were all inhibited by SR48692, the authors concluded that these effects were mediated by NTS1. FAK activation in response to NT was diminished by tyrosine kinase inhibitor (genistein), serine/threonine kinase inhibitor (H7) and an inhibitor of actin polymerization (cytochalasin D). Since these agents also inhibited NT-induced cell proliferation and since FAK activation is associated with increased oncogene expression, FAK activation may contribute to the NT-induced growth response. The authors speculated that NT operates as an autocrine growth regulator in this system since SR48692 inhibited basal growth in addition to NT-induced growth of these cells.

3.2. *Pancreatic cancer*

Ishizuka et al. were the first to report on the growth response to NT in pancreatic cancer MIA PaCa-2 cells [43]. The stimulatory effect of NT (10^{-12} to 10^{-8} M) on DNA synthesis correlated to its ability to elevate IP3 levels and to mobilize intracellular Ca^{2+}. However, at higher doses (>10^{-7} M), where NT stimulated cAMP formation, an inhibition of the growth response occurred. Since 8-Br-cAMP inhibited cell growth, it was speculated that NT enhanced cell growth at physiologic levels (via Ca^{2+}-mobilization) and that this response was inhibited at supra-physiologic levels (via cAMP). The authors proposed the involvement of a high affinity NT receptor (presumably Gq-coupled) and a low affinity NT receptor (presumably Gs-linked) in this mechanism of pathway cross-talk. However, other explanations involving only one NT receptor should be considered. Membrane binding studies by Yamada et al. suggest the presence of a single binding site in these cells, which has the character of NTS1. It could be that NTS1 couples to multiple G-proteins and evokes Ca^{2+} and cAMP responses with differing potency [85]. Even if NTS1 couples only to Gq, NT might exert downstream effects on Gs-linked systems (e.g., β2-adrenergic, PGE2), whose growth effects are potentiated by Gq-mediated superactivation of adenylyl cyclases that are sensitive to Ca^{2+} and PKC [57]. Finally, inhibition of NTs growth effect at high dose may be

due to receptor desensitization and/or downregulation and may have little to do with the rather modest effect (two-fold increase) that NT exerts on cAMP levels.

Studying Ca^{2+} responses to NT and its antagonist SR48692 in relation to the proliferation of pancreatic cancer MIA PaCa-2 and PANC-1 cells, Herzig et al. noted an important difference [41]. Whereas NT mobilized intracellular Ca^{2+} (and SR48692 inhibited this effect) in both cell lines, NT induced a proliferative response only in MIA PaCa-2 cells. Conversely, SR48692 inhibited basal growth in both cell lines, but NT reversed this effect only in MIA PaCa-2. Although the authors concluded that a Ca^{2+} response is not a sufficient prerequisite for NT-induced mitogenesis, an alternative explanation is that PANC-1 but not MIA PaCa-2 cells exhibit maximal growth in the presence of the 1% serum that was used as vehicle, making the effect of NT difficult to measure in PANC-1. Following up on this study, Ryder et al. found that NT (1–25 nM) induced rapid Ca^{2+} mobilization from intracellular stores, followed by Ca^{2+} influx in five different pancreatic cancer cell lines, including PANC-1 [69]. While responsiveness to angiotensin, bombesin, bradykinin and cholecystokinin varied in the cell lines, each gave a positive response to NT. In PANC-1 cells, NT dose–responsively induced phosphorylation of the MAP kinase ERK, and stimulated DNA synthesis (the cells were withdrawn from serum for these studies). Mek inhibitors (PD98059, U0126) blocked ERK activation in response to NT. Since SR48692 was shown to inhibit each of these responses, the authors concluded that NTS1 mediated the effects of NT on $[Ca^{2+}]_i$, ERK activation and DNA synthesis.

Ehlers et al. used MIA PaCa-2 cells to examine the effects of NT on the MAP kinases ERK and JNK, both of which are known to transmit mitogenic signals to the nucleus by activating transcription factors such as AP-1 [27]. NT (10 nM) was shown to activate ERK (maximal at 3 min) and JNK (maximal at 30 min), and these responses were followed by an increase in AP-1 binding activity (mostly c-Jun; maximal at 60 min). Since ERK and JNK can phosphorylate c-Jun, markedly stimulating transcription factor activity, these findings suggest that the mitogenic effect of NT involves activation of these pathways.

Guha et al. investigated NT signaling in PANC-1 cells and found that NT potently stimulated the ERK pathway through a PKC-dependent mechanism that did not involve transactivation of EGFR [36]. This conclusion was based on the finding that NT activated Raf-1 and enhanced the phosphorylation of Mek and ERK, but it did not phosphorylate EGFR or activate Ras. In fact, NT decreased the level of EGFR phosphorylation, possibly reflecting an action downstream that caused feedback inhibition. Also arguing against the involvement of EGFR was the finding that NT-induced ERK activation was not decreased by an inhibitor (GM 6001) of metalloproteases thought to generate EGFR ligands and was not sensitive to tyrosine kinase inhibitors (AG1478, PD158780) or src tyrosine kinase inhibitor (PP2). In contrast to the lack of dependence on EGFR, the requirement for PKC activity was absolutely clear. Raf-1 activation and ERK phosphorylation were both sensitive to PKC inhibitors (GF109203X, Ro31-8220) and to phorbol ester-induced PKC downregulation. NT also stimulated DNA synthesis and clonal growth in PANC-1 cells and these growth effects were PKC-dependent (inhibited by GF109203X) and Mek-dependent (inhibited by U0126). Since NT can activate PKC in these cells [36] and since PKC is known to activate Raf-1 [7], these findings suggest that NT acts in PANC-1 cells via a shortcut pathway involving a direct effect of PKC on Raf-1, followed by activation of Mek and ERK (Fig. 1). Although it is not known whether NT stimulates the PI3K/Akt pathway in PANC-1 cells, PI3K inhibitor (wortmannin) did not diminish ERK activation in response to NT [36].

Since many neuropeptides activate the ERK pathway by transactivating EGFR, Kisfalvi et al. carefully compared the effects of NT and EGF in pancreatic cancer cells [46]. NT stimulated ERK activation in PANC-1 and MIA PaCa-2 cells via a PKC-dependent (inhibited by Ro 31-8220) but EGF receptor-independent pathway (not inhibited by AG1478). In contrast, the response to EGF did not involve PKC. The effects of NT on ERK activation and DNA synthesis were synergistic with those of EGF. Whereas ERK activation in response to NT or EGF returned to baseline within 2 h, the combined effect was prolonged (>5 h). Similarly, the enhancement in DNA replication in response to NT plus EGF (4.5-fold) was greater than the additive effects of the individual agents (≅2.5-fold). Sustained activation of ERK was in fact necessary for the synergistic increase in DNA synthesis since addition of U0126 (Mek inhibitor) 2 h after NT and EGF (which converted sustained ERK activation to transient activation) greatly inhibited the DNA response. These findings support the idea that NT-induced ERK activation in PANC-1 cells involves a direct action of PKC on Raf-1 (scheme in Fig. 1).

The PKC dependence of NT growth signaling in pancreatic cancer cells led Guha et al. to search for potential mediators that were downstream of PKC [37]. Using PANC-1 cells, this group showed that NT (1–20 nM) induced a rapid (maximum, 10 min) and striking (10-fold) activation of PKD that involved translocation of the kinase to the plasma membrane. In the same dose range, NT enhanced DNA synthesis (≅2-fold). The effects of NT on PKD and on DNA synthesis were similarly sensitive to pretreatment with PKC inhibitors (GF-1 and Ro 31-8220). Thus, it is conceivable that PKD mediates some aspects of PKC-dependent mitogenesis in pancreatic cancers.

3.3. *Colon cancer*

Poinot-Chazel et al. [63] did some of the earliest studies investigating the mechanism by which NT promotes cell growth in CHO cells (transformed with human NTS1) and in colon cancer HT29 cells (known to express NTS1). NT (0.1–100 nM) enhanced ERK phosphorylation and ERK activity (maximum, 5 min), effects that were inhibited by SR48692. This was followed in 1–2 h by an induction of *Krox-24* protein expression. ERK activation and *Krox-24* induction in response to NT were strongly inhibited by PKC inhibitor (GF109203) and mimicked by PKC activator (PMA), indicating a dependence on PKC. Both responses were also partially inhibited by pertussus toxin (PTX), suggesting that NTS1 signaling involved both PTX-sensitive and PTX-insensitive G-proteins. The authors propose that growth stimulation by NT could involve the coupling of NT receptor to *Krox-24* induction via the MAP kinase cascade. This seems reasonable since *Krox-24* is an early response gene producing a zinc-finger transcription factor that targets several genes involved in cell division.

An extensive screening of colon cancer cell lines for growth responses to NT was performed by Maoret et al. [50]. NT stimulated growth of five different human cancer lines (SW480, SW620, HT29, HCT116, C1.19A) shown to express NTS1, whereas it had no effect in Caco-2 cells shown to lack NTS1. In SW480 cells, NT enhanced proliferation by two- to three-fold (EC50, ≅0.5 nM) and increased colony formation by ≅50%. Although SR48692 was shown to inhibit NT binding to cell membranes obtained from these colon cancers, its effects on cell growth could not be tested *in vitro* due to interference by solubilizing agents.

Martin et al. performed a rigorous biochemical analysis to probe the functional importance of NTS3/NTS1 heterodimers in HT29 cells [52]. Comparing HT29 cells to CHO cells expressing one or both receptors, the authors deduced that HT29 cells co-express NTS3 and NTS1 in a ratio of 20:1, and that the two receptors are complexed at the cell surface. The binding affinity of NTS1 measured in intact CHO cells was not changed much by its association with NTS3; however, the potency of NT to induce inositol phosphate (IP) formation or ERK phosphorylation was diminished in proportion to NTS3 expression. Thus, NTS3 (which could not promote either response) modulated the effects of NTS1 when co-expression was performed in CHO cells. For ERK phosphorylation, NTS3 shifted the NT dose–response curve to the right while increasing maximal stimulation two-fold. The authors suggested that NTS3 (by interacting with NTS1) generated a new mechanism of cell activation that could be necessary for the growth effect of NT. Although these results are enticing, it remains to be shown that NTS3 has any functional role at physiologic NT concentrations. In NTS1-expressing CHO cells (Martin's data), co-expression of NTS3 enhanced the absolute level of ERK activation in response to 10–100 nM NT but not to 0.1–1 nM NT. It also seems possible that the rightward shift in the NT dose–response curve demonstrated by Martin simply reflects the ability of NTS3 to compete for NT in a non-productive way. Further studies are warranted to sort out the precise role(s) of NTS3 in NT binding, signal transduction, desensitization and internalization.

3.4. *Prostate cancer*

Sehgal et al. performed the first work documenting a growth response to NT in androgen-dependent human prostate cancer LNCaP cells [73]. They found that while expression of NTS1 mRNA was independent of androgen status, a growth response to NT could only be shown in androgen-deprived cells. In the presence of NT (0.5–10 nM) and the absence of androgen for 12 days, cell growth was stimulated by ≅60%. Using an immunoassay to measure NT, they showed that the cellular production of NT and its secretion into conditioned media was stimulated by androgen-deprivation. However, the degradation of endogenous and exogenous NT by the cells was also promoted by androgen. Metalloprotease inhibitors decreased the androgen-associated loss of NT, suggesting that androgen-stimulated cells may become refractory to NT due to the induction of NT-degrading metalloprotease(s). The authors concluded that androgen-deprivation enhances both NT production and NT stability, leading to the development of an autocrine growth-regulatory mechanism.

To complement the work on androgen-dependent cells, Seethalakshmi et al. studied the PC3 cell line, a model of late stage androgen-independent cancer [72]. PC3 cells were found to express NTS1 mRNA and to bind ^{125}I-NT with high affinity. The receptor was similar to NTS1 in molecular size, reactivity with NT analogs and SR48692, and sensitivity to GTP analogs and NaCl. Unlike LNCaP cells, PC3 did not express NT precursor mRNA and did not contain, secrete or destroy NT. When added to PC3 cells, NT (0.1–1 nM) stimulated DNA synthesis and increased the number of cells, whereas SR48692 inhibited these effects. Since NT was active at near physiologic blood levels, these results support an endocrine or paracrine role for NT.

Because receptor tyrosine kinases are generally important for androgen-independent cancer cell growth, Hassan et al. investigated the interaction of NT with the EGF receptor (EGFR) and its associated signaling cascades in PC3 cells [40]. NT dose-dependently (0.1–30 nM) enhanced phosphorylation of EGFR, ERK and Akt (maximal, 3 min). There was an associated accumulation of EGF-like material in the medium and a two-fold increase in DNA synthesis. Each of these effects was inhibited by EGFR-tyrosine kinase inhibitor (AG1478), metalloendopeptidase inhibitor (phosphoramidon) and heparin but not by neutralizing anti-EGF antibody. Thus, NT appeared to transactivate EGFR via proteolytic liberation of cell-surface Hb-EGF or amphiregulin, rather than by release of EGF itself. PKC inhibitors blocked the effects of NT whereas PKC activator (PMA) mimicked NT, generating EGF-like activity and phosphorylating EGFR. PI3K inhibitors (wortmannin, LY294002) and Mek inhibitor (U0126) blocked the downstream effects of NT, including ERK phosphorylation and DNA synthesis. Although these signaling responses to NT did not require Ca^{2+} in the medium and were not blocked by calmodulin inhibitor, the growth effect of NT was Ca^{2+}-dependent. The authors concluded that NT stimulates mitogenesis in PC3 cells by a PKC-dependent ligand-mediated transactivation of EGFR, which leads to stimulation of the ERK pathway in a PI3K-dependent manner. They also suggested that PKC could play both causal and permissive roles, e.g., liberating EGF-like substances (causal) and facilitating the activation of Raf-1 (permissive).

Speculating that NT released from neurons might have a role in prostate cancer and realizing that NT is often co-stored with catecholamines, Mitra and Carraway studied the influence of a β2-adrenergic agonist on the growth effect of NT [57]. In the presence of isoproterenol, NT exerted an antimitogenic action in PC3 cells whereas in its absence, NT was mitogenic. The effect was attributed to the ability of NT to enhance adenylyl cyclase activation, doubling cAMP formation in response to Gs-activators [13]. NT did not alter cAMP degradation and did not affect the ability of dibutyryl cAMP to inhibit DNA synthesis. Thapsigargin and PMA mimicked the effect of NT on cAMP formation, suggesting that a Ca^{2+}-dependent and/or PKC-dependent superactivation of adenylyl cyclase(s) was involved. The crosstalk shown here in the NT and β-adrenergic signaling pathways implies that growth responses to NT may depend on the activity of catecholaminergic systems. This phenomenon may also apply to other regulators that utilize the cAMP signaling pathway such as PGE2. More effort seems warranted to define the growth

effects of NT in a more physiologic setting that includes inputs from other regulators.

Since some actions of NT depend on extracellular Ca^{2+} and are inhibited by Ca^{2+}-channel blockers, Carraway et al. tested blockers of voltage-gated and store-operated Ca^{2+}-channels for effects on NT binding and signaling in PC3 cells [10]. These agents dramatically enhanced NT binding to the cells and caused a parallel inhibition of NT-induced IP formation. Many of the agents exhibited these effects at doses known to inhibit store-operated Ca^{2+}-channels. The effects were receptor-specific and required intact cells, suggesting that they involved cellular metabolism. Nifedipine increased the apparent affinity of NTS1 ≅3-fold without changing the receptor number or the relative internalization rate. Although the results suggested that store-operated Ca^{2+}-channels might produce feedback effects on NTS1, further work indicated that the antioxidative nature of these drugs may have played a role since similar effects were produced by polyphenolic antioxidants [11]. Based on this, it was proposed that these agents disrupted NT signaling by inhibiting oxidative reactions or by scavenging ROS involved in regulating NTS1 function. Additional studies showed that a panel of lipoxygenase (LOX) inhibitors reproduced these effects, leading the authors to suggest that NTS1 function is regulated by lipid peroxidation or other LOX-dependent processes [12].

Hassan and Carraway also examined the involvement of LOX in NT-induced and EGF-induced DNA synthesis in PC3 cells [39]. NT and EGF stimulated arachidonic acid (AA) release from the cells, upregulated PLA2 and 5-LOX expression, and increased the formation of 5-HETE, which was shown to be mitogenic. Inhibitors of PLA2, DAG lipase and 5-LOX diminished the effects of NT and EGF on the ERK/Akt pathways, and on DNA synthesis. In a reciprocal manner, the ability of NT and EGF to enhance AA release was ERK-dependent. The authors proposed the existence of a feed-forward system involving cooperative interactions of the LOX pathway with the ERK/Akt pathways. The input of 5-LOX into NT-induced growth appeared to be both causal (via the generation of mitogenic 5-HETE) and permissive (via effects on NTS1 function). Although further work is required to fully understand the mechanisms involved, these findings are in agreement with a growing literature supporting the importance of 5-LOX in cancer cell growth [34].

Neuropeptides such as NT can substitute for androgen in sustaining the growth of prostate cancer cells. Lee et al. [47] have addressed the possibility that NT contributes to androgen-independent growth of LNCaP cells by activating the androgen receptor (AR). Using LNCaP cells transfected with reporter constructs, NT was shown to activate the promoter for prostate specific antigen (PSA), a known target of the activated AR. NT also stimulated the secretion of PSA by normal LNCaP cells. Further work in PC3 cells (which lack AR expression) and in PC3(AR)2 cells (transfected with the AR gene) suggested that AR was required for NT to stimulate the PSA promoter. Postulating that NT activated AR and that this was an indirect effect, Lee searched for possible mediators. Attention was focused on tyrosine kinases since they are known to activate AR. Further work identified FAK, Src and Etk as tyrosine kinases activated by NT in LNCaP cells. Then, using dominant negative constructs for these kinases transfected into LNCaP cells, Lee showed that the growth response to bombesin (and presumably NT) depends on the formation of an active signaling complex containing each kinase. The role of Src in bombesin-induced growth was also confirmed using Src inhibitor PP2. Although it is clear from this work that NT activates FAK, Src and Etk and that this signaling complex is required for NT-induced growth in transfected LNCaP cells, one can only speculate on the connection between this and the activation of AR. Obviously, the growth response to NT in PC3 cells does not require AR expression since these cells are AR negative. It could be interesting to compare the growth effects of NT in PC3 and PC3(AR)2 cells. One might also determine whether transfecting LNCaP cells with dominant negative AR alters the growth response to NT. Also, since Lee et al. showed that PKC inhibitors did not appreciably affect AR activation by NT, it could be worthwhile to test whether NT-induced LNCaP growth is PKC-dependent.

3.5. *Breast cancer*

Somai et al. found that breast cancer (MCF-7) cells express NTS1 and NTS3 mRNA, and bind ^{125}I-NT with Kd of ≅2 nM [77]. Exposing MCF-7 cells to NT agonist JMV-449 for 96 h caused a 60% increase in cell number via an anti-apoptotic effect that appeared to involve upregulation of Bcl-2 expression. There was an associated two-fold elevation in the levels of Bcl-2 mRNA and Bcl-2 protein that was blocked by Mek inhibitor (PD98059) but not by PKC inhibitor (D-sphingosine). The levels of p53 and caspase 3 did not change. The NT agonist also reduced by 65% the apoptotic response to serum withdrawal, and this effect was blocked by the Mek inhibitor. These results are consistent with the fact that NT can activate the MAP kinase (ERK) pathway and that ERK is known to mediate Bcl-2 promoter activation in some cells. The lack of dependence on PKC is surprising and could be examined using other PKC inhibitors.

4. Model for NT-induced growth signaling

Drawing from these studies, we have constructed a composite model of the signaling pathways implicated as potential contributors to NT-induced growth of cancer cells (Fig. 1). Our model assumes that the basic framework is similar in different cancers and what might vary is the activity level or expression level for the participants in each pathway. Thus, mechanistic differences reflect changes in pathway emphasis due to specific mutations that characterize each cancer. To initiate signaling, NT binds to Gq-coupled NTS1 whose effector (PLC) ostensibly causes an elevation in $[Ca^{2+}]_i$ and an activation of PKC (via IP3 and DAG, respectively). Since NTS3 is not G protein-coupled, it may not initiate signaling but it can modulate NTS1 function by forming heterodimers, and it may also effect the desensitization and internalization of NTS1.

The mechanism of growth stimulation by NT is similar to that for other neuroendocrine messengers in that a major step involves activation of the ERK pathway [35,54]. This cascade of kinases regulates a number of nuclear transcription factors that determine the synthesis of proteins involved in prolif-

eration and differentiation [75,76]. For NT, the pathway leading to ERK activation can differ, depending on the cancer. Some cancers are similar to prostate cancer PC3 cells, where ERK activation proceeds primarily via transactivation of EGFR, a mechanism that is common to several Gq-coupled receptor systems [20]. This generally involves a PKC-mediated shedding of cell-surface bound EGF-like substances (Fig. 1, path 1). However, in pancreatic cancer PANC-1 cells, NT appears to act independently of EGFR. Here, it seems that a direct PKC-dependent activation of Raf-1 occurs in response to NT (Fig. 1, path 2). This mechanistic difference may reflect the effects of an altered Ras activity in PANC-1 cells [36]. One might speculate that constitutively active Ras exerts feedback effects on EGFR and/or alters the requirements for Raf-1 activation. Since activation of Raf-1 involves: (a) translocation of the enzyme to membrane and (b) phosphorylation of the enzyme, it could be that PC3 and PANC-1 cells differ in regards to these steps. One possibility is that the altered Ras activity in PANC-1 cells promotes Raf-1 translocation, priming it for direct activation by PKC. In PC3 cells on the other hand, stimulation of EGFR (elevating Ras activity) may be required for PKC-mediated Raf-1 activation. Thus, PANC-1 cells may be a special case in which constitutive Ras activity nullifies the requirement for EGFR in the response to NT.

In general, mitogenesis is promoted by coordinate activation of the ERK/Akt pathways, which cooperate to regulate the expression of cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors in a manner that activates E2F transcription factors [56]. Thus, pharmacological inhibition of either the ERK or the Akt pathway generally inhibits the growth effects of Ras activators. For NT, the evidence suggests that it activates these two pathways in some cells by engaging EGFR, which presumably exerts its effects via Ras (Fig. 1). In PC3 cells, NT-induced ERK activation and DNA synthesis are PI3K-dependent [40]. Since PI3K/Akt activity has been shown to be required for cannabinoid-induced activation of Raf-1 in PC3 cells [70], we propose that PI3K, PDK1 and/or Akt facilitate Raf-1 activation in response to NT (Fig. 1). One might speculate that PI3K/Akt act to translocate Raf-1, facilitating its subsequent phosphorylation and activation by PKC. PI3K acts by converting PIP2 to PIP3 and its actions are subject to modulation by PTEN, a phosphatase that destroys the signaling product PIP3. Thus, the emphasis on this pathway depends on whether the cells express PTEN activity (LNCaP cells) or lack PTEN activity (PC3 cells).

The non-receptor Tyr-kinase FAK is involved in cell adhesion, cell migration and cell cycle progression. Although FAK is primarily regulated by integrin binding to the extracellular matrix, some GPCR agonists, including NT, have been shown to activate FAK. Thus, recruitment of FAK provides yet another mechanism for diversification of NT-induced growth responses (Fig. 1). Our model shows FAK downstream of EGFR since NT-induced FAK activation in lung cancer cells is inhibited by tyrphostin AG1478 [83] and

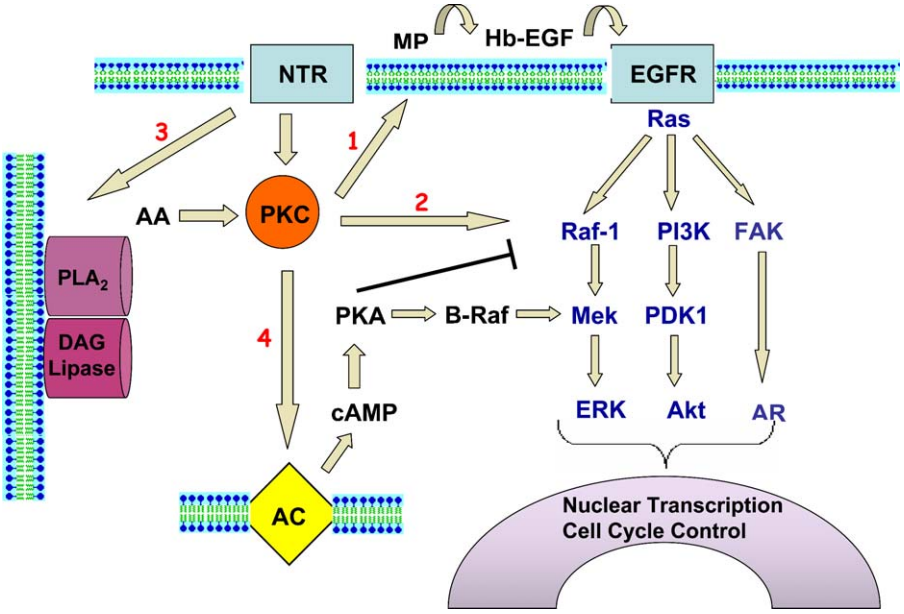

Fig. 1 – Diagram of pathways that appear to contribute to NT-induced cancer cell growth. NT interacts with NT receptor (NTR) to activate a set of PKC isotypes that may specify which paths (1, 2 or 3) the signal takes. This also depends on the level of expression for the effectors involved. Path 1: cell-surface shedding of EGF-like ligands via the action of metalloprotease(s) activates EGFR, which engages various kinase cascades shown to promote nuclear transcription and cell cycle control. The influence of PKC could be both causal (via path 1) and permissive (via path 2). Path 2: direct activation of Raf-1 by PKC can also occur independent of EGFR (the shortcut loop). Path 3: by stimulating store-operated Ca^{2+}-channels, NT activates PLA2 and DAG lipase to liberate AA, which is converted to LOX products. This is pictured to further enhance the activation of growth-regulatory PKCs. Path 4: NT can elevate cAMP levels in cancer cells and this is pictured as involving a superactivation of AC. This effect requires that there is a background of Gs activity, presumably from another GPCR coupled to Gs. Usually, the outcome is growth inhibition via PKA-mediated effects on Raf-1. However, in cases where B-Raf levels are elevated, growth enhancement occurs via effects on Mek.

genistein [48]. However, activation of FAK in response to NT also involves an elevation in $[Ca^{2+}]_i$ and a change in the actin cytoskeleton (not pictured in Fig. 1). Activated FAK forms a complex with Src and Etk, which is required for bombesin-induced growth in prostate LNCaP cells [47]. Since this complex seems to mediate bombesin-induced AR activation, it could be that AR participates in bombesin- (and presumably NT) induced growth of LNCaP cells. Although not yet proven, we indicate in Fig. 1 the possibility that AR has a role in transmitting the FAK signal to activate nuclear transcription factors.

The AA cascade participates in the regulation of cancer cell growth, and the LOX arm is particularly important in prostate cancer. The results of Hassan and Carraway indicate that AA release and 5-LOX activity are required for NT and EGF to stimulate the ERK/Akt pathways and DNA synthesis in prostate PC3 cells [39]. Thus, our model pictures NTR1 acting to liberate AA from membrane phospholipids by causing Ca^{2+}-mediated stimulation of DAG lipase and PLA2 (Fig. 1, path 3). Presumably, the metabolism of AA by 5-LOX produces oxidized products that can further stimulate the PKC pathway either directly or via effects on specific GPCRs.

The negative growth effect of cAMP seen in some systems is thought to involve inhibition of the ERK pathway at the level of Raf-1 [18]. This may explain the bell-shaped dose dependence of the NT growth effect in some cancers, since NT can promote cAMP formation. This relatively minor effect on cAMP levels (two-fold) can be greatly magnified (>500-fold) in the presence of a Gs agonist (e.g., isoproterenol), such that NT becomes growth inhibitory. Like growth stimulation, growth inhibition by NT appears to be PKC-mediated (not Gs-mediated) and seems to involve superactivation of adenylyl cyclase (Fig. 1, path 4). Adding another degree of complexity is the fact that cAMP can produce both negative and positive growth effects in various cell systems [80], which can depend on cell type [86], cell density [82] and the availability of Ca^{2+} [87]. Some of these variables may be working by way of altering the level of expression for isotypes of PKA [6] and B-Raf [82] that can mediate ERK activation to stimulate growth (see Fig. 1). For example, the fact that cAMP is growth stimulatory in prostate LNCaP cells but growth inhibitory in PC3 cells has been attributed to enhanced expression of B-Raf isotype in LNCaP [17]. Preliminary results also indicate that the ability of NT to stimulate DNA synthesis in PC3 cells decreases with increasing cell density (Hassan, unpublished results). Whether this contact inhibition is due to changes in cAMP formation or changes in the cAMP response machinery is not known. These observations suggest that the effects of NT on the ERK pathway may depend on inputs that can stimulate cAMP formation and conditions that can influence the expression and activity of various effectors involved in the cAMP pathway (Fig. 1).

Some investigators believe that it may be improper to rigorously assign specific roles to growth factor pathways, not only because of system redundancy and signaling crosstalk but also because the end effects of pathway stimulation can be interdependent. For example, metabolic effects might be expected to influence any growth effects that arise from the regulation of DNA synthesis, cell cycle, apoptosis and cytoskeletal interactions. Therefore, it may be expedient to adopt a holistic view, not only with respect to intracellular mechanisms by which NT enhances cancer cell growth *in vitro* but also in regards to mechanisms in animals bearing tumors. We elaborate on this idea in Section 7 which presents a brief discussion of how NT might initiate multiple mechanisms that could cooperatively influence tumor growth *in vivo*.

5. Effects of NT receptor agonists and antagonists on cancer growth *in vivo*

5.1. *Lung cancer*

Moody et al. found that SR48692 (given orally in PEG-400) inhibited the proliferation of human SCLC cells (NCI-H209) xenografted in nude mice [59]. Over the 5-week period, tumor growth was inhibited 20% and 99% at a dose of 0.04 and 0.4 mg/kg/d, respectively. Prior work by this group showed that SCLC cells produce NT immunoreactivity, express NT receptor and display enhanced clonal proliferation in response to NT [21]. In total, these findings strongly support the idea that SR48692 can inhibit the autocrine action of NT produced by these tumors *in vivo*.

5.2. *Pancreatic cancer*

MIA PaCa-2 cells express NTS1 mRNA and bind ^{125}I-NT with high affinity (IC50, ≅2 nM). Pretreatment with SR48692 inhibits this binding as well as the ability of NT to stimulate Ca^{2+} mobilization, IP3 formation and cell growth *in vitro*. However, Sumi et al. found that despite this potential, NT (0.6 mg/kg; 3 times/day given s.c.) exerted only a marginal effect on MIA PaCa-2 tumor growth in athymic nude mice [81]. Since the intestinal mucosa of the same animals gave a strong trophic response, the dose of NT appeared to be sufficient. On the other hand, Iwase et al. showed that this same dose of NT significantly enhanced MIA PaCa-2 tumor growth in nude mice [44]. They also found that NT antagonist SR48692 (2 mg/kg given s.c. in 10% DMSO) inhibited the response to NT, but it had no effect on basal tumor growth in these animals. These findings suggest that endogenous NT does not play a major role in the growth of MIA PaCa-2 cells *in vivo*. Apparently, MIA PaCa-2 cells do not produce NT and do not depend on circulating levels of NT in mice fed normal chow. However, it is still possible that the elevated levels of NT in animals given a high fat diet might contribute importantly to the growth of such tumors.

5.3. *Colon cancer*

NT receptor antagonist SR48692 (2 mg/kg/d given s.c. in 10% DMSO) was found by Iwase et al. to inhibit the growth stimulatory effect of NT (0.6 mg/kg) on xenografted human colon cancer (LoVo cells) in nude mice [45]. Treatment with NT enhanced tumor growth, weight, DNA and protein content. SR48692 blocked NT-induced growth but had no effect on basal growth of the tumor. In contrast to the *in vivo* results, NT had no effect on LoVo cell growth *in vitro*. Consistent with this, LoVo cells and the derived tumors did not bind ^{125}I-NT and showed no NTS1 expression by Northern blot analysis. These

results suggest that the trophic effects of NT on LoVo cancers *in vivo* may be indirect, possibly involving the release of other growth-promoting factors.

Maoret et al. examined the effects of NT and NT antagonist on the growth of colon cancer SW480 cells xenografted into nude mice [50]. Continuous infusion of NT (Alzet pump, 0.54 μmol/kg/d) increased tumor growth to 255% of control. Conversely, SR48692 (given i.p. in tween 80 at 0.17 and at 1.7 μmol/kg/d) reduced tumor volume by 26% and 33%, respectively. Since SW480 cells do not produce NT, the authors reasoned that NT released from endogenous sources such as the intestine contributes to the growth of this colon cancer. Given that fat is the strongest stimulus for intestinal NT release, it is possible that NT may contribute to the epidemiological association between dietary fat intake and colorectal cancer incidence [50]. Further examination of their data suggests to us that the major effect of SR48692 was to delay tumor development since tumor growth rates towards the end of the experiment were similar to control. Thus, one wonders if these tumors become refractory to SR48692 in time and whether they would have developed fully, given sufficient time, despite the effects of SR48692.

Collectively, these studies indicate that tumor growth *in vivo* can be driven by tumor-derived NT (e.g., lung cancers) and by endogenous NT (e.g., colon cancers). In some studies, SR48692 dramatically inhibited the early phase of tumor growth but had less effect towards the end of the experiments. Similarly, we found that growth of prostate PC3 xenografts in SR48692-treated nude mice and in NT knockout-nude mice was slowed compared to controls primarily during the early stage of tumor development (Carraway, unpublished results). Although there are many possibilities, this behavior might be expected if the growth-promoting effects of NT depend on cell density. Indeed, the proliferative effect of NT in PC3 cells *in vitro* is most easily demonstrated in sparsely populated cells, suggesting that the cells become refractory to NT as cell density increases (Hassan, unpublished results). The effect does not seem to involve a change in NT receptor expression. Another explanation is that once a critical mass of cells is obtained, autocrine factors such as Hb-EGF drive cell growth independently of NT.

6. NT analogs for diagnostic imaging and therapeutic targeting of cancer

In order to gauge the potential value of *in vivo* NT receptor scintigraphy in the diagnosis of pancreatic cancers, Reubi et al. have used receptor autoradiography to measure NT receptor expression in pancreatic tissue from human patients [64]. NT receptor was found in 75% of all ductal (exocrine) pancreatic adenocarcinomas while endocrine pancreatic cancers were NT receptor negative. These results show that NT receptor might be a marker for such cancers since normal tissue and tissue from patients with pancreatitis were also NT receptor negative. This group has also succeeded in labeling DTPA-derivatized NT ligands with ^{111}In, a commonly used radioisotope in diagnostic medicine. These findings led them to suggest that NT receptor scintigraphy might possibly be used in the detection of pancreatic cancer. The fact that NT receptor is largely internalized upon the binding of NT-related ligands indicates that NT receptor might also serve to target cancer cells with various therapeutic agents, including radioactive isotopes and toxins.

Mazor et al. labeled NT with Eu^{3+} via the chelator DTPA attached to the ε-amino group of Lys^6 naming the conjugate Eu-NT [53]. This derivative was then tested as a fluorescent probe for receptor binding in colon cancer HT29 cells. The Kd for binding of Eu-NT was ≅10-times that determined using ^{125}I-Tyr^3-NT and the EC50 for inducing Ca^{2+} transients was ≅50-times that for NT. The high signal to noise ratio of Eu-NT suggested that similar derivatives (with the chelate at the N-terminus of NT) could be highly useful alternatives to radioactivity. Aiming to label NT with ^{111}In to enable imaging of NT receptors in mice bearing HT29 tumors, de Visser et al. characterized six NT analogs containing N-terminal DTPA and a number of stabilizing amino acid substitutions [24]. The most promising derivative (#2530: DTPA-(Pip)Gly-Pro-(PipAm)Gly-Arg-Pro-Tyr-Tle-Leu-OH) displayed an increased stability in human plasma (recovery: 96% versus 2% after 4 h) and high affinity binding and internalization by NT receptor in HT29 cells *in vitro* (IC50: ≅3 times that for NT). Biodistribution studies in nude mice bearing HT29 tumors indicated that ^{111}In-labeled #2530 (0.1 μg/mouse) accumulated within tumors in a specific manner (1.1% ID/g at 4 h). The tumor/blood ratio was >50. The authors concluded that this DTPA-conjugated analog was an excellent candidate for scintigraphic imaging of tumors overexpressing NT receptor. They further suggested that a DOTA-conjugated analog labeled with β-emitters (^{177}Lu, ^{90}Y) might be useful for radionuclide therapy.

The effects of amino acid substitutions on the biostability and receptor binding of five analogs of [^{99m}Tc]-NT^{7-13} were investigated by Bruehlmeier et al. [4] and Blauenstein et al. [3]. Peptide labeling with ^{99m}Tc was by virtue of (NαHis)Ac placed at the N-terminus. Stability in human plasma was most affected by replacing Ile^{12} with Tle (40-fold increase in $T_{1/2}$), although additional stability was conferred by placing Lys(χCH_2NH)-Arg or (N-Me)-Arg-Lys in the Arg^8-Arg^9 position corresponding to NT. All of these analogs displayed high affinity for NTS1 (Kd, <1.8 nM) when tested for binding to intact HT29 cells. Using mice bearing HT29 tumors, biodistribution studies were performed for three analogs containing Tle^{12} and varying substitutions at Arg^8-Arg^9. While specific uptake into tumor was observed for all analogs (2.9–3.9% ID/g at 1.5 h), distribution into other tissues varied. The parent Arg^8-Arg^9 analog gave far more uptake into liver, lung and kidney than the (N-Me)-Arg^8-Lys^9 and the Lys^8(χCH_2NH)-Arg^9 analogs. The more selective uptake appeared to relate to the greater stability of these peptides, although the ability to compete with endogenous NT for binding sites in other tissues may have also been a factor. The authors concluded that they had identified two NT analogs that could label HT29 tumors in mice with a greater selectivity relative to other tissues. Garcia-Garayoa et al. performed additional studies using one of these preferred analogs ((NαHis)Ac-N-Me-Arg-Lys-Pro-Tyr-Tle-Leu) in mice bearing colon cancer (HT29) and prostate cancer (PC3) xenografts [33]. Tumors were easily visualized in scintigraphic images 1.5 h after injection of this ^{99m}Tc-labeled NT-analog (0.2 pmol) into mice. Although the peptide accumulated non-specifically in kidney and liver (2–6% ID/g), specific uptake was

demonstrated into tumor (≅4% ID/g) and intestine (≅1.5% ID/g). This group also succeeded in labeling this NT analog with ^{185}Re and ^{187}Re, radionuclides that have therapeutic applications.

Using NT$^{6-13}$ as a template, Achilefu et al. synthesized more than 24 NT analogs with amino acid substitutions at positions corresponding to Arg^{8}, Ile^{12} and Leu^{13} in NT and with DTPA at the N-terminus to enable labeling with ^{111}In [1]. The stability of each analog was determined during incubation with human serum. Substitution of Tle for Ile^{12} strikingly enhanced peptide recovery (five-fold), whereas replacement of Arg^{8} with Gly(PipAm) had a minor effect and replacement of Leu^{13} with Cha or tBuAla had no effect. Of the analogs that were >90% stable for 4 h, peptide #17 (DTPA-Gly(Pip)-Pro-Gly(PipAm)-Arg-Pro-Tyr-Tle-Leu-OH) exhibited the highest binding affinity for HT29 cell membranes (IC50, 0.3 nM) as compared to NT (0.2 nM). Biodistribution studies using peptide #17 in mice bearing HT29 tumors indicated specific uptake into tumor (2.18% ID/g at 4 h). The authors concluded that that the features of peptide #17 might be exploited to selectively deliver diagnostic and therapeutic drugs to target tissues.

The first human studies were reported by Buchegger et al. who employed the ^{99m}Tc-labeled NT-analog ((NαHis)Ac-Lys-(χCH$_2$-NH)-Arg-Pro-Tyr-Tle-Leu) to perform scintigraphy over 4 h in four patients presenting with ductal pancreatic adenocarcinoma [5]. Injection of 20 μg permitted visualization of the tumor in patient #4, the only patient with a NT receptor-positive tumor. Radiopeptide half lives in whole body and blood were 1.7–6.3 h in keeping with work in animals. At surgery (≅20 h post-injection), radioactive uptake into tumor (% ID/g) was 0.09 and the tumor to blood ratio was 9.2. The authors concluded that the results in humans were encouraging and in keeping with predictions obtained in mice.

These studies highlight the remarkable progress that has been achieved in the design of stable analogs of NT that can be used for scintigraphy and other diagnostic and therapeutic applications. These substances appear to be rather non-toxic compounds that are stable for sufficient time *in vivo* to penetrate and accumulate within tumors. Despite the high non-specific uptake into liver and kidney for many of them, these analogs can be used to visualize tumors in animals and humans. In order to capitalize on these promising results, future efforts could be directed towards improving the efficiency and selectivity of tumor uptake. For example, by altering the radionuclide/ligand ratio, one might enhance the signal imparted to the tissue. It may also be possible to use adjuvants to specifically stimulate ligand uptake by tumor cells [11].

7. Speculative comments and future directions

Here we speculate about the role of the NT system in the development and progression of epithelial cancers. Key to our model is the involvement of NT in inflammation (Fig. 2). Genetic and epigenetic events alter proto-oncogenes and tumor suppressor genes, eventually leading to cancer. Recent insights indicate that inflammation can alter genetic stability and that chronic inflammation could be a forerunner of neoplastic transformation. Inflammatory cells generate cytokines, matrix metalloproteases and ROS that activate growth signaling and stimulate oxidative reactions that impart genomic damage. Assuming that chronic inflammation initiates some cancers, then what evidence supports the

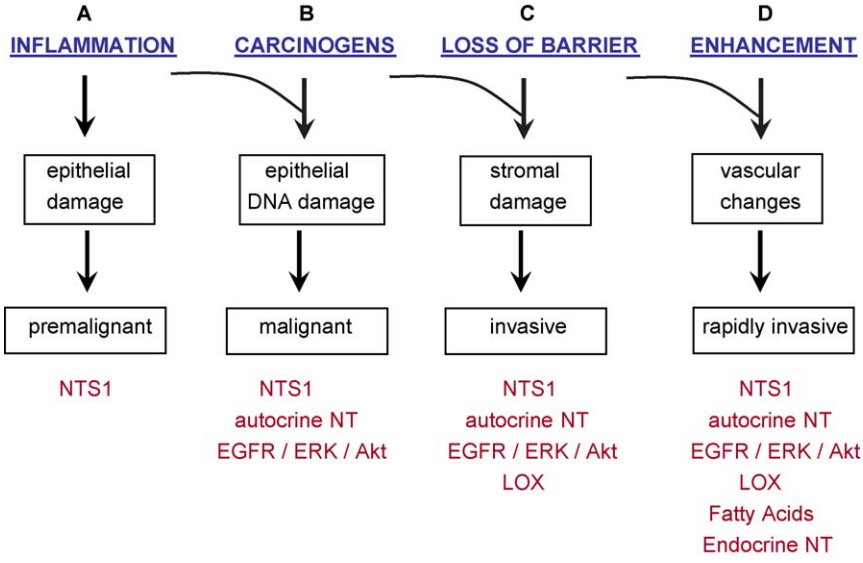

Fig. 2 – Scheme showing proposed relationship between cancer progression and the status of the NT system. Chronic inflammation produces cytokines, enzymes and ROS that damage epithelial cells leading to a premalignant state (A). Genetic predisposition and carcinogens facilitate the transformation to a malignant state (B). Loss of the epithelial barrier alters the stroma, giving rise to an invasive state (C). Enhancers such as dietary fat lead to vascular changes that enhance growth and promote motility (D). Although NTS1 expression might increase progressively from A to D, we speculate that NT signaling is enhanced primarily by upregulation of its signaling partners (EGFR, ERK, Akt and LOX) and perhaps by an increased release of NT in response to fat. The order of signaling partner expression undoubtedly varies for different cancers.

involvement of NT? During *C. deficile* toxin-induced inflammation of the colon, NTS1 is dramatically upregulated and administration of SR48692 inhibits aspects of the inflammatory response [16]. NT also stimulates non-transformed human colonic epithelial NCM460 cells to release the inflammatory cytokine IL-8 by a NF-κB-dependent mechanism [88]. Since IL-8 is chemotactic for inflammatory cells and NF-κB regulates the expression of many genes involved in inflammation, this suggests that upregulation of the NT system could lead to a cycle of inflammation and ultimately to cancer. The scheme in Fig. 2 proposes that NTS1 is expressed at the pre-malignant stage and that NT signaling is enhanced in a stepwise manner by the upregulation of each of its signaling partners.

Assuming that NT has a hormonal role as a tissue growth factor, one might speculate that it acts on a number of inputs (both directly and indirectly) in a coordinated fashion. Thus, NT might enlist the help of secondary regulators to orchestrate a series of changes involving multiple tissues and processes to support a growth event. For example, it would be beneficial to coordinate changes in metabolism, blood flow and substrate availability with changes in growth stimulation. Viewing tumor growth from this perspective suggests that many of the effects of NT *in vivo* could be indirect and several findings support this idea. NT receptor is largely absent from intestinal mucosa [74], yet NT given to animals very effectively stimulates growth of this tissue [84]. NT enhances the growth of colon cancer (LoVo cells) in mice despite the absence of NT receptor in the tumors and the inability of NT to enhance LoVo cell growth *in vitro* [45]. While the incidence and size of chemically induced gastric tumors in rats are increased by NT [61], these tumors may not express NT receptor given that human gastric tumors lack NT receptor [65]. One possible explanation for such findings is that some of the growth effects of NT *in vivo* are indirect, perhaps involving the release of other mitogens or the stimulation of rate-limiting processes such as blood flow.

The importance of the vascular supply to tumor development is well established [32] and suggests that the delivery of oxygen, nutriment and other blood-borne factors may be rate-limiting for growth. As compared to blood serum, NT has a minor effect on the growth of PC3 cells *in vitro*. DNA synthesis is greatly enhanced in the presence of serum factors, the majority of the activity residing in the large-molecular protein fractions (Hassan, unpublished results). NT given to rats potently enhances vascular permeability, causing proteins to enter tissue spaces [9]. This effect involves the degranulation of mast cells and the release of various mediators [8]. Tumors often possess large numbers of mast cells and display signs of increased permeability, such as fibrin deposition in the interstitial spaces [66]. Thus, we suggest that some of the growth effects of NT *in vivo* may stem from its actions on the circulation and the reticuloendothelial system. By promoting vasodilation and releasing VEGF to stimulate angiogenesis, NT could enhance the delivery of oxygen and nutriment for growth. By stimulating mast cells, NT could enhance vascular permeability to mitogenic proteins. By targeting resident macrophages, NT could promote the local production of mitogens and survival factors. Other more complex mechanisms could involve actions of NT at distant sites (e.g., liver) known to regulate general metabolism and to synthesize important mediators. Obviously, the effects of NT on the digestion and absorption of fats could also contribute since tumor growth is highly dependent on fatty acid metabolism [42,71].

8. Conclusions

We have made considerable progress in the identification and the imaging of cancers that overexpress NT receptors, as well as in the description of signaling events involved in the direct effects of NT on cancer cells *in vitro*. However, the paucity of information concerning mechanisms that operate *in vivo* suggests that additional studies are warranted and could produce some interesting and unexpected findings.

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Neurotensin receptor binding and neurotensin-induced growth signaling in prostate cancer PC3 cells are sensitive to metabolic stress

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Abstract

Neurotensin (NT) stimulates the proliferation of prostate cancer PC3 cells, which express high levels of its G protein-coupled receptor NTS1. To shed light on mechanisms that might serve to coordinate mitogenic responses to metabolic status, we studied the effects of metabolic inhibitors on NTS1 function. We also related these effects to cellular ATP levels and to the activation of AMP-activated protein kinase (AMPK). Glycolytic and mitochondrial inhibitors, at concentrations that reduced cellular ATP levels, altered NT binding to the cells, inhibited NT-induced inositol phosphate formation, and inhibited NT-induced DNA synthesis. For eight of the nine inhibitors, the potencies to alter NT receptor function correlated to the potencies to decrease cellular ATP levels. In keeping with its known role to oppose metabolic stress, AMPK was activated by the metabolic inhibitors. Accordingly, the AMPK activator AICAR elevated cellular ATP levels and produced effects on NTS1 function that were opposite to those for the metabolic inhibitors. These results indicate that metabolic stress inhibited NTS1 function by a mechanism that involved a fall in cellular ATP levels and that was opposed by activation of AMPK. In a broader context, these findings are compatible with the idea that one means by which cells might coordinate mitogenic signaling to metabolic status could involve changes in growth factor receptor function.

Keywords: Neurotensin; G protein-coupled receptor; Metabolism; Receptor binding; Growth signaling; Cancer cell growth; Metabolic stress

1. Introduction

Metabolic sensors, such as AMP-activated protein kinase (AMPK), sense the energy charge in cells and regulate metabolic enzymes involved in energy production and utilization [1,2]. As might be expected, the AMPK cascade has also been shown to coordinate cell proliferation with the availability of fuel and oxygen [3,4]. This led us to hypothesize that the responsiveness of cells to mitogenic signals could depend on the cellular energy status, and that some of this regulation might involve changes in mitogen receptor function. To address this hypothesis, we have employed the human prostate cancer PC3 cell line, which exhibits growth responses to neurotensin (NT) mediated by its type 1 G protein-coupled receptor NTS1 [5–7].

NT is a neuroendocrine peptide which promotes the growth of the intestinal mucosa *in vivo* [8] and the proliferation of dispersed hepatocytes in culture [9]. The significance of NT in the regulation of tissue growth has been reviewed by Evers [10]. NT receptor binding activity [11] and NTS1 mRNA [12] are grossly overexpressed by a variety of primary human cancers as compared to normal tissues. In addition, human cancer cell lines, including those from lung [13,14], pancreas [15,16], colon [17,18] and prostate [19], express high levels of NTS1 and exhibit growth responses to sub-nanomolar concentrations of NT. These findings and the epidemiological association of cancer incidence with high fat intake around the world [20] suggest that NT, whose release from the gut is enhanced during fat digestion [21,22], could be an important contributor in particular to the fat-induced component of tumor growth in humans. The significance of NT in the regulation of cancer cell growth has been reviewed [23].

Prostate cancer (PC) is the most commonly diagnosed cancer in Western men and the second leading cause of cancer-related death [24]. Although PC generally presents as an androgen-dependent disease that can be successfully treated by androgen ablation therapy, it inevitably progresses to an independent

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stage that leads to the demise of the patient [25]. Investigating factors that might contribute to this transition, Sehgal et al. showed that the androgen-dependent human prostate cancer LNCaP cell line exhibited an autocrine growth response to NT only in the androgen-deprived condition [19]. Vias and coworkers found that long-term anti-androgen treatment of LNCaP cells produced a sub-line exhibiting upregulated expression of NT and NT receptors, along with an enhanced growth rate and invasiveness that was sensitive to siRNA knockdown of NT [26]. Seethalakshmi et al. showed that the human androgen-independent PC3 cell line, which was derived from PC metastasized to bone, expressed very high levels of NT receptor NTS1 and gave growth responses to NT at concentrations near to postprandial blood levels in humans [27]. These and other studies [5,6] support the notion that NT is a potential autocrine, paracrine and endocrine regulator of PC growth in humans, particularly after androgen ablation therapy and during the devastating final stages of the disease.

Since 1956, when Otto Warburg noted that cancer cells exhibit unusual glycolytic activity [28], there has been an increasing appreciation for the metabolic differences between normal and cancerous cells and the hope that metabolic vulnerabilities will be identified that might serve as the basis for the development of new anti-cancer therapies [29,30]. For example, glycolytic and mitochondrial inhibitors have shown some promise as anti-cancer agents for *in vitro* and *in vivo* work [31,32]. Metabolic inhibitors and other conditions that deplete cellular ATP and elevate AMP levels can initiate the activation of AMPK, which has an ability to modulate ATP-consuming and ATP-generating pathways to maintain cellular homeostasis [33–35]. The activation of AMPK that occurs in response to metabolic stress has been shown to protect cells from the ensuing apoptosis [36,37]. On the other hand, treatment of cells with the AMPK activator AICAR under non-stressful conditions was found to induce apoptosis in human prostate cancer cells [4,38]. Thus, AMPK can be protective in the context of metabolic stress by opposing the insult to the cellular energy store. However, due to its inhibitory effects on anabolic pathways involved in cell membrane and protein synthesis, AMPK can also exert negative effects on the health and proliferation of cells.

The aim of the present work was to evaluate the effects of metabolic inhibitors on NTS1 function in PC3 cells, and to determine how they relate to cellular ATP levels and to the activation of AMPK. We measured the effects of nine inhibitors, one glycolytic and eight mitochondrial, on cellular ATP levels and we correlated these to the changes in NT binding and NT-induced IP formation. The magnitude of these effects and the potencies of the various inhibitors were compared. We also investigated the involvement of AMPK by assessing the effects of metabolic inhibitors on the phosphorylation of AMPK, and by determining the effects of AMPK activator AICAR on NTS1 function. Our results indicated that metabolic stress altered NTS1 function in PC3 cells by a mechanism that was associated with a fall in cellular ATP levels and that was opposed by the activation of AMPK. Although cellular responses to mitogens are undoubtedly regulated at multiple levels in their signaling cascades in order to coordinate growth responses to metabolic status, the present findings suggest that some of this regulation (at least in regard to the effects of NT) could occur at the level of the growth factor receptor.

2. Materials and methods

2.1. Materials

[^{125}I]-sodium iodide (2000 Ci/mmol), [1,2-^{3}H(N)]-*myo*-inositol (60 mCi/mmol) and [methyl-^{3}H]-thymidine were obtained from Perkin Elmer Life Science (Boston, MA). Antibodies to ERK, phospho-ERK, AMPK and phospho-AMPK were from Cell Signaling Technology (Beverly, MA). HRP-linked secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was from Toronto Research Chemicals, Canada. Troglitazone and ciglitazone were from Cayman Chemical (Ann Arbor, MI). Oligomycin was from Calbiochem (San Diego, CA). Myxothiazole, rotenone, antimycin A, dicumarol, 2-deoxyglucose (2-DG), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and all other chemicals were from Sigma (St. Louis, MO).

2.2. Tissue culture

The human prostate cancer PC3 cell line, obtained from American Type Culture Collection (Manassas, VA), was maintained in F12K medium as described [27]. LNCaP^{CS} cells, a clone derived from the human prostate LNCaP cell line, were a gift from Shuk-mei Ho, Department of Environmental Health, University of Cincinnati Medical School and were maintained in phenol red-free RPMI medium as described [39]. For all studies, cells were grown in 24 well culture plates. For IP studies, cells were labeled with ^{3}H-inositol using medium 199 (Difco), which has a low inositol content.

2.3. Glycolytic and mitochondrial inhibitors

2-DG is a competitor for glucose transport and an inhibitor of the first two enzymes in the glycolytic pathway, hexokinase and phosphoglucose isomerase [40]. Rotenone is a classical inhibitor of mitochondrial complex I activity [41]. The thiazolidinediones, troglitazone and ciglitazone, are antidiabetic drugs that were recently found to inhibit complex I activity [42] and to activate AMPK [1]. Treatment of PC3 cells with thiazolidinediones was shown to induce apoptosis in a Bcl-2 dependent manner [43]. Dicumarol is an inhibitor of complex III activity, which was shown to enhance the cytotoxicity of cisplatin in a p53-dependent manner [44]. Antimycin A and myxothiazole are classical inhibitors of complex III, and oligomycin is a specific inhibitor of complex V (ATP synthase) [45,46]. CCCP is a protonophore whose major effect is to dissipate the mitochondrial hydrogen ion gradient, uncoupling oxidative phosphorylation [47].

2.4. Effects of the agents on cell viability

To examine the possibility that the agents affected cell viability, we tested their effects in a trypan blue exclusion assay.

Cells were treated in an identical fashion as described for the experiments reported in Results. To control for the growth studies (Fig. 1B and C), cells were incubated with 0.5 mM 2-DG, 1 μM CCCP or vehicle control for 24 h at 37 °C. For the studies on NT binding and IP formation (Figs. 2 and 3), cells were incubated with 10 mM 2-DG, 10 μM oligomycin, 10 μM myxothiazole, 10 μM CCCP, 20 μM antimycin A, 20 μM rotenone, 20 μM troglitazone, or vehicle control for 40 min at 37 °C. The medium was removed and the cells were incubated with 0.4% trypan blue for 15 min. After washing in Locke, the percentage of stained cells was determined by counting multiple fields of 200 (method A), and by dividing the total number of stained cells by the total cells determined by measuring protein content (method B). The percentage of stained cells for the control and experimental samples ($n=3$) was <1% (method A) and <0.1% (method B). Thus, none of these treatments seriously altered the viability of the cells.

2.5. Binding to cultured cells

HPLC-purified monoiodinated NT (^{125}I-NT) at 2000 Ci/mmol was prepared and binding was performed as described by us [48]. In brief, cells in 24-well plates were washed with HEPES-buffered Locke-BSA (Locke): 148 mM NaCl; 5.6 mM KCl, 6.3 mM HEPES; 2.4 mM $NaHCO_3$; 1.0 mM $CaCl_2$; 0.8 mM $MgCl_2$; 5.6 mM glucose; 0.1% BSA; pH 7.4. Stock solutions (5–10 mM) of agents in dimethyl sulfoxide (DMSO) were prepared just before use and were diluted in Locke to give ≤1% DMSO. Equilibrium binding at 37 °C was performed for 30 min using 10^5 cpm/ml ^{125}I-NT in 1.0 ml Locke. The reaction was stopped on ice, the medium was aspirated and cells were washed in ice-cold saline. Cellular binding was determined as radioactivity per μg protein in cells extracted in 0.3 M NaOH. Specific binding, displaceable by 1 μM NT (20,000-fold excess), was 95% of total binding. Binding displacement curves were constructed and binding parameters were determined by Scatchard analysis [49]. Cell surface binding and internalization were assessed by washing cells at room temperature for 2 min with 1 ml of 0.2 M acetic acid, 0.5 M NaCl [50].

The binding parameters for the ligands used to measure bombesin receptor binding and EGF receptor binding in PC3 cells were described by us [49]. In brief, equilibrium binding was performed in a manner identical to that for NT using HPLC-purified ^{125}I-labeled ligands (>1000 Ci/mmol; 10^5 cpm/ml). Specific binding was defined as that displaceable by 1 μM unlabeled ligand (10,000-fold excess).

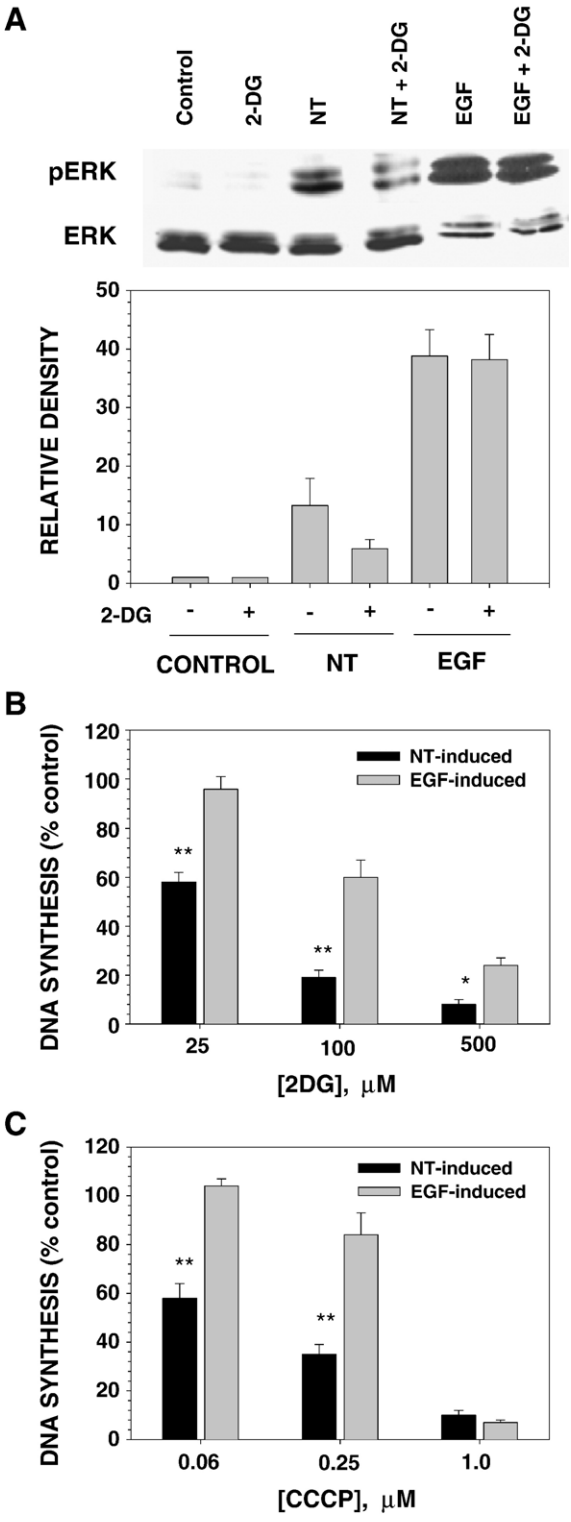
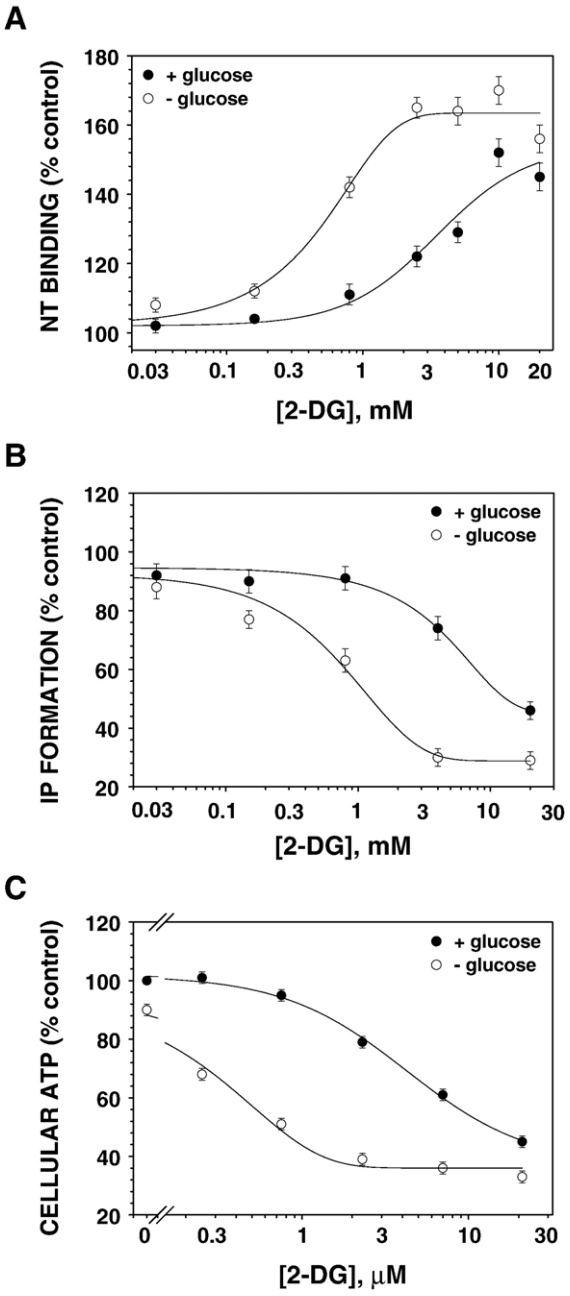


Fig. 1. Glycolytic inhibitor 2-DG blocked NT-induced ERK phosphorylation but not EGF-induced ERK phosphorylation (A), and also inhibited NT-induced DNA synthesis more than EGF-induced DNA synthesis (B). Mitochondrial blocker CCCP inhibited NT-induced DNA synthesis more than EGF-induced DNA synthesis (C). In (A), quiescent PC3 cells were preincubated with 10 mM 2-DG in Locke for 30 min, and then stimulated with NT (30 nM) or EGF (3 nM) for 5 min. Cell lysates were subjected to western blotting using phospho-specific and enzyme-specific antibodies to ERK. The relative densities for the pERK signals were normalized and plotted in the bar graph (mean±range, $n=2$). In (B) and (C), quiescent cells were preincubated with control (DMSO) or the indicated concentrations of 2-DG and CCCP for 30 min, followed by stimulation with NT (30 nM) or EGF (10 nM) for 24 h. The incorporation of [^{3}H]-thymidine into DNA was measured and the data were expressed as % control. Data (mean± SEM) represent at least 3 experiments. **Result for NT was significantly different from corresponding result for EGF ($p<0.01$).

2.6. IP formation

Formation of [^{3}H]-IP was measured as described by us [49]. Briefly, PC3 cells in 24-well plates were incubated 48 h with *myo*-[^{3}H]-inositol (2.5 μCi/ml) in medium 199, 0.2% fetal calf serum. Cells were treated with Locke, 15 mM LiCl for 10 min to inhibit phosphatases. Then, they were preincubated at 37 °C with test agents or vehicle (DMSO) in Locke, 15 mM LiCl for 10 min. The reaction was initiated by adding NT or vehicle (Locke). After 30 min, medium was aspirated, ice-cold 0.1 M formic acid in methanol was added and plates were placed at −20 °C overnight. IP was adsorbed to AG-1X8, which was washed in 5 mM *myo*-inositol and eluted in 1.5 M ammonium formate. Scintillation counting was performed in Ecoscint (National Diagnostics).



2.7. Cellular ATP measurement

Cells in 24-well plates were incubated at 37 °C with test agents for the indicated times, the medium was aspirated, 0.3 ml of ice-cold 1.2% TCA was added and the plates were placed at −20 °C. After 16 h, aliquots were diluted 15-fold into PBS and 20 μl samples were added to separate wells of a 96-well luminometer plate. The plate was placed in a Beckman LD400 luminometer that was programmed to add 100 μl luciferase bioluminescent reagent (Enliten, Promega) and to integrate luminescence over 10 s.

2.8. Western blotting

Western blot analysis was performed as described by us [6]. In brief, PC3 cells in 60-mm dishes were withdrawn from serum for 24 h. Cells, washed in Locke, were exposed to stimuli at 37 °C for times indicated. After washing with ice-cold PBS containing phosphatase inhibitors, cells were placed on ice and scraped into 2× SDS buffer (plus inhibitors) and sonicated. Equal amounts of protein were separated by SDS–PAGE and transferred onto PVDF (Immobilon P, Millipore). Blots were incubated with the primary antiserum in blocking buffer for 18 h at 4 °C. After washing, blots were incubated with HRP-linked secondary antibody for 1 h at 20 °C, and ECL was performed using supersignal west pico reagent (Pierce, Rockford, IL). After staining, blots were stripped and reprobed using different antibodies for comparison and normalization. Note that the results in Fig. 1A were normalized based on protein loading rather than on total ERK staining. This was necessary because strong phosphorylation of ERK (most evident in the response to EGF) diminished the signal obtained with the ERK antibody, most likely due to a lesser affinity for the phosphorylated protein.

2.9. DNA synthesis assay

The assay was performed as described [6]. Briefly, 10^4 cells were plated per well in 24-well plates. After 24 h, cells were serum-starved for 48 h, then fresh RPMI medium was added containing inhibitors or vehicle control, followed in 30 min with the stimuli (NT or EGF). After 24 h, [^{3}H]-thymidine (1 μCi) was

Fig. 2. Glycolytic inhibitor 2-DG increased specific NT binding (A), decreased NT-induced IP formation (B), and decreased cellular levels of ATP (C) with a potency that was enhanced in the absence of glucose. In (A), PC3 cells were equilibrated in Locke or Locke minus glucose for 20 min. Then, 2-DG was added at the indicated concentrations. After 10 min, ^{125}I-NT (10^5 cpm; 50 pM) was added and specific NT binding was measured 30 min later. Non-specific binding was <5% of total binding. The EC50 in the absence and presence of glucose was 0.5 mM and 3 mM, respectively. Data (mean±SEM) were from 3 experiments. In (B), glucose was withdrawn from half of the cells during the LiCl treatment period (10 min). Then, the cells were pretreated for 10 min with 2-DG at concentrations indicated, NT was added (a maximal dose, 30 nM), and IP formation was measured after 30 min. The IC50 in the absence and presence of glucose was 0.6 mM and 4 mM, respectively. Data (mean±SEM) were from 3 experiments. In (C), cells were pretreated in Locke or Locke minus glucose for 20 min. Then, 2-DG was added and after 40 min, the medium was aspirated and the cells were extracted with 1.2% TCA. Cellular ATP was measured and expressed as % appropriate control. The IC50 in the absence and presence of glucose was 0.75 mM and 4 mM, respectively. The data (mean±range) were from a typical experiment that was repeated.

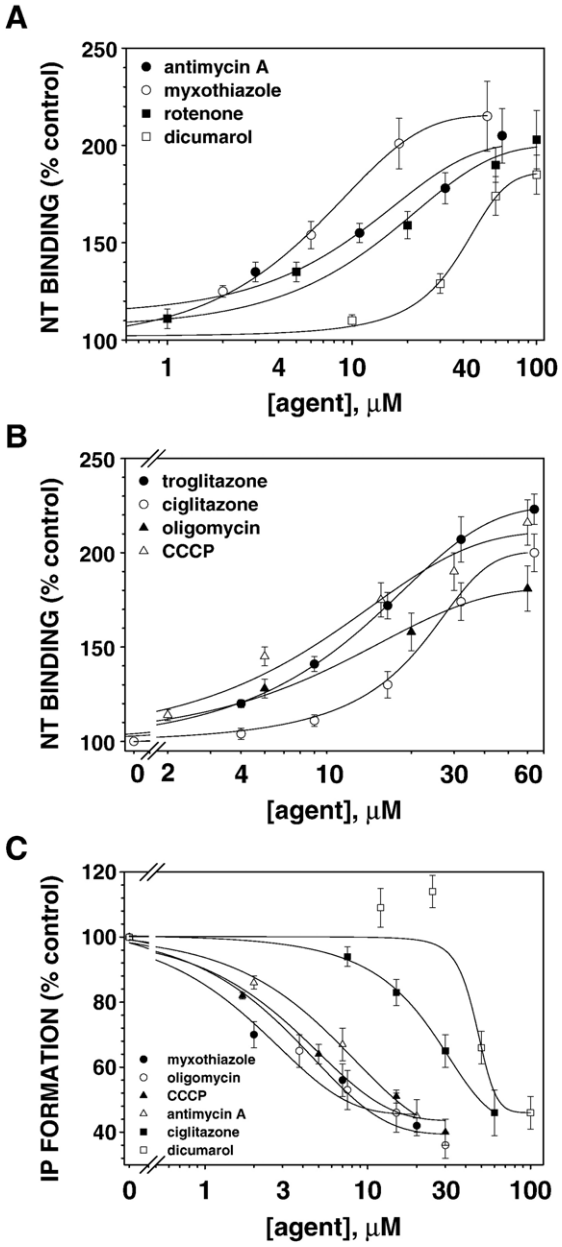


Fig. 3. Mitochondrial inhibitors increased specific NT binding (A, B) and decreased NT-induced IP formation (C) dose-responsively. In (A) and (B), PC3 cells were incubated for 10 min in Locke plus control (DMSO) or agents at indicated concentrations. Then, ^{125}I-NT (10^5 cpm/ml) was added and specific NT binding was measured at equilibrium (30 min). Non-specific binding was <5% of total binding. Data (mean±SEM) were from at least 3 experiments. In (C), cells were pretreated for 10 min with the indicated concentrations of agents, NT was added (a maximal dose, 30 nM), and IP formation was measured after 30 min. Data (mean±SEM) were from at least 3 experiments.

added for 3 h. DNA was precipitated with 6% TCA, washed in PBS and ethanol, solubilized in 0.3 N NaOH, 0.1% SDS and counted by liquid scintillation spectrometry.

2.10. Statistics

Data were calculated as mean±SEM for n independent observations. Statistically significant differences were assessed using Student's t-test for single comparisons and using ANOVA (Graph Pad Software, San Diego, CA) for multiple comparisons. Statistical significance was defined as $p<0.05$.

3. Results

3.1. Metabolic stress inhibited NT-induced growth signaling

Treatment of PC3 cells with NT is known to stimulate PLC-mediated inositol phosphate (IP) production, mobilize intracellular Ca^{2+} and activate PKC in a number of cell systems [51]. In PC3 cells, these events lead to the release of EGF-like substance (s), the transactivation of EGFR and the activation of ERK and AKT, culminating in a growth response [6]. To assess the effects of metabolic stress on the growth response to NT, we examined the effect of the glycolytic inhibitor 2-DG on NT-induced ERK activation and NT-induced DNA synthesis in PC3 cells. In preliminary experiments, we determined the optimal doses of 2-DG that could compete with the 5.6 mM glucose present in the medium. Under these conditions, 2-DG did not affect the viability of the cells (see Section 2.4). Pretreatment of cells with 10 mM 2-DG dramatically inhibited the stimulatory effect of NT on ERK phosphorylation (Fig. 1A) and pretreatment with 0.1 mM 2-DG strongly inhibited NT-induced DNA synthesis (Fig. 1B). In contrast, these doses of 2-DG were less effective in inhibiting the stimulatory effects of EGF in these assays (Fig. 1A and B). Similarly, the mitochondrial inhibitor CCCP was more potent in inhibiting the growth effect of NT than that of EGF (Fig. 1C). Given that NT and EGF utilize the same downstream pathways to stimulate growth, the enhanced sensitivity of the NT response to metabolic inhibition suggested that one of the early steps in NT signal transduction, such as

Table 1
Activity of metabolic inhibitors on NT binding, NT-induced IP formation and ATP levels in PC3 cells

Agent	NT binding[a]	IP formation[b]	ATP level[c]
	EC50 (μM)	IC50 (μM)	IC50 (μM)
2-DG (+glucose)	5000	6000	5000
2-DG (−glucose)	550	600	550
Myxothiazole	7	3	9
Antimycin A	13	7	14
Rotenone	18	8	21
CCCP	9	5	62
Oligomycin	20	4	30
Troglitazone	16	8	28
Ciglitazone	25	30	36
Dicumarol	50	50	62

[a] The ability to enhance NT binding was tested in PC3 cells. Since the maximal increase observed was ≅120%, EC50 was defined as the [agent] that increased NT binding by 60%. Given are means determined using data from at least 3 experiments.

[b] The ability to inhibit NT-induced IP formation was tested in PC3 cells. Since the maximal inhibition observed was ≅70%, IC50 was defined as the [agent] that decreased IP by 35%. Given are means determined using data from at least 3 experiments.

[c] The ability to decrease [ATP] was tested in PC3 cells. The IC50 was defined as the [agent] that decreased ATP levels by 35%. Given are means determined using data from at least 3 experiments.

Table 2
Effects of metabolic inhibitors on IP formation in response to NT, bombesin and ATP

Agent	Dose[a] (μM)	IP formation in response to stimulus (% control)[b]		
		NT	Bombesin	ATP
Rotenone	2	85±3*	65±3**	64±3**
	20	55±3**	51±3**	47±2**
CCCP	2	76±4**	64±3**	65±2**
	20	46±3**	52±3**	49±2**
Myxothiazole	2	68±4**	63±3**	nd
	20	40±3**	42±3**	nd
Antimycin A	2	83±4*	65±3**	nd
	20	52±3**	49±2**	nd

[a] The doses of each agent were chosen based on the dose–response curves (Fig. 3C).
[b] PC3 cells were pretreated 10 min with the agents at concentrations indicated. NT, bombesin or ATP was added and IP formation was measured after 30 min at 37 °C. The doses of NT (30 nM), bombesin (2 nM) and ATP (2 μM) were chosen to stimulate basal IP formation 4-to 6-fold under control conditions. Results (mean±SEM) are from at least 3 experiments. nd, not determined.
* Result was significantly different from control ($p<0.05$).
** Result was significantly different from control ($p<0.01$).

binding to NTS1 or activation of PLC, might be sensitive to metabolism.

3.2. Metabolic stress elevated NT binding to PC3 cells

When PC3 cells were incubated with ^{125}I-NT (10^5 cpm/ml) in Locke (5.6 mM glucose), equilibrium was achieved after 30 min at 37 °C and specific binding was 18±3 cpm/μg protein (mean±SEM, $n=9$) in agreement with our earlier work [52]. Withdrawing glucose from the cells for 60 min (30 min pretreatment plus 30 min binding reaction) enhanced specific NT binding by 15±4% ($p<0.05$; $n=9$). This result suggested that NT binding was sensitive to metabolic stress and led us to examine the effects of metabolic inhibitors known to affect glycolysis and mitochondrial oxidative phosphorylation.

Pretreatment of cells with the glycolytic inhibitor 2-DG for 2 to 30 min caused an increase in specific NT binding that rose to a maximal value within 10 min and then plateaued (results not shown). Since the binding reaction required 30 min to reach equilibrium, the effect of 2-DG was maximal within 40 min. Specific NT binding was increased by as much as 70% in a dose-dependent manner (Fig. 2A) without an effect on non-specific binding (results not shown). The potency of 2-DG was enhanced ≅7-fold by removing glucose from the buffer, indicating that 2-DG was in metabolic competition with glucose.

Inhibitors of mitochondrial oxidative phosphorylation also increased specific NT binding when preincubated with the cells for 10 min, and their effects were dose-dependent (Fig. 3A and B). Specific NT binding was increased by as much as 125% (Fig. 3A and B) without an effect on non-specific binding. The effective agents included complex I inhibitors (rotenone, troglitazone, ciglitazone), complex III inhibitors (antimycin A, myxothiazole, dicumarol) and ATP synthase inhibitor (oligomycin). The protonophore (CCCP), which dissipates the mitochondrial H^+ gradient and uncouples oxidative phosphorylation, also enhanced specific NT binding (Fig. 3B). The order of potency was myxothiazole>CCCP>antimycin A>troglitazone>rotenone>oligomycin>ciglitazone>dicumarol (Table 1). These treatments with the glycolytic and mitochondrial inhibitors did not affect the viability of the cells (see Section 2.4).

3.3. Metabolic inhibitors diminished NT-induced IP formation

Although metabolic inhibitors enhanced NT binding, they reduced the ability of NT to stimulate PLC. In agreement with our prior work [49], stimulation of PC3 cells with a maximal dose of NT (30 nM) increased IP formation 4.2±0.3-fold (mean±SEM, $n=8$). Pretreatment of the cells with the glycolytic inhibitor 2-DG dose-dependently reduced the ability of NT to stimulate IP formation (Fig. 2B). The potency of 2-DG was enhanced ≅9-fold by removing glucose from the buffer, indicating that 2-DG was in metabolic competition with glucose.

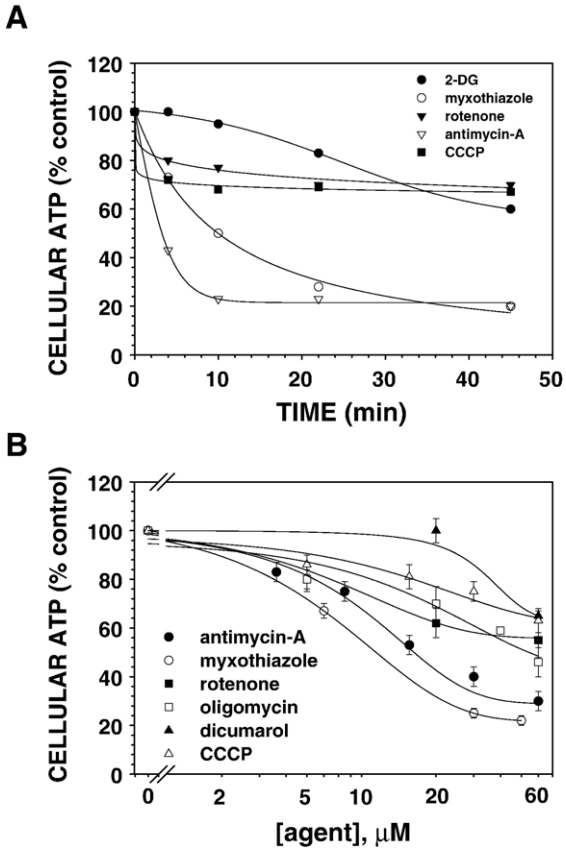


Fig. 4. Metabolic inhibitors decreased cellular ATP levels time-dependently (A) and dose-dependently (B). PC3 cells were treated with indicated agents in Locke for the times indicated and cellular ATP levels were measured. In (A), cells were incubated with Locke (0.25% DMSO) or 2-DG (10 mM), myxothiazole (25 μM), rotenone (25 μM), antimycin A (25 μM) or CCCP (25 μM) for the times indicated. Cellular ATP levels were expressed relative to control. Data were from a typical experiment that was repeated. In (B), cells were incubated for 40 min in Locke with agents at indicated concentrations, cellular ATP was measured and expressed relative to DMSO control. Data (mean±SEM) were from at least 3 experiments.

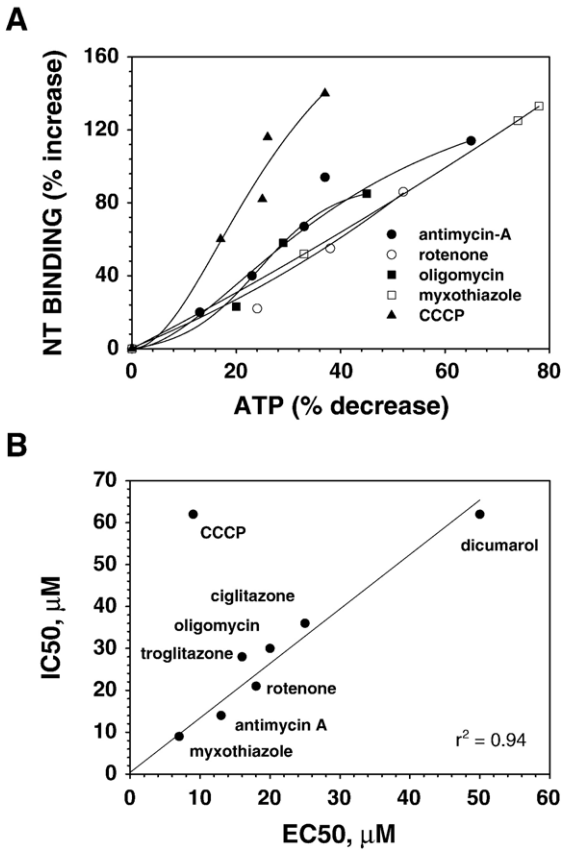


Fig. 5. For the effects of the mitochondrial inhibitors, the % increase in NT binding was linearly related to the % decrease in cellular ATP (A), and the EC50 for the increase in NT binding was linearly related to the IC50 for the decrease in cellular ATP (B). In (A), cells were treated with increasing doses of each inhibitor as in Figs. 2 and 3. Cellular ATP and NT binding were measured after 40 min. The % decrease in cellular ATP and the % increase in NT binding were calculated relative to controls. Plotted are mean values determined in at least 3 experiments. In (B), the ability of mitochondrial inhibitors to enhance NT binding (EC50) was correlated to the ability to decrease cellular ATP (IC50). The values shown were determined from studies such as those in Figs. 2 and 3, and are presented in Table 1. The line drawn was obtained by excluding CCCP from the linear regression analysis and it yielded a correlation coefficient $r^2 = 0.94$.

The effects of 2-DG on NT binding and IP formation occurred in the same dose range (Table 1).

Mitochondrial blockers also diminished NT-induced IP formation (Fig 3C). The order of potency was myxothiazole > oligomycin > CCCP > antimycin A > rotenone ≅ troglitazone > ciglitazone > dicumarol (Table 1). In comparison to the potency order for the effects on NT binding, these results agreed quite well with the exception of oligomycin (Table 1). For the other inhibitors, the EC50's for the effect on NT binding correlated to the IC50's for the effect on IP formation ($r^2 = 0.87$). Thus, the sensitivities of the two assays to the effects of the mitochondrial inhibitors were not precisely the same but the dependence was similar for seven out of eight. This suggested that both effects might have been caused by a change in the state of NTS1 (e.g., NTS1 may have shifted to a state that was uncoupled from PLC and displayed higher binding ability).

Further study, however, demonstrated that metabolic blockers not only inhibited IP formation in response to NT but also that induced by bombesin and ATP, agonists for other Gq-coupled receptor systems (Table 2). These results indicate that metabolic stress exerted a general effect on agonist-stimulated IP formation. Possible mechanisms include (a) an uncoupling of

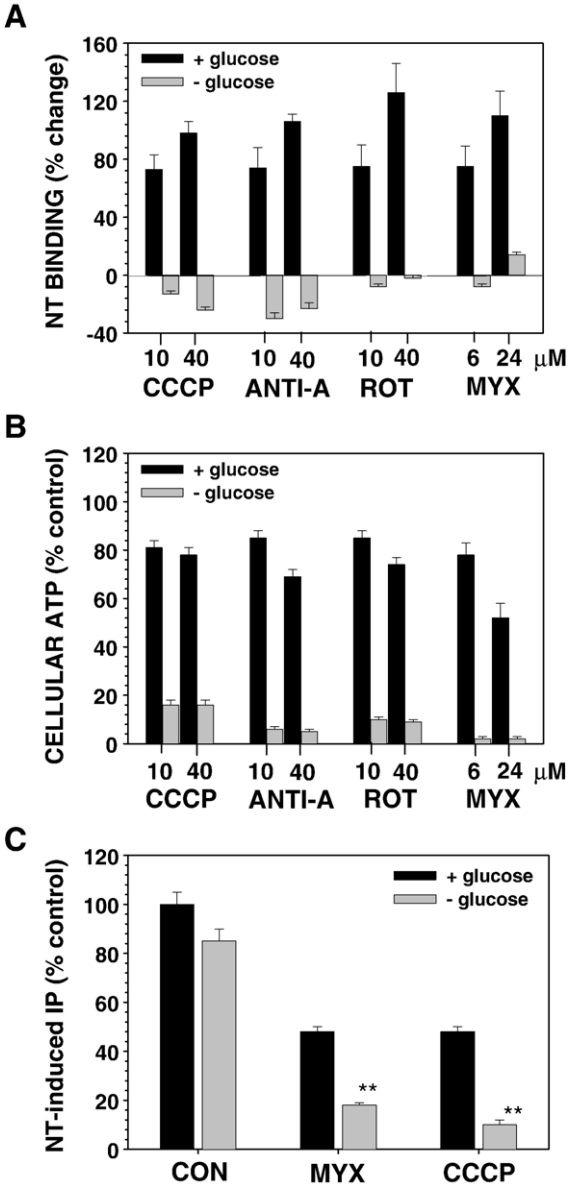


Fig. 6. Inhibition of both glycolysis and oxidative phosphorylation greatly suppressed NT binding (A), reduced cellular ATP levels to <15% control (B), and increased the inhibitory effects of mitochondrial blockers on NT-induced IP formation (C). In (A), cells were preincubated 20 min in Locke (+glucose) or in Locke minus glucose (−glucose). The mitochondrial inhibitors were added at concentrations indicated and after 10 min, ^{125}I-NT was added. Specific NT binding was measured at equilibrium (30 min). The % increase or the % decrease in NT binding was determined relative to control. Data (mean±SEM) were from 3 experiments. In (B), cells were treated as in (A), and cellular ATP was measured after 40 min and expressed as % control. Data (mean±SEM) were from 3 experiments. In (C), glucose was withdrawn from half the cells for 10 min. DMSO (0.1%), myxothiazole (5 μM) and CCCP (10 μM) were added and after 10 min, cells were stimulated with 30 nM NT for 30 min. IP formation was measured and expressed relative to the +glucose control. Data (mean±SEM) were from 2 experiments. **Result for −glucose was significantly different from result for +glucose ($p < 0.01$).

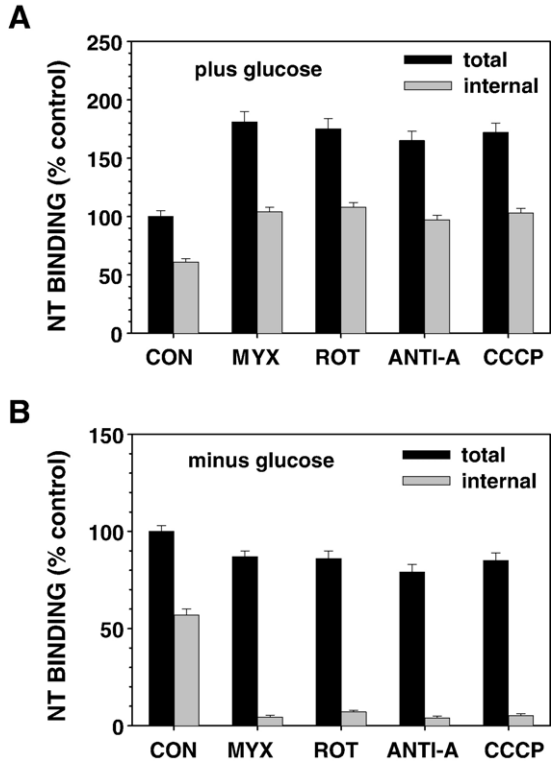


Fig. 7. When performed alone (A), glucose deprivation or treatment with mitochondrial inhibitors did not change the percentage of internalized NT binding but when combined (B), they severely and specifically inhibited internalization. Identical treatments were performed in the presence (A) and absence (B) of glucose. Cells were pretreated with Locke or Locke minus glucose for 15 min. Agents were added: DMSO (0.3%), myxothiazole (10 μM), rotenone (30 μM), antimycin A (10 μM), CCCP (15 μM). After 10 min, cells were incubated with ^{125}I-NT (10^5 cpm/ml) for 30 min. Total cellular binding and internalized binding were measured and the results were expressed as percentage of the total control binding. NT binding to the cell surface is shown as the difference between total and internal binding. Data (mean±SEM) were from 2 experiments.

PLC from Gq-coupled receptors; (b) an inhibition of PLC activity due to decreased levels of substrate PIP2; and (c) some other effects. If the altered binding and IP formation were due to a single effect, then one might propose that there was a general effect on G protein receptor coupling to PLC, which gave rise to a unique binding response for NTS1. Alternatively, there could be two effects: one involving a general inhibition of IP formation in response to Gq-coupled agonists; and the other involving a specific enhancement of NT binding. However, it seemed likely that these two effects were coupled in some way, given that they were strongly correlated.

3.4. Metabolic inhibitors decreased cellular ATP levels

Incubation of PC3 cells with metabolic inhibitors caused a decrease in cellular ATP levels that occurred within the 40 min over which the changes in NT binding and IP formation were seen (Fig. 4A). The effects of 2-DG on ATP levels were dose-related and its potency was enhanced ≅5-fold in the absence of glucose (Fig. 2C). Increasing doses of 2-DG decreased cellular ATP until it reached a plateau of 30–40% control. The incremental decreases in ATP levels were associated with incremental increases in NT binding and incremental decreases in NT-induced IP formation (compare Fig. 2A, B and C). For example, in the presence of glucose, 5 mM 2-DG caused a 35% fall in ATP, a 34% rise in NT binding, and a 30% fall in IP formation. In the absence of glucose, 0.55 mM 2-DG caused a 35% fall in ATP, a 34% rise in NT binding, and a 32% fall in IP formation. Thus, the effects of 2-DG on cellular ATP and on NT binding were related quantitatively. These results suggested that NT binding and NT-induced IP formation were influenced by the glycolytic rate as reflected in the cellular ATP level.

Similarly, increasing doses of the mitochondrial inhibitors decreased cellular ATP levels, which plateaued at 20–70% control depending on the agent (Fig. 4B). The order of potency was myxothiazole > antimycin A > rotenone > troglitazone > oligomycin > ciglitazone > CCCP ≅ dicumarol (Table 1). As the dose of each inhibitor was increased, there was nearly a linear relationship between the decrease in ATP level and the increase in NT binding (Fig. 5A). For the agents other than CCCP, a dose that caused a 35% fall in cellular ATP led to a 55–75% increase in NT binding. Furthermore, for all agents except CCCP, the EC50 for the

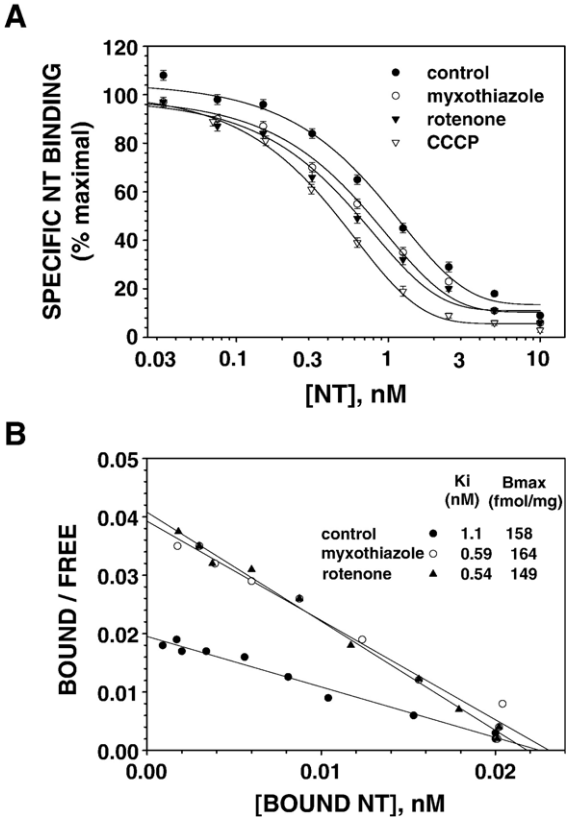


Fig. 8. Binding displacement curves (A) and Scatchard plots (B) for NT binding to PC3 cells in the presence and absence of mxyothiazole (10 μM), rotenone (30 μM) and CCCP (20 μM). (A) Log dose–response plots show displacement of specific ^{125}I-NT binding by NT in which NT binding was expressed as % control. The inhibitors shifted the curves to the left. Data were from a typical experiment that was repeated. (B) Scatchard plots for a typical experiment showing that myxothiazole (10 μM) and rotenone (30 μM) increased NT binding affinity without much effect on NT receptor number.

Table 3
Effects of metabolic inhibitors on NT binding parameters in PC3 cells

Agent [a]	Dose [b] (μM)	Bmax [c] (fmol/mg)	Ki for NT [c] (nM)
Control		158±6	1.05±0.08
Myxothiazole	10	156±11	0.54±0.06*
Rotenone	30	162±8	0.54±0.05*
CCCP	20	155±12	0.39±0.06**

[a] PC3 cells were pretreated 10 min with the agents or vehicle control, ^{125}I-NT (10^5 cpm, 50 pM) was added and specific binding was measured for 30 min at 37 °C.
[b] The doses used were those that increased NT binding ≅2-fold (Fig. 3).
[c] Scatchard analyses were performed using 10 concentrations of NT and results for Bmax and Ki (mean±SEM) were obtained in 3 experiments for each agent.
* Result was significantly different from control ($p<0.05$).
** Result was significantly different from control ($p<0.01$).

increase in NT binding correlated ($r^2=0.94$) to the IC50 for the decrease in ATP (Fig. 5B). These results suggested that the fall in cellular ATP might possibly have caused the rise in NT binding. On the other hand, a dose of CCCP that decreased ATP by 35% increased NT binding by 140% (Fig. 5A). This finding suggested that NT binding was not only a function of mitochondrial ATP production but also might have depended on the mitochondrial membrane potential which would be strongly affected by CCCP [47].

3.5. NT binding required some ATP

Having shown that blocking either glycolysis or mitochondrial oxidative phosphorylation increased NT binding, we next determined the outcome of inhibiting both metabolic pathways. This was done by comparing the effects of mitochondrial blockers on NT binding in the presence and absence of glucose, using doses of each agent that fell in the middle of the dose–response curve (Fig. 3A and B). The results in Fig. 6A show that mitochondrial inhibitors decreased NT binding to cells that had been withdrawn from glucose, whereas they increased NT binding to cells in the presence of glucose. This was likely due to the associated dramatic fall in cellular ATP levels that resulted from the concomitant inhibition of glycolysis and mitochondrial oxidative phosphorylation (Fig. 6B). As might have been predicted from the striking effect on NT binding, the withdrawal of glucose greatly enhanced the inhibitory effects of mitochondrial blockers on NT-induced IP formation (Fig. 6C).

Since receptor-mediated endocytosis normally accounts for the majority of NT binding to PC3 cells [49] and since ATP is required for this process [53], we determined the percentage of NT binding that was internalized by the cells under these conditions. This was done by comparing the effects of mitochondrial blockers on NT internalization in the presence and absence of glucose, using doses of each agent that fell in the middle of the dose–response curve (Fig. 3A and B). The results in Fig. 7A show that, in the presence of glucose, internalization as a percentage of total NT binding was similar for control cells (61±3%) and cells incubated with mitochondrial inhibitors (range, 58–62%). Also note that NT binding to the cell surface and the internalization of NT were both increased by mitochondrial inhibitors (Fig. 7A). In contrast, in the absence of glucose, internalization represented 57±3% of total NT binding for control cells but only 5–8% for cells incubated with mitochondrial inhibitors (Fig. 7B). However, NT binding to the cell surface (Fig. 7B, total minus internal) was still increased by the inhibitors, even though NT internalization was dramatically reduced.

These results indicate that mild metabolic stress (ATP levels 20–100% control) enhanced NT binding, while severe metabolic stress (ATP levels <20% control) reduced NT binding. Whereas mild metabolic stress elevated both NT binding to the cell surface and NT internalization, severe metabolic stress increased NT binding to the cell surface while dramatically reducing NT internalization.

3.6. NTS1 affinity versus NTS1 number

In order to determine if the metabolic inhibitors altered NT binding by changing receptor affinity or receptor number, we tested their effects on the NT displacement curve, using doses of each agent that elevated NT binding ≅2-fold (Fig. 3A and B). The results in Fig. 8A show that metabolic inhibitors increased the steepness of the NT displacement curve. When NT displacement data were expressed as % maximal binding, the curves were generally shifted to the left by a factor of 2 to 3 in the presence of the inhibitor (e.g., Fig. 8A). Scatchard analyses indicated that the receptor affinity was increased, without a change in the receptor number (e.g., Fig. 8B). The results summarized in Table 3 show that the shift in NT binding affinity was a general property for this set of inhibitors. These results

Table 4
Effects of metabolic inhibitors on PC3 cell binding for bombesin and EGF receptors

Agent	Dose [a]	Specific binding (% control) [b]	
		Bombesin receptor [c]	EGF receptor [d]
2-DG	1 mM	107±5	101±4
	4 mM	108±3	96±5
Antimycin A	10 μM	112±5	93±6
	40 μM	121±4*	85±5**
CCCP	10 μM	110±3**	77±2*
	40 μM	102±6**	62±3*
Oligomycin	10 μM	100±5	75±3*
	40 μM	102±6	62±3*
Rotenone	10 μM	104±5	71±3*
	40 μM	116±3**	54±2*
Myxothiazole	10 μM	114±2*	80±1*
	40 μM	120±2*	76±4*

[a] The doses used were those that effectively increased NT binding (Figs. 2 and 3).
[b] Specific binding of each ^{125}I-ligand (10^5 cpm/ml) was measured in PC3 cells using conditions identical to those for NT binding: equilibrium at 37 °C for 30 min. Binding was expressed as % control (mean±SEM) for at least 3 independent experiments.
[c] The ligand, ^{125}I-[Nle^{14}]-bombesin, gave 1016±64 fmol/mg binding at 10^5 cpm/ml.
[d] The ligand, ^{125}I-EGF, gave 151±11 fmol/mg binding at 10^5 cpm/ml.
* Result was significantly different from control ($p<0.01$).
** Result was significantly different from control ($p<0.05$).

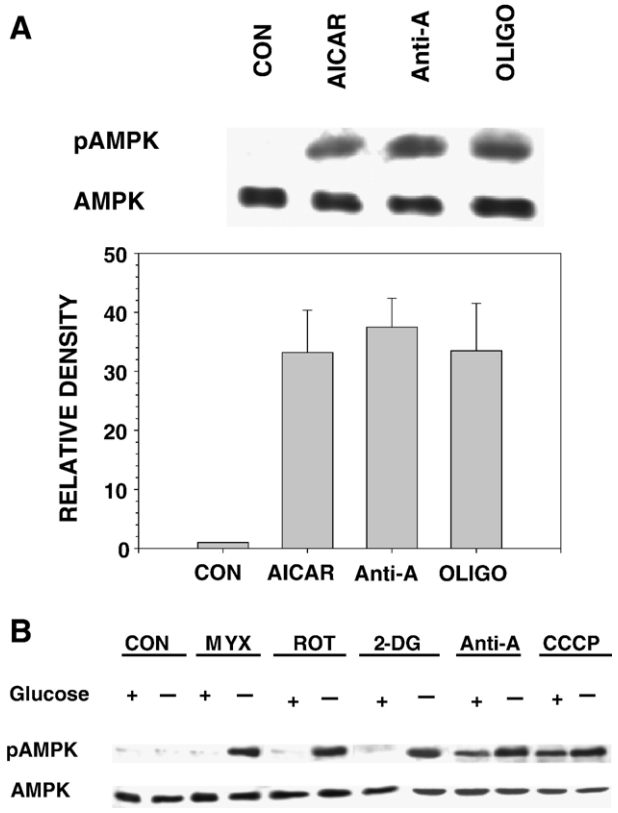


Fig. 9. Metabolic inhibitors stimulated phosphorylation of AMPK (A) and this response was enhanced in the absence of glucose (B). In (A), quiescent cells were equilibrated with Locke for 5 min and then treated with control (0.5% DMSO), AICAR (3 mM), antimycin A (30 μM) or oligomycin (50 μM) for 60 min. The relative densities for the pERK signals were plotted in the bar graph (mean±range, $n=2$). In (B), quiescent cells were equilibrated with Locke or Locke minus glucose for 5 min. Then, they were treated with control (0.5% DMSO), myxothiazole (50 μM), rotenone (50 μM), 2-DG (5 mM), antimycin-A (30 μM) or CCCP (20 μM) for 60 min. Cell lysates with equal protein content were subjected to western blotting using phospho-specific and enzyme-specific antibodies to AMPK.

were consistent with the hypothesis that mild metabolic stress increased NT binding by shifting NTS1 to a higher affinity state, not by increasing the number of receptors at the cell surface.

3.7. Receptor specificity

To determine if these effects were specific to NTS1, we tested metabolic inhibitors for effects on PC3 cell binding of ligands for other receptors using assays validated by us [49]. Table 4 shows that 2-DG, antimycin A, CCCP, oligomycin, rotenone and myxothiazole did not greatly enhance ligand binding to bombesin and EGF receptors. Although 4 of the 6 inhibitors tested increased bombesin binding slightly (≤20%), EGF binding was actually decreased by these agents. These results show that the robust elevation in cell binding caused by these metabolic inhibitors was specific to NTS1, although smaller elevations also occurred for the bombesin receptor.

3.8. Cell specificity

To determine if the effects of metabolic inhibitors were specific to PC3 cells, we assessed the effects of 2-DG and antimycin A on NT binding to another prostate cancer cell line LNCaP^{CS}. The level of NT receptor expression in LNCaP^{CS} (3 cpm/μg) was less than that in PC3 (18 cpm/μg). In the

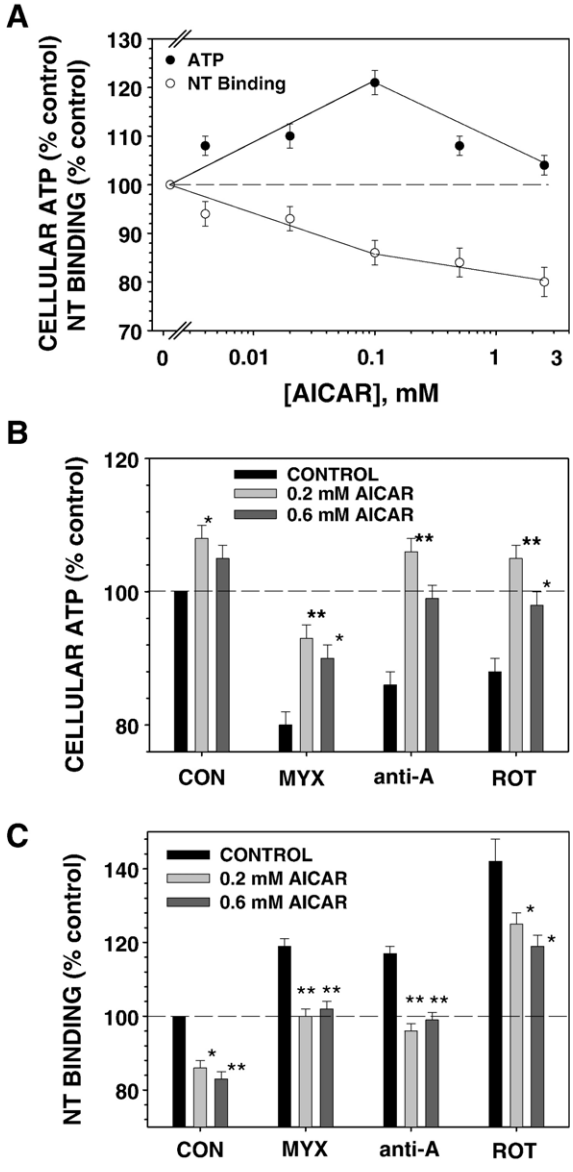


Fig. 10. Treatment of PC3 cells with AICAR increased ATP levels and decreased NT binding (A), and reversed the effects of mitochondrial inhibitors on cellular ATP (B) and on cellular NT binding (C). In (A), PC3 cells were preincubated in Locke with indicated concentrations of AICAR for 15 min. Then, ^{125}I-NT (10^5 cpm/ml) was added and after 30 min, NT binding and cellular ATP levels were measured. In (B) and (C), PC3 cells were preincubated with indicated concentrations of AICAR for 15 min. Then, mitochondrial inhibitors were added: control (0.1% DMSO), myxothiazole (4 μM), antimycin A (8 μM) or rotenone (16 μM). Then, ^{125}I-NT (10^5 cpm/ml) was added and after 30 min, NT binding and ATP levels were measured. Data (mean±SEM, $n=3$) were from at least 2 experiments. *Result was significantly different from corresponding control ($p<0.05$). **Result was significantly different from corresponding control ($p<0.01$).

presence of 2 mM 2-DG, specific NT binding expressed as % control was (mean±SEM, $n=3$ experiments): PC3 (128±7) and LNCaP^{CS} (119±6). For 60 μM antimycin A, specific NT binding was: PC3 (201±11) and LNCaP^{CS} (183±8). These results indicate that 2-DG and antimycin A enhanced NT binding similarly in these cancer cell lines.

3.9. Possible involvement of AMPK

Metabolic stress has been shown to activate AMPK in many cell systems, and this has been associated with a recruitment of glucose transporters [54], a stimulation of energy-producing processes [55,56], and an inhibition of energy-consuming processes [3]. Thus, we hypothesized that AMPK would be activated by treatment of PC3 cells with metabolic inhibitors and that its activation would oppose the effects of metabolic stress. To address this issue, we compared the effects of metabolic inhibitors (used at doses that maximally enhanced NT binding) with the effect of a maximal dose of AICAR (3 mM) on the activation of AMPK in PC3 cells. The results in Fig. 9A show that phosphorylation of AMPK was stimulated to a similar extent by antimycin A and oligomycin as it was by the positive control AICAR. We also demonstrated that the responsiveness of the cells to myxothiazole, rotenone, 2-DG, antimycin A and CCCP was enhanced in the absence of glucose (Fig. 9B). These results were consistent with our finding that the decrease in cellular ATP levels induced by the mitochondrial inhibitors was dramatically enhanced by the withdrawal of glucose (Fig. 6B).

Having shown that AMPK was activated in response to metabolic inhibitors, we then determined the effects of AMPK activator AICAR on cellular ATP levels and NTS1 function. Since AMPK generally opposes metabolic stress, we predicted that AICAR would elevate cellular ATP levels and this would lead to a decrease in NT binding. As shown in Fig. 10A, treatment of PC3 cells with less than maximal doses of AICAR (0.004–2.5 mM) elevated cellular ATP levels and decreased NT binding. In addition, pretreatment of cells with doses of AICAR (0.2 and 0.6 mM) shown to elevate ATP not only reversed the effects of myxothiazole (4 μM), antimycin A (8 μM) and rotenone (16 μM) on cellular ATP levels (Fig. 10B), but also reversed the effects of these inhibitors on cellular NT binding (Fig. 10C). Thus, the effects of AICAR on cellular ATP levels and also on NTS1 function were opposite to those seen in response to the metabolic inhibitors.

4. Discussion

Our results demonstrate that metabolic stress, induced by inhibiting glycolysis or mitochondrial oxidative phosphorylation, influenced NTS1 function in PC3 cells as indicated by the effects on NT binding, NT-induced IP formation and NT-induced growth responses. Interestingly, work by Li et al. has shown that acute as well as chronic inhibition of mitochondrial function suppressed NT secretion in the endocrine BON cell line [57]. Taken together, these findings suggest that NT release and NT receptor function both require the production of ATP, and this could provide a mechanism for coordinating NT-induced growth effects to the cellular energy status.

In PC3 cells, some of this regulation appeared to be at the level of NTS1, since NT binding was altered within minutes by the metabolic inhibitors and since the changes in NT binding were correlated to the fall in cellular ATP levels. In keeping with this was the finding that the growth responses to NT were more sensitive to the effects of 2-DG and CCCP than were the growth responses to EGF, despite the involvement of the same downstream signaling mediators. The evidence also pointed towards some regulation that might have occurred at the level of phosphoinositide metabolism, since metabolic stress not only inhibited IP formation in response to NT but also that in response to bombesin and ATP, which are agonists for other Gq-coupled receptors. However, this non-selectivity might also be attributed to a general effect on Gq-coupled receptor function (e.g., an uncoupling from PLC or an inhibition of GTP exchange).

Consistent with its role to oppose metabolic stress, AMPK was activated in response to treatment of the cells with metabolic inhibitors. Accordingly, the AMPK activator AICAR elevated cellular ATP levels and produced effects on NTS1 function that were opposite to those for the metabolic inhibitors. Since AICAR has been shown to promote glucose uptake [58] and to stimulate glycogenolysis [56] and fatty acid oxidation [55], these effects might possibly explain our finding that AICAR rapidly elevated ATP levels in PC3 cells. Nevertheless, our results demonstrate a consistent relationship between NTS1 function and the AICAR-induced rise in cellular ATP levels, as well as the stress-induced fall in cellular ATP. In total, these findings are compatible with the idea that NTS1 function is sensitive to metabolism.

Moderate levels of metabolic stress (cellular ATP levels 20–100% control), induced by inhibiting glycolysis or mitochondrial oxidative phosphorylation, rapidly enhanced NT binding by an effect that involved an increase in receptor affinity without a change in receptor number or a change in the percentage of receptor internalization. For each glycolytic or mitochondrial inhibitor, there was a dose-dependent correlation between the decrease in cellular ATP levels and the increase in NT binding. For eight of the nine inhibitors, the IC50 for the effect on ATP correlated to the EC50 for the effect on NT binding. Although this suggested that the drop in ATP level might have induced the change in NT binding, it was still possible that these were strongly coupled epi-phenomena. One of the inhibitors, CCCP, altered NT binding more than could be explained simply by the change in cellular ATP. This suggested that NT binding was not only sensitive to cellular ATP levels but could also be affected by changes in the mitochondrial membrane potential [47]. Indeed, our prior work has shown that aromatic antioxidants such as dihydropyridines [49] and polyphenols [52], which have the potential to disrupt membrane potentials [59,60], mimicked the effects seen here with CCCP.

Severe metabolic stress (cellular ATP levels <20% control), induced by inhibiting glycolysis and mitochondrial oxidative phosphorylation concurrently, decreased total cellular NT

binding by an effect that involved an inhibition of receptor internalization. Under these conditions, NT binding to the cell surface was elevated, in keeping with the increased receptor affinity, but receptor internalization was inhibited by >90%. These findings are consistent with the fact that receptor-mediated endocytosis has a general requirement for ATP [53]. When cellular levels of ATP fall to <20% control, this represents a major metabolic crisis that in other cells has been shown to induce apoptosis and/or necrosis [31]. In line with this, our results indicate that, under these conditions, the ability of NTS1 to promote IP formation was severely inhibited. These findings are in accord with those of Navarro et al., who showed that internalization of NTS1 was required for NT-induced growth signaling in HT-29 cells [61].

In addition to altering NTS1 binding, metabolic blockers inhibited NT-induced IP formation. For each metabolic blocker, the increase in NT binding was associated with a parallel inhibition of NT-induced IP formation, and for eight of the nine agents, the drug potencies in these two assays were correlated. Although this suggests that these two effects were linked, they could also be independent effects that exhibited similar drug dependencies. The latter idea is supported by the fact that the dramatic effects on binding were seemingly specific to NTS1, whereas the effects on IP formation were more general (responses to bombesin, ATP and NT were inhibited). Metabolic stress could have exerted a generalized inhibitory effect on IP formation in several ways. It could have diminished the levels of PLC substrate PIP2 by inhibiting the ATP-dependent kinase reactions involved in its synthesis. However, metabolic stress inhibited IP formation under conditions where the estimated cellular ATP concentration did not fall to less than 1 mM, which is about 50 times the Km's reported for these enzymes [62]. Alternatively, metabolic stress might have had a general effect on Gq-coupled receptor systems (e.g., uncoupling PLC or inhibiting GTP exchange). Assuming that this latter possibility holds, then one might explain the apparent specificity of the effect on NT binding by postulating that the uncoupled state of NTS1 was unique in displaying a greatly enhanced affinity for its ligand. Further support derives from our finding that metabolic stress elevated bombesin binding, albeit to a lesser degree than NT binding.

At first glance, enhanced receptor binding in the face of inhibited receptor function would seem unusual. However, there is precedence for this kind of behavior in that tyrosine kinase inhibitors were shown to shift the EGF receptor to a dimer state that exhibited a greatly enhanced ability to bind EGF and a diminished tyrosine phosphorylation activity [63]. Since NTS1 can heterodimerize with NTS3 in some cells, decreasing the ability of NT to stimulate IP formation [64], it might be worthwhile to examine the effects of metabolic inhibitors on the interaction of NTS1 with NTS3 in PC3 cells.

The events that link metabolic stress to the alterations in NTS1 function have not yet been identified. AMPK, a metabolic sensor of the AMP to ATP ratio [65], which has been implicated in the regulation of glucose and lipid metabolism in response to metabolic stress [54,66,67], came to mind as a likely participant. Although in the current study we showed that AMPK was phosphorylated during treatment of PC3 cells with metabolic inhibitors, it is not yet known if AMPK has a direct role in regulating NTS1 function. AMPK can be activated in an allosteric manner by increasing levels of cellular AMP [35] and also by phosphorylation within its activation loop by AMPK kinases [68]. AICAR becomes an AMPK activator after its intracellular phosphorylation to form the AMP mimetic ZMP [65]. Here, we found that 3 mM AICAR stimulated the phosphorylation of AMPK in PC3 cells, which agrees with Rattan et al., who showed AMPK activation by 0.1–2 mM AICAR [4]. Consistent with the role of AMPK in opposing metabolic stress, we found that the effects of AICAR were opposite to those of metabolic inhibitors. Thus, at doses in the range of 0.04 to 2.5 mM, AICAR elevated cellular ATP levels and inhibited NT binding. We also found that AICAR reversed the effects of myxothiazole, antimycin A and rotenone on cellular ATP levels and on NT binding. Our findings suggest that the short-term effects of AICAR in PC3 cells were protective against stress, which is consistent with the work of Buzzai et al. who showed that AICAR reversed the sensitivity of glioblastoma cells to glucose deprivation [30].

Because of its devastating effects in PC patients, the transition to androgen independence is a key process that is of great interest to physicians and scientists. Recently, Higuchi et al. found that by depleting mitochondrial DNA, they could generate LNCaP clones that displayed diminished mitochondrial respiration along with enhanced androgen independence and tumorigenicity [25]. These effects were reversed by reconstituting the mitochondrial DNA, and the authors concluded that androgen independence and mitochondrial function were closely associated. Mutations in mitochondrial DNA are common in PC and investigators have speculated that they might contribute to tumor susceptibility [69]. The leading explanations for a link between mitochondrial loss of function and tumorigenesis include (a) the attendant increase in glycolysis leads to upregulation of growth regulators; (b) the metabolic stress leads to induction of hypoxia inducible factor under normoxic conditions; and (c) the metabolic stress alters the production of mitogenic reactive oxygen species. Whatever the explanation, it is clear that the state of the metabolic pathways utilized by PC cell lines can markedly influence the growth and tumorigenicity of the cells.

The present findings indicate that PC3 cells utilize both glycolytic and mitochondrial pathways to maintain their ATP levels. Whereas inhibition of either pathway could only reduce cellular ATP by about 70%, inhibition of both pathways lowered ATP by more than 90%. NT binding, NT-induced IP formation and growth signaling were all sensitive to the ATP-lowering effects of metabolic inhibitors. Accordingly, the inhibition of NT-induced IP formation by myxothiazole or CCCP was strongly potentiated by the withdrawal of glucose. These results suggest that combination therapy using glycolytic and mitochondrial inhibitors might provide an effective means of suppressing the growth of PC *in vitro* and *in vivo*.

5. Conclusions

This study shows that NT-induced growth signaling in PC3 cells is sensitive to metabolic stress, and that this regulation

occurs under conditions that decrease cellular ATP levels in parallel with changes in cellular NT binding and NT-induced IP formation. Thus, the cellular energy status appears to influence NTS1-mediated mitogenic signaling by altering the ability of NTS1 to stimulate IP formation, an effect that could involve modulation of the receptor state (e.g., uncoupling G protein and/or PLC) or modulation of the levels of PLC substrate PIP2.

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Protein kinase C inhibitors alter neurotensin receptor binding and function in prostate cancer PC3 cells

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Abstract

Prostate cancer PC3 cells expressed constitutive protein kinase C (PKC) activity that under basal conditions suppressed neurotensin (NT) receptor function. The endogenous PKC activity, assessed using a cell-based PKC substrate phosphorylation assay, was diminished by PKC inhibitors and enhanced by phorbol myristic acid (PMA). Accordingly, PKC inhibitors (staurosporine, Go-6976, Go-6983, Ro-318220, BIS-1, chelerythrine, rottlerin, quercetin) enhanced NT receptor binding and NT-induced inositol phosphate (IP) formation. In contrast, PMA inhibited these functions. The cells expressed conventional PKCs (α, βI) and novel PKCs (δ, ε), and the effects of PKC inhibitors on NT binding were blocked by PKC downregulation. The inhibition of NT binding by PMA was enhanced by okadaic acid and blocked by PKC inhibitors. However, when some PKC inhibitors (rottlerin, BIS-1, Ro-318220, Go-69830, quercetin) were used at higher concentrations (>2 μM), they had a different effect characterized by a dramatic increase in NT binding and an inhibition of NT-induced IP formation. The specificity of the agents implicated novel PKCs in this response and indeed, the inhibition of NT-induced IP formation was reproduced by PKCδ or PKCε knockdown. The inhibition of IP formation appeared to be specific to NT since it was not observed in response to bombesin. Scatchard analyses indicated that the PKC-directed agents modulated NT receptor affinity, not receptor number or receptor internalization. These findings suggest that PKC participates in heterologous regulation of NT receptor function by two mechanisms: a) — conventional PKCs inhibit NT receptor binding and signaling; and b) — novel PKCs maintain the ability of NT to stimulate PLC. Since NT can activate PKC upon binding to its receptor, it is possible that NT receptor is also subject to homologous regulation by PKC.
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Keywords: Neurotensin; Prostate cancer; Protein kinase C; G protein receptor binding; G protein receptor signaling

1. Introduction

Neurotensin (NT), a regulatory peptide found in the nervous system and in endocrine cells of the intestinal mucosa [1, 2], has multiple roles as a neurotransmitter and hormone [3–5]. Considerable evidence suggests that NT could contribute to the growth of normal and neoplastic cells [6,7]. The high affinity G protein-coupled NT receptor NTS1 is overexpressed in many primary human tumors and cell lines, including human prostate cancer PC3 cells [8]. We showed that stimulation of PC3 cells with NT induced a growth response that involved protein kinase C (PKC)-dependent transactivation of the EGF receptor and activation of downstream mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase [9]. The growth effects of NT in other systems and its ability to activate MAPK [10], as well as to enhance cyclic AMP formation [11] and to phosphorylate GSK-3α/β [12] were also PKC-dependent. In a number of these cells, NT was shown to activate phospholipase C (PLC) [13,14], stimulating the formation of inositol phosphate (IP) and the mobilization of intracellular Ca^{2+} [15]. Since PKC activity is dependent on the levels of Ca^{2+} and DAG, NT could activate both conventional and novel PKCs, and our recent work indicates that NT enhanced PKC isotype phosphorylation assessed by western blotting in PC3 cells (S Hassan, unpublished results). Not only

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might PKC mediate some of the effects of NT, but the activation of PKC could possibly exert feedback effects on NT receptor binding and signaling. Despite these findings, there is a paucity of information concerning the relationships between PKC activity and NT receptor function.

The responsiveness of cells to signaling inputs is regulated by a variety of mechanisms that serve to maintain homeostasis and coordinate signaling events. Some of the effects of NT have been shown to display homologous desensitization, such that there is a diminished response to a second stimulus given after the initial one. Heterologous desensitization has also been noted using stimuli that do not activate NT receptors [16]. These findings suggest that NT receptors could be subject to regulation by second messenger-dependent kinases, which could alter the availability of or the activity of the receptor. For the G protein-coupled receptors that have been studied extensively, such as the rhodopsin receptor and the β2-adrenergic receptor, there is considerable evidence suggesting that receptor phosphorylation is an important mechanism for regulating receptor responsiveness [17,18]. One generality that has emerged from the work in the laboratory of Robert Levkowitz and others is that G protein receptor kinases usually mediate phosphorylation events that are associated with agonist-induced (homologous) receptor desensitization, whereas signaling kinases such as PKC and PKA usually promote receptor phosphorylations involved in heterologous desensitization [18,19]. Endocytosis by β-arrestin and clathrin dependent mechanisms can also be part of the desensitization process [20]. In regards to NTS1, however, these mechanisms have not been well studied. Agonist dependent phosphorylation of HA-tagged NTS1 has been demonstrated in HEK-293 cells and the importance of certain C-terminal serine residues has been studied [21]. However, the kinases involved and the relationship to the desensitization of NT-induced signaling have not been studied. In addition, the effects on the parameters of receptor binding and internalization have not been defined.

In order to better understand the potential role of PKC in regulating NT receptor function, we set out to determine which PKC isotypes were expressed in PC3 cells and to study the effects of PKC activation and inhibition on NT receptor binding and signaling. The PKC family of serine/threonine kinases phosphorylate proteins at sites resembling the consensus motif RXXS/TXR [22,23] and phospho-specific antibodies towards similar motifs have been used to measure PKC substrate phosphorylation in cells [24,25]. Subcategories of PKC isotypes have been defined, including the conventional PKCs (PKCα, βI, βII and γ), the novel PKCs (PKCδ, ε, θ and η) and the atypical PKCs (PKCζ, μ and ι) based on their activity requirements [26,27]. Conventional PKCs are activated by Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DAG), whereas novel PKCs respond to PS and DAG, and atypical PKCs respond only to PS. Phorbol 12-myristate 13-acetate (PMA), which can mimic the effects of DAG, has been used to activate and/or downregulate conventional and novel isoforms of PKC. The phosphorylation of PKCs and their translocation from cytosol to membrane compartments that occur following acute treatment with PMA have been accepted as indicators of PKC activation. Chronic treatment with PMA on the other hand has generally been found to downregulate PKC expression. Many of the PKC inhibitors that have been used to investigate the involvement of PKC in cellular processes act by blocking the ATP binding site [23]. The most potent of these include the structurally related compounds staurosporine, bisindolylmaleimide I (BIS-1), Ro-328220, Go-6983 and Go-6976 [28]. These compounds exhibit a rather broad isotype specificity [29], except for Go-6976 which preferentially inhibits conventional PKCs, and Go-6983 which does not inhibit PKCμ [30]. Although rottlerin was originally described as a specific PKCδ inhibitor [31], further study indicated that it did not inhibit the enzyme directly [29] and that it could act as a mitochondrial uncoupler [32]. Less potent PKC inhibitors that act by mechanisms that are not well defined include chelerythrine [33] and quercetin [34].

In the work reported here, we first examined the effects of PKC-directed agents on PKC substrate phosphorylation in PC3 cells, establishing that these cells displayed a high level of constitutive PKC activity that responded to PKC activation and inhibition. Then, we investigated the effects of PKC activation, PKC inhibition and PKC isotype knockdown on NT receptor function. Two categories of PKC inhibitors were defined which had dramatically different effects on NT receptor binding and signaling. Based on the selectivity of these inhibitors and the effects of PKC isotype knockdown, we concluded that conventional and novel PKCs played opposite roles in regulating NT receptor function.

2. Materials and methods

2.1. Materials

[^{125}I]-sodium iodide (2000 Ci/mmol), [1,2-^{3}H(N)]-myo-inositol (60 mCi/mmol) and [methyl-^{3}H]-thymidine were obtained from Perkin Elmer Life Science (Boston, MA). The phospho-specific antiserum to the PKC substrate motif (#2261) was from Cell Signaling Technology (Beverly, MA). The antibodies towards PKCα, PKCβI, PKCβII, PKCδ, PKCε, PKCγ and HRP-linked secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). SuperSignal chemiluminescent substrate was obtained from Pierce (Rockford, IL). Staurosporine was from Biomol (Plymouth Meeting PA). Bisindolylmaleimide I (BIS-1), bisindolylmaleimide V (BIS-V), chelerythrine chloride, Go-6976, Go-6983 and Ro-318220 were from Calbiochem (San Diego, CA). Phorbol-12 myristate 13-acetate (PMA), rottlerin, quercetin and all other chemicals were from Sigma (St. Louis, MO).

2.2. Tissue culture

PC3, PC3M, DU145, MatLyLu and HT29 cells, obtained from American Type Culture Collection (Manassas, VA), were maintained in F12K medium (PC3, PC3M) and DMEM medium (DU145, MatLyLu, HT29), supplemented with 10% fetal bovine serum [8]. PC3, PC3M (its highly metastatic clone), and DU145 are androgen-independent cell lines derived from

human prostate adenocarcinomas. MatLyLu was derived from a rat prostate carcinoma and HT29 from a human colon carcinoma. LNCaP^{CS} cells, a clone derived from the human prostate LNCaP cell line, were a gift from Shuk-mei Ho, Department of Environmental Health, University of Cincinnati Medical School. The conditions for growing these cells have been described by us [35]. For western blots, cells were grown in 60 mm dishes; for all other studies, cells were grown in 24-well culture plates. For IP studies, cells were labeled with ^{3}H-inositol using medium 199 (Difco), which has a low inositol content.

2.3. Binding to cultured cells

HPLC-purified monoiodinated NT (^{125}I-NT) at 2000 Ci/mmol was prepared and binding was performed as described by us [13]. In brief, cells in 24-well plates (80–90% confluent) were washed with hepes-buffered Locke-BSA (Locke): 148 mM NaCl; 5.6 mM KCl, 6.3 mM hepes; 2.4 mM $NaHCO_3$; 1.0 mM $CaCl_2$; 0.8 mM $MgCl_2$; 5.6 mM glucose; 0.1% BSA; pH 7.4. Stock solutions (2–10 mM) of the PKC inhibitors in dimethyl sulfoxide (DMSO) were stored at −20°C, except for quercetin (prepared just before use), and were diluted in Locke to give ≤1% DMSO. Equilibrium binding at 37 °C was performed for 30 min using 10^5cpm/ml ^{125}I-NT in 1.0 ml Locke. The reaction was stopped on ice, the medium was aspirated and cells were washed in ice-cold saline. Cellular binding was determined as radioactivity per μg protein in cells extracted in 0.3 M NaOH. Specific binding, displaceable by 1 μM NT (20,000-fold excess), was 95% of total binding. Binding displacement curves were constructed and binding parameters were determined as described [35]. Cell-surface binding and internalization were assessed by washing cells at room temperature for 2 min with 1 ml of 0.2 M acetic acid, 0.5 M NaCl.

The binding parameters for the ligands used to measure bombesin (BOM) receptor binding and EGF receptor binding in PC3 cells were described by us [13]. In brief, equilibrium binding was performed in a manner identical to that for NT using HPLC-purified ^{125}I-[Nle^{14}]-BOM (10^5 cpm, 50 pM) and ^{125}I-EGF (10^5 cpm, 50 pM). Specific binding was defined as that displaceable by 1 μM BOM (20,000-fold excess) or 0.1 μM EGF (2000-fold excess).

2.4. IP formation

Formation of [^{3}H]-IP was measured as described by us [13]. Briefly, PC3 cells in 24-well plates were incubated 48 h with myo-[^{3}H]-inositol (2.5 μCi/ml) in medium 199, 1% fetal calf serum. Cells were pretreated with Locke, 15 mM LiCl for 10 min to inhibit phosphatases. In some cases, they were concomitantly pretreated with test agents or vehicle (DMSO) in Locke, 15 mM LiCl as indicated. The reaction was initiated by adding NT, BOM or vehicle (Locke). After 30 min at 37 °C, the medium was aspirated, ice-cold 0.1 M formic acid in methanol was added and the plates were placed at −20 °C overnight. IP was adsorbed to AG-1X8, which was washed in 5 mM myo-inositol and eluted in 1.5 M ammonium formate. Scintillation counting was performed in Ecoscint (National Diagnostics).

2.5. Inhibition of PKCδ and PKCε in PC3 cells through stable transfection of shRNA expression plasmids

Plasmid constructs (Open Biosystems, Huntsville, AL) designed to constitutively express shRNAs that specifically target either PKCδ (Open Biosystems catalog number RHS1764-9492328 or RHS1764-9098879) or PKCε (RHS1764-9493023) or a control plasmid encoding a non-silencing shRNA (RHS1707) were purchased from the UMASS shRNA Core Facility. The parent plasmid (pSM2a) contains the bacterial puromycin resistance gene under the control of the PGK promoter and the mir-30 gene containing gene-specific synthetic microRNA sequences under the control of the U6 promoter to drive microRNA production [36]. PC3 cells were transfected with individual plasmids on 100 mm tissue culture dishes using FuGene transfection reagent (Roche) according to the manufacturer's instructions, and stably transfected cells were selected by the addition of puromycin (1.0 μg/ml) to the culture medium. Visible clones were isolated after 2–3 weeks of selection using cloning cylinders, propagated, and screened by western blot analysis to examine PKCδ and PKCε expression levels. Clones displaying significant inhibition of PKCδ or PKCε were grown under continued puromycin selection for use in NT binding and other experiments. PKCδ was significantly inhibited by only one of two shRNAmir constructs tested (RHS1764-9098879).

2.6. Western blotting

Western blot analysis was performed as described by us [9]. In brief, PC3 cells in 60-mm dishes were withdrawn from serum for 24 h. Cells, washed in Locke, were exposed to stimuli at 37 °C for times indicated. After washing with ice-cold PBS containing phosphatase inhibitors, cells were placed on ice and scraped into 2× SDS buffer (plus inhibitors) and sonicated. Equal amounts of protein were separated by SDS-PAGE using polyacrylamide minigels and electroeluted onto PVDF (Immobilon P, Millipore). Blots were incubated with primary antibodies in blocking buffer for 18 h at 4 °C. After washing, blots were incubated with HRP-linked secondary antibodies for 1 h at 20 °C, and ECL was performed using supersignal west pico reagent (Pierce, Rockford, IL) and films were scanned for computerized densitometric analysis. After staining with one antibody, blots were stripped and re-probed using different antibodies for comparison and normalization.

3. Results

3.1. PC3 cells displayed constitutive PKC activity and PMA-induced PKC activity

The growth-promoting effects of NT in PC3 cells are PKC-dependent. In order to assess the importance of PKC for NT receptor binding and signaling, we first determined the basal level and stimulated level of PKC activity in the cells by

performing western blotting using a phospho-specific antiserum directed towards PKC substrates exhibiting the motif R/K-X-S-Hyd-R/K (where Hyd = hydrophobic amino acid). PKC isotype specificity considerations predicted that this assay would primarily detect substrates phosphorylated by conventional PKCs (α, βI, βII, γ), and might respond to novel PKCε substrates but would react poorly with novel PKCδ substrates [37].

Untreated cells (results not shown), and cells incubated with 0.1% DMSO (vehicle control) for 30 min, exhibited at least ten positive bands with molecular sizes ranging from 40 kDa to 140 kDa (Fig. 1A, control). In contrast, cells pretreated for 30 min with 1 μM concentrations of staurosporine, BIS-1, Go-6983 or Ro-318220 displayed less intense labeling of as many as eight of these bands, which was most evident for the 40 kDa, 50 kDa and 140 kDa bands (Fig. 1A). The inhibitory effects of Go-6976 and chelerythrine were seen at higher concentrations (5–10 μM), whereas rottlerin (10 μM) and quercetin (15–40 μM) gave only a slight inhibition (Fig. 1A and C). That these effects were due to the inhibition of constitutive PKC activity was shown by stimulating the cells with PKC activator PMA, which further enhanced the labeling of these bands as well as others not seen under basal conditions (Fig. 1B, control). As expected, the effects of PMA were attenuated (Fig. 1B and D) by pretreating the cells with staurosporine (1 μM), BIS-1 (1 μM), Ro-318220 (1 μM), Go-6976 (1–10 μM), Go-6983 (1–10 μM) or chelerythrine (5 μM). In contrast, rottlerin (1–10 μM) and quercetin (15–40 μM) inhibited only slightly (Fig. 1B and D). These findings suggested that the identified bands represented PKC substrates which were constitutively phosphorylated by PKCs that were active under basal conditions and that could be further activated by PMA. The effects of the inhibitors were consistent with the prediction that this assay detected primarily substrates that could have been phosphorylated by conventional PKCs (α, βI, βII, γ) or novel PKCε but not novel PKCδ.

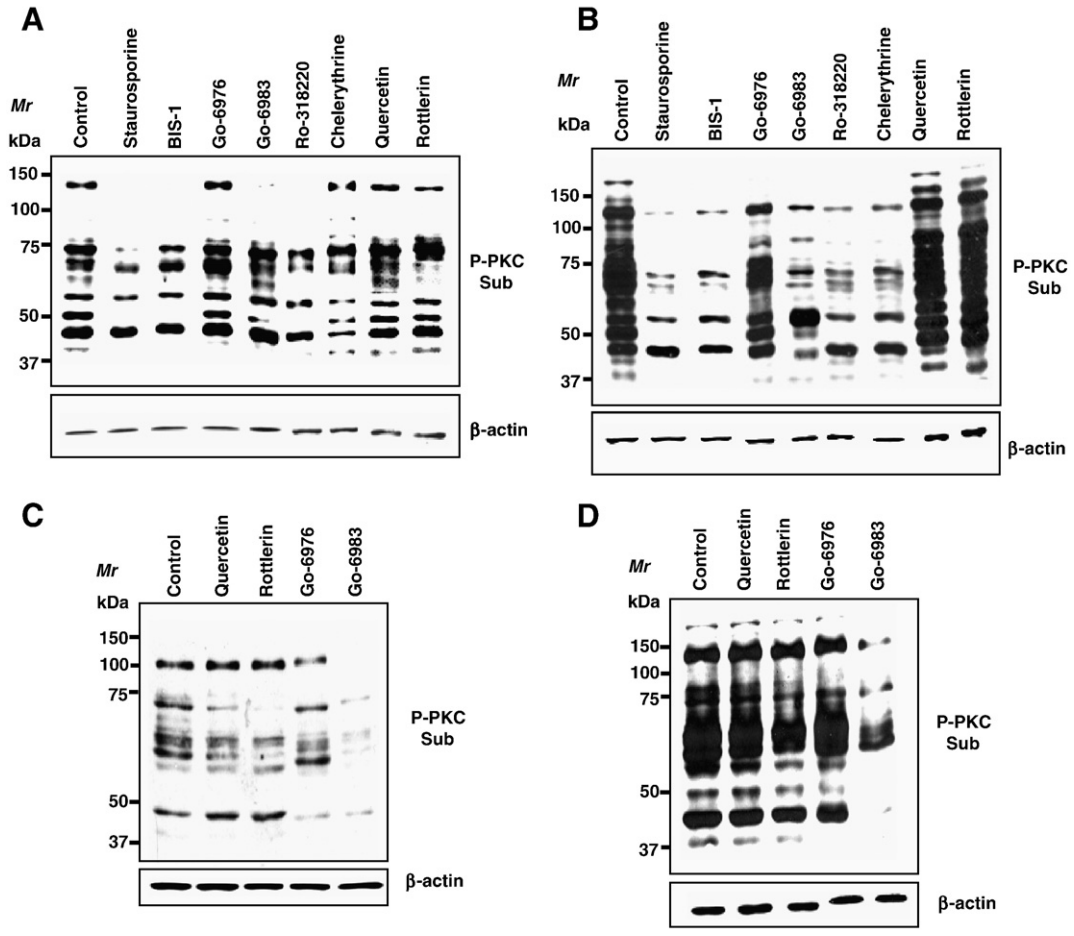

Fig. 1. PC3 cells displayed constitutive PKC activity (A and C) and PMA-induced PKC activity (B and D) that was sensitive to PKC inhibitors. Endogenous PKC substrate phosphorylation was assessed by western blotting using a phopho-specific antiserum to the motif R/K-X-S-Hyd-R/K. In A, quiescent cells were incubated with staurosporine (1 μM), BIS-1 (1 μM), Go-6976 (1 μM), Go-6983 (1 μM), Ro-318220 (1 μM), chelerythrine (5 μM), quercetin (15 μM) rottlerin (1 μM), or 0.1% DMSO (control) for 30 min. In B, cells pretreated as in A were then stimulated with 100 nM PMA for 5 min. In C, quiescent cells were incubated with quercetin (40 μM), rottlerin (10 μM), Go-6976 (10 μM), Go-6983 (10 μM), or 0.1% DMSO (control) for 30 min. In D, cell pretreated as in C were then stimulated with 100 nM PMA for 5 min. Cell extracts containing equal amounts of protein were subjected to western blotting using the PKC substrate-specific antiserum. Shown are typical results that are representative of at least three experiments each.

3.2. PKC activator PMA inhibited NT binding and NT-induced IP formation in PC3 cells

To determine whether PKC activity could regulate NT receptor function, we first tested the effect of PKC activator PMA on NT binding in intact PC3 cells. Specific binding of ^{125}I-NT (10^5 cpm/ml) to the cells, measured at equilibrium (30 min), was 21.5±1.9 cpm/μg protein (n=9), which was consistent with our prior work [13]. Incubation of the cells with PMA for 15 min decreased NT binding by as much as 42% with an IC50≅1 nM (Fig. 2A). Pretreating the cells with the inhibitor of protein phosphatases (okadaic acid) enhanced the effect of PMA on NT binding (Fig. 2B). Pretreating the cells with PKC inhibitors (staurosporine, BIS-1, Go-6983 and Go-6976), using the dose (1 μM) shown in Section 3.1 to block the PMA-induced phosphorylation of PKC substrates, prevented the effect of PMA on NT binding (Fig. 2C). These results indicated that PMA-induced PKC activity decreased NT binding by an effect involving protein phosphorylation.

Next, we tested the effect of PMA on NT-stimulated PLC activity in PC3 cells. Stimulation of the cells with 30 nM NT (a maximal dose) increased IP formation 3.9±0.4 fold (mean± SEM, n=8) in keeping with our prior work [13]. As could be predicted from its inhibitory effect on NT binding, PMA diminished NT-induced IP formation and exhibited an IC50≅ 1 nM (Fig. 4A). Taken together, these results indicated that PMA-induced PKC activity inhibited both NT receptor binding and NT receptor-mediated activation of PLC.

3.3. PKC inhibitors enhanced NT binding and NT-induced IP formation in PC3 cells

The studies in Section 3.1 indicated that PC3 cells displayed constitutive PKC activity. To determine whether this constitutive PKC activity regulated NT receptor function, we tested the effects of PKC inhibitors on NT binding and NT-induced IP formation. Six PKC inhibitors (staurosporine, Go-6976, Go-6983, chelerythrine, BIS-1, and Ro-328220) elevated NT binding when used at the low micromolar concentrations shown in Section 3.1 to inhibit PKC substrate phosphorylation. Thus, at concentrations ≤2 μM, these agents elevated NT binding by 20–40% (Fig. 3A and B). NT binding was also elevated 20–40% by rottlerin (0.5–2 μM) and quercetin (5–20 μM), which inhibited PKC substrate phosphorylation to a lesser degree (Fig. 3B and 1C).

In keeping with their ability to enhance NT binding, the PKC inhibitors also enhanced NT-induced IP formation by 15–30% when used in the same concentration range (Fig. 4B). The negative control compound for PKC inhibitors (BIS-V) had little effect on NT binding or NT-induced IP formation at concentrations up to 10 μM (results not shown). Taken together, these findings suggested that the ≅30% elevation in NT binding and NT-induced IP formation involved an inhibition of the constitutive PKC activity displayed by these cells. In keeping with this, NT binding was decreased by ≅20% by protein phosphatase inhibitor okadaic acid, which would be expected to enhance the effects of the constitutive PKC activity (Fig. 2B).

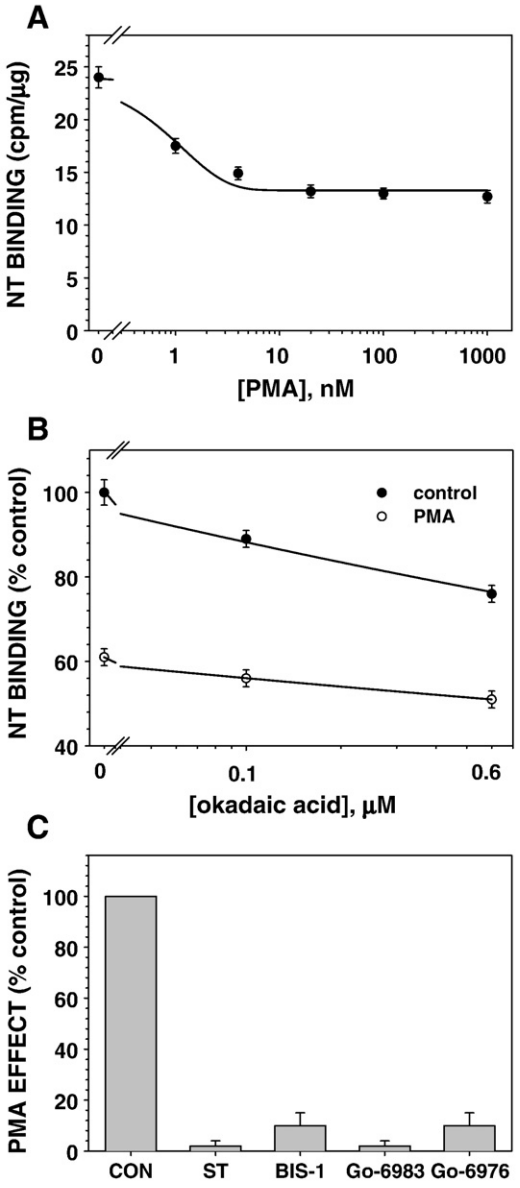

Fig. 2. PKC activator PMA decreased NT binding to PC3 cells (A), and its effect was enhanced by protein phosphatase inhibitor okadaic acid (B) and inhibited by pretreatment with PKC inhibitors (C). Specific NT binding was measured to intact cells at 37° C. In A, cells were pretreated with indicated concentrations of PMA for 15 min prior to measuring NT binding. In B, cells were pretreated with okadaic acid in the presence and absence of 100 nM PMA for 20 min prior to measuring NT binding. In C, cells were pretreated with staurosporine (1 μM), BIS-1 (1 μM), Go-6983 (1 μM), Go6976 (1 μM) or 0.05% DMSO (vehicle control) for 20 min. Then, 10 nM PMA was added for 10 min and NT binding was measured. For each experiment, the data (mean±SEM) are representative of at least three experiments.

3.4. Higher doses of some PKC inhibitors further enhanced NT binding and inhibited NT-induced IP formation

At concentrations >2 μM, five PKC inhibitors (Go-6983, BIS-1, rottlerin, Ro-318220 and quercetin) dramatically enhanced NT binding (Fig. 3A and B). Thus, these agents displayed the ability to increase NT binding by as much as 200%. Associated with this response was a dose-dependent inhibition

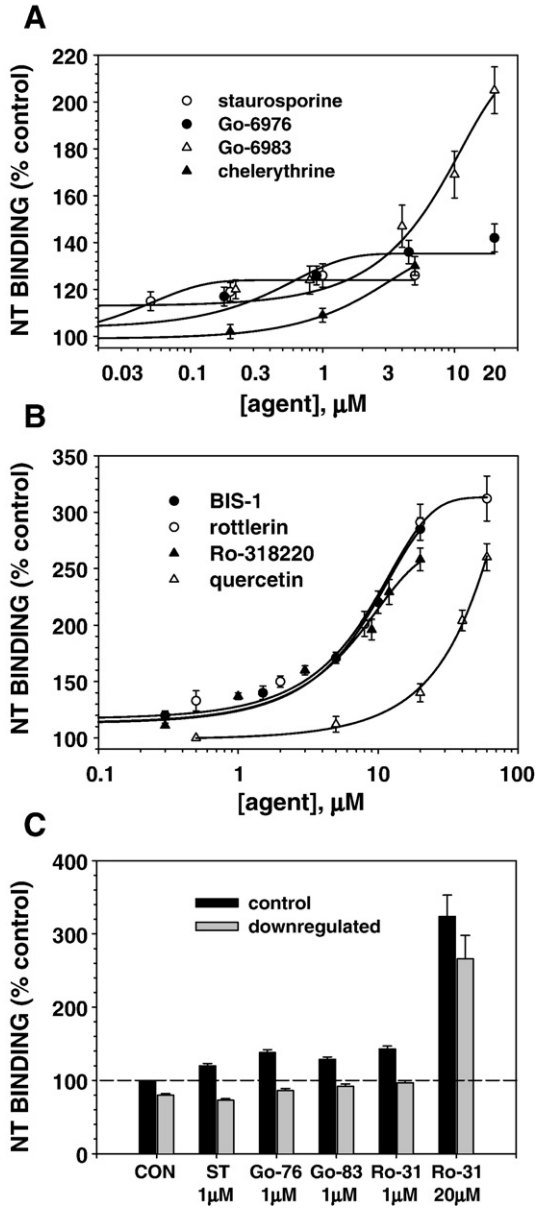

Fig. 3. PKC inhibitors enhanced NT binding to PC3 cells (A, B), and the response to low doses of PKC inhibitors was blocked by downregulation of PKC (C). Specific NT binding was measured to intact cells. In A and B, sub-confluent cells were pretreated with the indicated concentrations of agents for 15 min prior to measuring NT binding. The data (mean±SEM) were from at least 4 experiments. In C, sub-confluent cells were pretreated with 1 μM PMA (downregulated) or 0.01% ethanol (control) for 24 h. After washing in Locke, cells were pretreated with staurosporine (ST), Go-6976 (Go-76), Go-6983 (Go-83), Ro-318220 (Ro-31) or 0.05% DMSO (CON) for 15 min prior to measuring NT binding. The data (mean±SEM; $n=4$) from two experiments were expressed as % control. Note that PKC downregulation blocked the response to the low dose (1 μM) of each inhibitor and diminished the response to the high dose (20 μM) of Ro-31220.

of NT-induced IP formation by as much as 70% (Fig. 4C). In contrast, three PKC inhibitors (staurosporine, Go-6976 and chelerythrine) were unable to further elevate NT binding beyond ≅40%, even at higher doses (Fig. 3A). In addition, staurosporine and Go-6976 did not inhibit NT-induced IP formation (Fig. 4C).

These results indicated that we had defined two categories of PKC inhibitors differing in their ability to modulate NT receptor function. The agents in category I (staurosporine, Go-6976 and chelerythrine) produced only response I (a modest enhancement of NT binding and NT-induced IP formation). In contrast, the agents in category II (Go-6983, BIS-1, rottlerin, Ro-318220 and quercetin) produced two responses in a dose-dependent manner. At low concentrations response I was obtained, whereas at higher concentrations response II (a dramatic increase in NT

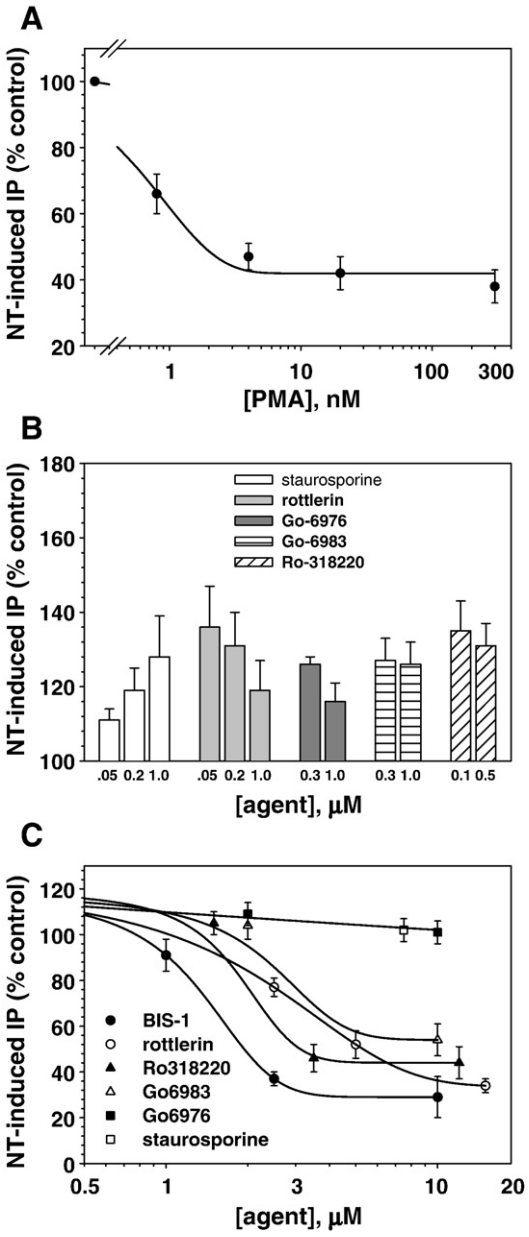

Fig. 4. NT-induced IP formation was inhibited by PKC activator PMA (A), whereas it was enhanced by low doses (B) and inhibited by high doses (C) of PKC inhibitors. IP formation was measured in response to 30 nM NT. The effect of NT (≅4-fold elevation) was calculated as the increment above the appropriate control. In A, cells were pretreated with indicated concentrations of PMA for 15 min prior to stimulation with NT. In B and C, cells were pretreated with the indicated agents for 15 min prior to stimulation with NT. For each panel, the data (mean±SEM) are representative of at least three experiments.

binding and an inhibition of NT-induced IP formation) was seen. Three of the agents in category II were known to inhibit conventional PKCs more than novel PKCs when used at low concentrations [30,38]. This suggested that our findings might be attributed to a disproportionate inhibition of PKC isotype activity, and we postulated that inhibition of conventional PKCs induced response I, whereas an inhibition of novel PKCs brought about response II. However, not all of the data fit this model and it was still possible that other targets were involved, especially in response II.

3.5. PKC isotype expression and downregulation

PC3 cells expressed conventional PKC subtypes (α, βI) as well as novel PKC subtypes (δ, ε) as determined by western blotting (Fig. 5). Pretreating the cells with 1 μM PMA for 24 h downregulated the expression of conventional PKCα and PKCβI more than novel PKCδ and PKCε (Fig. 5). Using this method to downregulate PKC expression, we then tested the effects of PKC inhibitors on NT binding. The results in Fig. 3C show that PKC downregulation blocked the modest elevation in NT binding in response to 1 μM doses of staurosporine, Go-6976, Go-6983 and Ro-318220, but only reduced (by 25%) the dramatic response to 20 μM Ro-318220. These results provided further support for the idea that low doses of PKC inhibitors caused a modest increase in NT binding by inhibiting conventional PKCα and PKCβI, whereas high doses of PKC inhibitors (from category II) caused a dramatic increase in NT binding by inhibiting novel PKCδ and PKCε. Thus, the partial inhibition of the response to 20 μM Ro-318220 (Fig. 3C) was consistent with the partial downregulation of PKCδ and PKCε (Fig. 5). However, it was also possible that targets other than PKC mediated part of this response.

3.6. Effect of PKCδ and PKCε knockdown on NT binding

To determine if the effects of category II inhibitors on NT binding were mediated by PKCδ or PKCε, RNAi technology

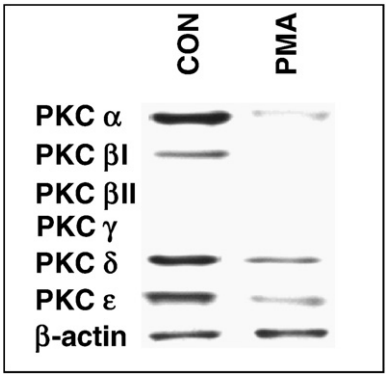

Fig. 5. Western blot assessing the expression of PKC isotypes in control PC3 cells and in cells pretreated with PMA. Cells grown in 60 mm dishes were withdrawn from serum for 24 h and then pretreated with 0.1% DMSO or 1 μM PMA for 24 h. Whole cell extracts were prepared, 40 μg protein was subjected to SDS-PAGE and western blotting was performed using antisera specific for PKCα, PKCβI, PKCβII, PKCγ, PKCδ and PKCε. β-actin was used as the loading control. The results shown are representative of two experiments.

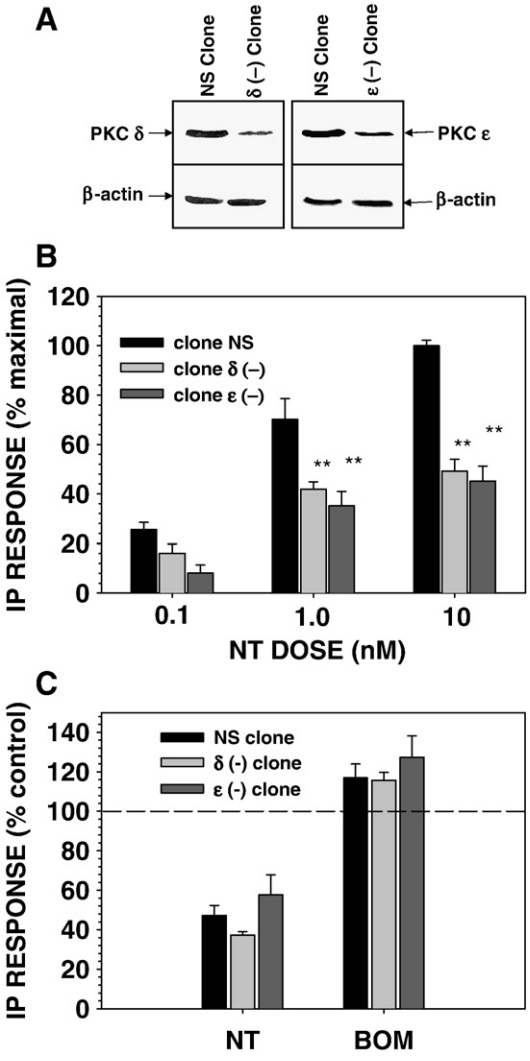

Fig. 6. Effect of PKCδ and PKCε knockdown on PKC expression (A), NT-induced IP formation (B) and on the ability of BIS-1 to alter NT-induced and BOM-induced IP formation (C). In A, an equal number of cells from each clone was extracted, 40 μg protein was subjected to SDS-PAGE and western blotting was performed for PKCδ and PKCε. β-actin was the loading control. The results, representative of multiple experiments using 2 to 5 clones each, indicate >50% knockdown of PKCδ and PKCε. In B, equal numbers of cells from each clone were stimulated with the indicated doses of NT. IP formation was measured and the responses were calculated as % maximal. In C, cells pretreated 15 min with 2.5 μM BIS-1 or vehicle control, were stimulated with 10 nM NT or 3 nM BOM. The IP responses were calculated as % control, which gave ≅4-fold elevation (NT) and ≅10-fold elevation (BOM). In B and C, the data (mean± SEM) are representative of at least three experiments. ** $p<0.01$.

was used to individually knockdown these novel PKC isotypes. A puromycin resistance gene was incorporated into the DNA construct to facilitate clone selection, and several clones were obtained that displayed >50% knockdown of PKCδ and PKCε (Fig. 6A). Based on results from western blotting, one clone representing each treatment was selected for further analysis: nonsense (NS clone); PKCδ(−) clone; and PKCε(−) clone. Basal NT binding, determined when the cells were ≅90% confluent and expressed as cpm/μg protein (mean±SEM, $n=4$), did not differ for the 3 clones (NS clone, 18.0±1.4; PKCδ(−) clone, 15.8±1.2; and PKCε(−) clone, 15.4±1.1). Assessing the

effects of BIS-1 and rottlerin on cellular NT binding in these clones indicated that they were also not significantly different (Table 1). These results indicated that PKCδ knockdown or PKCε knockdown by itself was not sufficient to alter basal NT binding or the effects of BIS-1 and rottlerin on NT binding. One possible explanation was that knockdown of both PKCδ and PKCε (and perhaps PKCα and PKCβI) was necessary for the effect.

3.7. Effect of PKCδ and PKCε knockdown on NT-induced IP formation

NT was less effective in elevating IP formation in the PKCδ(−) and the PKCε(−) clones as compared to the NS clone (Fig. 6B). This was not due to a general effect on PLC activity or the level of phospholipid substrate since the IP response to 3 nM BOM was not inhibited in the PKCδ(−) clone (% control response, 95±2; $n=6$) and was enhanced in the PKCε(−) clone (% control response, 148±13; $n=6$). Consistent with the importance of both PKCδ and PKCε in this regulation, BIS-1 further inhibited the NT response and further enhanced the BOM response, even in the PKCδ(−) and PKCε(−) clones (Fig. 6C). These results were in keeping with the hypothesis that PKCδ and PKCε activity maintained the ability of NT receptor to stimulate PLC.

3.8. PMA decreased and PKC inhibitors increased both cell-surface NT binding and internalization in PC3 cells

Since internalization of the NT receptor occurs during NT binding to PC3 cells [13], we sought to determine if activation or inhibition of PKC altered this process. NT receptor internalization, determined as the percentage of NT binding that resisted acid washing of the cells, was 69±3% (mean±SEM, $n=5$) under control conditions. Pretreatment of the cells with PKC activator PMA decreased both cell-surface NT binding and internalized NT binding by ≅50% (Fig. 7A). Although there was a tendency for PMA to increase the percentage of internalization (Fig. 7A legend), the results did not differ significantly (e.g., control, 68±2% vs 25 nM PMA, 72±2%; $p>0.1$).

Table 1
Effect of BIS-1 and rottlerin on NT receptor binding in PKC knockdown and control clones of PC3 cells

Cell clone	NT binding (% DMSO control)			
	BIS-1		Rottlerin	
	0.5 μM	2.5 μM	0.5 μM	2.5 μM
NS control	155±14	216±20	150±16	181±17
PKCδ(−)	168±13	254±17	145±13	222±18
NS control	140±13	225±19	140±14	236±19
PKCε(−)	167±12	268±21	177±15	244±18

NT receptor binding to each PC3 cell clone was measured in the presence of BIS-1 or rottlerin at the indicated concentrations as compared to the vehicle (DMSO) control. The cells (80–90% confluent) were pre-incubated for 15 min with the agents in Locke prior to the binding reaction. Control NT binding for each clone was similar when expressed as cpm/μg protein: NS control, 18.0±1.4; PKCδ(−); 15.8±1.2; PKCε(−), 15.5±1.1 ($n=4$). For each clone, the effects of BIS-1 and rottlerin were expressed as % control NT binding (mean±SEM; $n=3$ experiments). The results for the different clones were not significantly different.

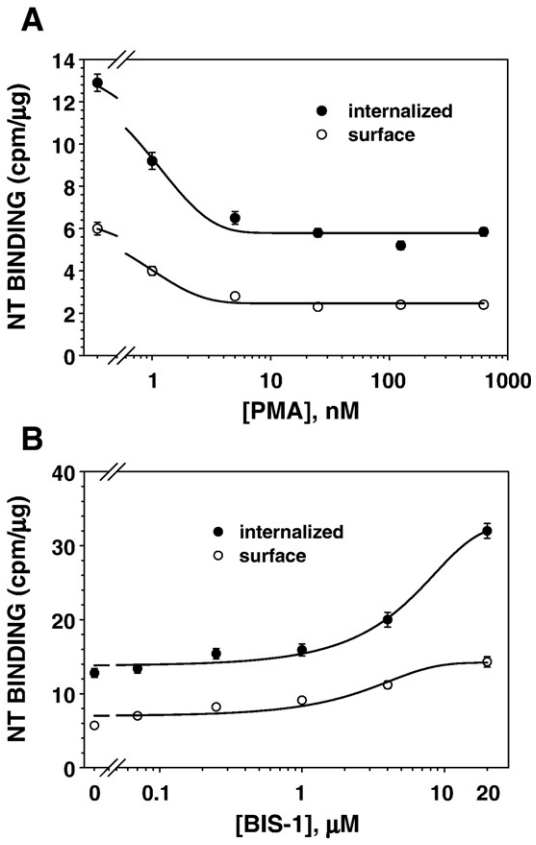

Fig. 7. NT binding to PC3 cells was diminished by PMA (A) and enhanced by BIS-1 (B), without an effect on the percentage of ^{125}I-NT internalized by the cells. Cells were pretreated 10 min with indicated concentrations of PMA, BIS-1 or vehicle control. After the binding reaction, cell-surface and internalized binding were determined by acid washing. The data (mean±SEM) were pooled from three experiments. In A, internalization expressed as percentage of total binding (mean±SEM) was: 68±2, 70±2, 70±2, 72±2, 68±2 and 71±2 at the 0, 1, 5, 25, 125 and 625 nM doses of PMA. In B, % internalization was 69±3, 66±2, 65±2, 64±2, 64±2 and 69±2 at the 0, 0.07, 0.25, 1, 4 and 20 μM doses of BIS-1.

Pretreatment of the cells with PKC inhibitor BIS-1 increased both cell-surface NT binding and internalized NT binding by as much as 2.5-fold (Fig. 7B). There was a tendency for BIS-1 to decrease the percentage of internalization (Fig. 7B legend); however, the results did not differ significantly (e.g., control, 69±3% vs 1 μM BIS-1, 64±2%; $p>0.05$). These results indicated that the changes in NT binding induced by PKC activation or PKC inhibition could not be attributed solely to any effects on NT receptor internalization.

3.9. Clathrin-coated vesicle-mediated endocytosis

To assess the importance of clathrin-coated vesicle-mediated endocytosis in the effects of PKC inhibitors, cellular NT binding was measured in hypertonic medium (Locke containing sucrose), which is known to block clathrin-coated vesicle-mediated endocytosis of ligands and receptors [39]. Sucrose

dose-dependently inhibited NT binding to the cells, with 0.33 M sucrose giving about 45% inhibition (Fig. 8A). However, sucrose unexpectedly inhibited both NT binding to the cell-surface and NT internalization, such that the internalized percentage was only slightly decreased (Fig. 8B). This was in contrast to the results for BOM receptor which was used as the positive control [40], where sucrose inhibited BOM internalization, enhanced BOM cell-surface binding and decreased the internalized percentage (Fig. 8B). In addition, when the NT binding reaction was done in 0.33 M sucrose, the response to BIS-1 was enhanced and the effect of PMA was diminished (Table 2). These findings suggested that hyperosmolar sucrose stimulated PKC activity and indeed, we found that cellular PKC substrate phosphorylation was enhanced by exposing the cells to 0.33 M sucrose for 15 min (Fig. 8C).

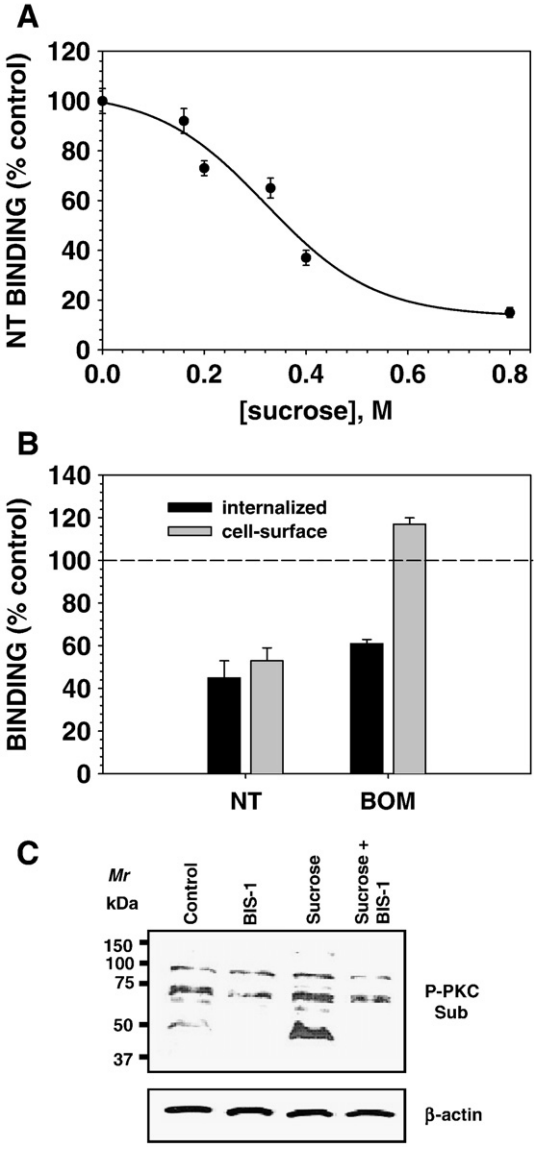

Fig. 8. Effect of hypertonic sucrose on NT receptor binding (A), NT receptor and BOM receptor internalization (B) and PKC substrate phosphorylation (C) in PC3 cells. In A, total cellular NT binding was measured in Locke containing varying concentrations of sucrose and the results were expressed relative to control. In B, the effect of 0.33 M sucrose on cell-surface and internalized binding for the NT receptor and the BOM receptor is shown. After binding was performed with ^{125}I-NT or ^{125}I-BOM, internalized and cell-surface radioactivity was measured by acid washing. The data (mean±SEM) show % binding obtained in sucrose relative to Locke control. Internalization of NT receptor expressed as percentage of total binding was 70±2 (Locke) and 65±2 (sucrose), while that for the BOM receptor was 68±1 (Locke) and 54±1 (sucrose). In C, quiescent cells, pretreated with 1 μM BIS-1 or vehicle control for 30 min, were stimulated with Locke or 0.33 M sucrose for 15 min. Cell lysates were subjected to western blotting using the PKC substrate specific antiserum. β-actin was used as loading control. Shown are typical results representing 3 experiments.

Table 2
Effects of PKC-directed agents on internalized and cell-surface NT binding in Locke and hypertonic sucrose

Agent	Concentration (μM)	Internalized NT binding (% control)[a]		Cell-surface NT binding (%control)[a]	
		Locke	Sucrose	Locke	Sucrose
DMSO	(0.1%)	100	100	100	100
BIS-1	0.6	126±4	153±5[b]	117±4	141±4[b]
	3.0	157±13	216±18[b]	145±6	194±10[b]
PMA	1	70±6	95±5[b]	48±6	77±11[b]

[a] The effect of each agent on NT receptor binding was tested in Locke or in Locke containing 0.33 M sucrose. The cells (80–90% confluent) were pre-incubated for 15 min with the agents or the vehicle control (0.1% DMSO) in Locke prior to the binding reaction. The medium was changed to Locke or sucrose containing the agents, and ^{125}I-NT was added. After 30 min, the cells were placed on ice and NT internalization and NT binding to the cell-surface were measured using the acid washing technique. The data (mean±SEM; $n=4$ experiments) were expressed relative to the appropriate vehicle control. For the control condition, sucrose reduced internalized NT binding and cell-surface NT binding to a similar extent (45–55%).

[b] Results in sucrose differed significantly from those in Locke ($p<0.05$).

We interpreted these findings to indicate that clathrin-coated vesicle-mediated endocytosis participated in NT receptor internalization, but the effect of BIS-1 on NT receptor binding persisted despite inhibition of this process with sucrose. Furthermore, our results suggested that sucrose decreased cellular NT binding at least partly by activating PKC, which is consistent with work in NIH/3T3 cells showing that hyperosmolality increased PKC activity within 10 min [41].

3.10. PMA increased and PKC inhibitor decreased the IC50 for NT binding in PC3 cells

To determine whether the effects of PKC-directed agents on NT binding reflected changes in receptor affinity or receptor number, NT displacement data were analyzed. The PKC activator PMA shifted the NT displacement curve to the right (Fig. 9A). The average IC50 (mean±SEM) was increased from 1.07±0.05 nM (control) to 2.17±0.17 nM (0.5 μM PMA; $p<0.01$) in 3 experiments. In contrast, the PKC inhibitor Ro-318220 shifted the NT displacement curve to the left (Fig. 9B). The average IC50 was decreased from 1.35±0.22 nM (control) to 0.55±0.09 nM (1 μM Ro-318220; $p<0.01$) in 3 experiments. Scatchard analyses (Fig. 9C and D) indicated that the changes in NT binding were due to the demonstrated changes in receptor affinity and could not be attributed to changes in receptor

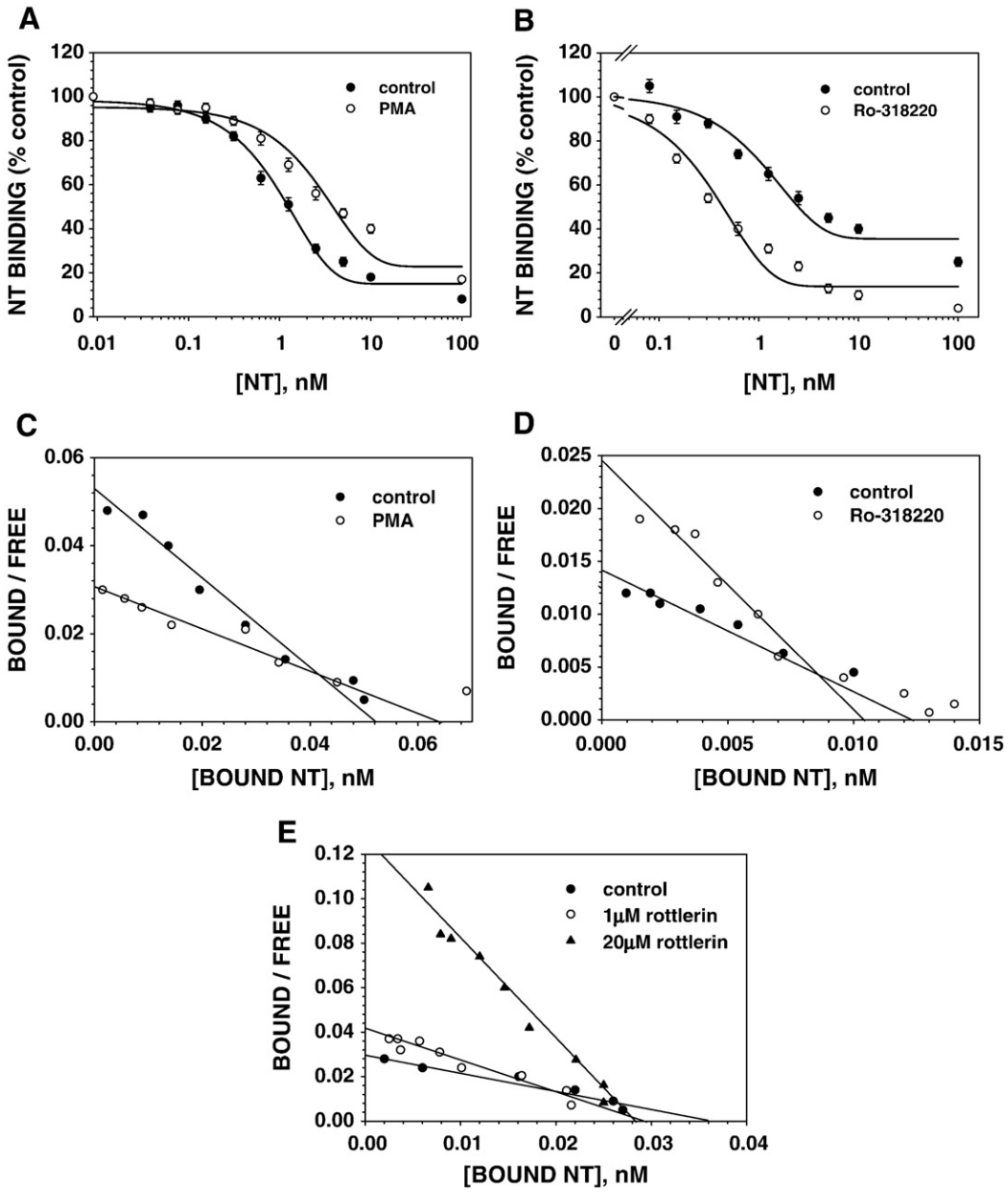

Fig. 9. Binding displacement curves (A, B) and Scatchard plots (C, D, E) for NT binding to PC3 cells in the presence and absence of PKC-directed agents. Cells were pretreated with agents indicated or control for 30 min. Then, specific NT binding was measured in the presence of indicated concentrations of NT at equilibrium (30 min). NT binding was decreased 35% by 100 nM PMA, whereas it was increased 54% by 1 μM Ro-318220. In A and B, log dose–response plots are shown in which NT binding was expressed as percentage of control. The IC50 was shifted to the right by PMA (A) and to the left by Ro-318220 and rottlerin (B). Results are from typical experiments that were repeated twice. C, D and E are Scatchard plots for typical experiments, showing that the changes in NT binding were due to shifts in receptor affinity (slope of line) rather than receptor number (intercept at *x*-axis). In C, note that two components were seen for PMA and the line drawn estimates only the high affinity sites. In C, Ki was 1.0 nM (control) and 2.1 nM (PMA); Bmax was 260 fmol/mg (control) and 320 fmol/mg (PMA). In D, Ki was 1.0 nM (control) and 0.42 nM (1 μM Ro-318220); Bmax was 203 fmol/mg (control) and 186 fmol/mg (1 μM Ro-31882). In E, Ki was 1.2 nM (control), 0.69 nM (1 μM rottlerin) and 0.23 nM (20 μM rottlerin); Bmax was 246 fmol/mg (control), 219 fmol/mg (1 μM rottlerin) and 199 fmol/mg (20 μM rottlerin).

number. Studies comparing the effects of inhibitors from categories I and II showed that 1 μM Go-6976 gave results that were similar to those for 1 μM Ro-318220 (results not shown). In addition, Fig. 9E shows that the effect of the high dose (20 μM) of the category II inhibitor rottlerin on the binding parameters was similar to that of the low dose (1 μM), except that there was a greater shift in the Ki to even higher affinity, without an increase in receptor number.

3.11. Receptor specificity

To determine if these responses were specific to the NT receptor, we tested PKC inhibitors for effects on BOM receptor binding and EGF receptor binding in PC3 cells [13]. Since the BOM [42] and EGF receptors [43] are known to be regulated by PKC, this provided another test regarding the specificity of these agents. Under control conditions, BOM receptor binding

Table 3
Effects of PKC-directed agents on BOM-induced IP formation in PC3 cells

Agent	Dose (μM)	IP formation[a] (% control)
PMA	0.001	43±4[c]
	0.04	25±3[c]
	0.1	18±2[c]
Staurosporine	0.2	129±7[b]
	0.8	120±5
	3.2	117±7
Go-6976	0.4	138±5[b]
	2	107±5
	10	109±8
Go-6983	0.4	129±6[b]
	2	129±6[b]
	10	119±5
BIS-1	0.1	117±5
	0.5	124±5[b]
	2.5	138±5[b]
Ro-318220	0.2	125±6
	0.8	129±6[b]
	3.2	78±6
Rottlerin	0.3	146±9[b]
	1	81±6
	10	45±6[c]

[a] Cells were pretreated with the indicated concentrations of each agent or vehicle control for 15 min, then stimulated with 2 nM BOM for 30 min, and IP formation was measured. The increment in IP formation was expressed as % control (mean±SEM) for at least three experiments.
[b] BOM-induced IP formation was significantly increased ($p<0.05$).
[c] BOM-induced IP formation was significantly decreased ($p<0.01$).

was 112±14 cpm/μg ($n=6$) and EGF receptor binding was 35.7±2.7 cpm/μg ($n=6$) in agreement with our prior work. Pretreatment of the cells with PKC inhibitors from category I (staurosporine) and category II (BIS-1 and rottlerin) modestly elevated BOM receptor binding (<20% increase) and EGF receptor binding (<30% increase). These agents were effective in the low micromolar range and their effects did not increase dramatically at higher doses. These results indicated that the robust elevation (>200% increase) in NT receptor binding caused by category II PKC inhibitors was specific to the NT receptor, whereas the smaller elevation (<30%) was also seen for the BOM and EGF receptors.

The PKC-directed agents were also tested for the ability to alter IP formation in response to BOM since it was known to stimulate PLC [13]. IP formation in PC3 cells was enhanced 4.7±1.1 fold by 1 nM BOM and 14.5±2.5 by 10 nM BOM. Therefore, we used 2 nM BOM as the stimulus to test the effects of the agents. As expected, the PKC activator PMA inhibited the response to BOM, exhibiting an IC50 near 1 nM (Table 3). When used at nanomolar and low micromolar doses, the PKC inhibitors (staurosporine, Go-6976, Go-6983, BIS-1, Ro318220 and rottlerin) enhanced BOM-induced IP formation by 20–40% (Table 3). These results, which were similar to those for NT, suggested that the constitutive PKC activity in PC3 cells inhibited PLC activation in response to both NT and BOM. This interpretation is consistent with work showing that BOM receptor function is subject to PKC regulation [44].

Although the enhancement of the IP response to BOM fell off at higher doses of rottlerin (Table 3) as was seen for NT (Fig. 4C), in general the results for BOM differed from those for NT. For example, doses of BIS-1, Ro-318220 and Go-6983 that inhibited NT-induced IP formation by >40% (Fig. 4C) did not inhibit BOM-induced IP formation (Table 3). These results indicated that the inhibition of NT-induced IP formation by the PKC inhibitors in category II was relatively specific to NT.

3.12. Cell specificity

To determine if the effects of PKC inhibitors were specific to PC3 cells, we assessed the effect of BIS-1 on NT binding in other cancer cell lines, including prostate carcinomas (PC3M, DU145, LNCaP^{CS}, MatLyLu) and a colon carcinoma (HT29). The level of NT receptor expression in these cell lines was previously described by us [35]. As compared to the vehicle control (0.06% DMSO), specific binding in the presence of 6 μM BIS-1 (mean±SEM; $n=3$ experiments) was: PC3 (186±11); HT29 (188±12); MatLyLu (217±13); PC3M (227±12); LNCaP^{CS} (162±15) and DU145 (276±18). These results indicate that 6 μM BIS-1 enhanced NT binding similarly in a number of cancer cell lines.

4. Discussion

Here, we report for the first time that NT receptor function in a number of prostate cancer cell lines is regulated by endogenous PKC activity. Focusing on PC3 cells, we demonstrated the expression of constitutive PKC activity that, under basal conditions, suppressed NT receptor binding and NT-induced IP formation. Thus, eight different PKC inhibitors (staurosporine, BIS-1, Ro-318220, Go-6976, Go-6983, chelerythrine, rottlerin and quercetin) enhanced NT binding and NT-induced IP formation by 20–40% at low micromolar concentrations that for most of these agents were shown to inhibit conventional PKC substrate phosphorylation in the cells. Not only was NT receptor function inhibited in a tonic manner by the basal PKC activity but pharmacologic activation of PKC was also shown to further inhibit NT receptor function. Thus, PKC activator PMA further inhibited NT binding and NT-induced IP formation. Our results established that NT receptor function was regulated by a heterologous desensitization process that appeared to involve primarily conventional PKC activity. However, our studies also uncovered a second mechanism by which novel PKC activity appeared to maintain (perhaps via effects on G protein coupling) the ability of NT receptor to activate PLC. Overall, our findings imply that, depending on the predominant PKC isotypes involved, heterologous regulation of NT receptor function can serve to either inhibit or maintain NT receptor function. Since NT-induced growth signaling in PC3 cells is PKC-dependent [9] and is associated with enhanced PKC isotype phosphorylation (S Hassan, unpublished data), our findings are also compatible with the existence of homologous regulation of NT receptor function via feedback effects of the PKC isotypes activated by NT. However, further studies are needed before a definitive conclusion can be drawn.

Although we have not yet identified all of the PKC isotypes involved, our studies point towards roles for both conventional (Ca^{2+}-sensitive) PKCs and novel (Ca^{2+}-insensitive) PKCs, with the latter exerting more striking effects. This was suggested initially by the fact that rottlerin, a PKCδ (novel)-specific inhibitor, was one of the most potent and efficacious agents to enhance NT receptor binding (Fig. 3B), whereas the conventional-specific inhibitor Go-6976 was much less effective (Fig. 3A). However, the results were puzzling since some of the inhibitors with broad specificity (BIS-1, Ro-318220, Go-6983) were quite effective while others (staurosporine, cherlerythrine) were rather ineffective (Fig. 3A and B). Furthermore, the effects of some of the agents on NT-induced IP formation were dose-dependently biphasic, while others were monophasic (Fig. 4B and C). Careful analysis finally led us to conclude that there were two different responses: response I was characterized by a modest enhancement (≅40%) in NT receptor binding and NT-induced IP formation; response II involved a dramatic enhancement (≅200%) in NT receptor binding associated with an inhibition of NT-induced IP formation. Response I was brought about by most of the PKC inhibitors at the low micromolar concentrations that were shown to inhibit conventional PKC substrate phosphorylation. On the other hand, response II could only be induced by inhibitors in category II (rottlerin, BIS-1, Ro-318220, Go-6983 and quercetin) and only at higher concentrations (>2 μM).

It seems probable that PKC inhibition was involved in both responses I and II, and that the outcome depended on the differential effects on specific PKC isotypes. Western blotting demonstrated that the major isotypes present were the conventional PKCs (α, βI) and the novel PKCs (δ, ε). The results for Go-6976 (specific for conventional PKCs) were consistent with the idea that inhibition of PKCα and/or PKCβI produced response I. The results for BIS-1 and Ro-318220 (known to require 5–16-fold higher concentrations to inhibit novel PKCs) and for rottlerin (putative PKCδ inhibitor) suggested that response II involved an inhibition of PKCδ and/or PKCε. To explain the results for the other inhibitors (which had broad specificity), one might propose that staurosporine and chelerythrine were unable to gain access to or sufficiently inhibit PKCδ and/or PKCε and thus, did not produce response II. Consistent with this interpretation, we found that knockdown of PKCδ or PKCε reproduced at least one aspect of response II, the inhibition of NT-induced IP formation. Although the PKCδ(−) and PKCε(−) clones did not exhibit the enhanced NT binding that is characteristic of response II, this apparent difference in PKC isotype dependence could have been due to differences in the assay conditions. For example, the NT binding assay (in contrast to the IP assay) did not involve stimulating the cells with high levels of NT that might have induced homologous desensitization. Another explanation is that knockdown of both PKCδ and PKCε (and possibly PKCα and PKCβI) might have been required to reproduce the effects of category II inhibitors on NT binding. Finally, it might have been that some of the effects of category II inhibitors on NT binding involved targets other than PKC.

The simplest interpretation of our results at this time is that inhibition of conventional PKCα and PKCβI produced response I, whereas the additional inhibition of novel PKCδ and PKCε produced response II. Thus, when BIS-1 was used at low doses that preferentially inhibited conventional PKCs, NT binding and IP formation were modestly enhanced. It seems likely that the increase in NT receptor binding led to the enhanced IP response. Therefore, a reasonable model is that conventional PKCs modulate NT binding to regulate receptor function. On the other hand, when BIS-1 was used at higher doses that inhibited novel as well as conventional PKCs, NT binding was dramatically enhanced and IP formation was inhibited. Since the inhibition of IP formation was specific to the NT receptor, it seems likely that this involved some form of G protein uncoupling. For example, novel PKC activity might maintain NT receptor–effector function by keeping G protein receptor kinases in check. Whatever the mechanism, this model is consistent with most of the data. For example, knockdown of PKCδ or PKCε would be expected to inhibit NT-induced IP formation but it would not necessarily cause the dramatic increase in NT receptor binding. This is because the uncoupled receptor would still be subject to binding inhibition imposed by constitutive PKCα and PKCβI activity.

The mechanistic studies reported here show that the modulation of NT receptor binding by the PKC-directed agents could not be attributed to changes in receptor number or receptor internalization. However, PKC activation or inhibition induced a remarkable change in receptor affinity that was consistent with the idea that PKC activity shifted the NT receptor to a low affinity state. Thus by blocking the constitutive PKC activity of the cells, PKC inhibitors shifted the NT receptor to a higher affinity state resulting in increased binding activity. Consistent with this, NT binding to the cell-surface and NT internalization were increased similarly and dose-responsively by BIS-1. The enhancing effects of BIS-1 persisted despite the inhibition of clathrin-coated vesicle-mediated endocytosis by sucrose, indicating that the mechanism was not likely to involve a change in receptor trafficking. Although it seems clear that a shift in NT receptor affinity is the basis for the regulation of NT binding by PKC, we do not know if the effects of PKC are direct or indirect. NT has been shown to induce phosphorylation of HA-tagged NT receptor in HEK-293 cells [21], but it is not known whether PKC participated in this or if PKC can under any conditions phosphorylate NT receptor. Since PKC inhibitors had only modest effects on BOM receptor binding and EGF receptor binding, the dramatic effects on the behavior of the NT receptor appeared to be receptor-specific. We demonstrated the effect of BIS-1 on NT receptor binding in a number of cancer cell lines, indicating that this behavior may apply generally to cells expressing the NT receptor.

It is interesting to note that the ability to alter NT receptor function was related to the chemical structures of the PKC inhibitors that were analogs of staurosporine. The agents in category II that induced dramatic effects on NT binding (BIS-1, Go-6983 and Ro-318220) were derivatives of maleimide, whereas the agents in category I that induced moderate effects on NT binding (staurosporine and Go-6976) were derivatives of carbazole. It is tempting to speculate about how the chemical nature of these derivatives might relate to their differing effects on

NT receptor function. In comparison to the carbazoles, the maleimides offer a more highly conjugated system that could promote redox reactions, one possibility involving donation of the central imide hydrogen. Interestingly, the maleimide derivative with a CH_3 group instead of hydrogen at this position (BIS-V) was inactive as a PKC inhibitor [45] and unable to modulate NT receptor function. This suggested that an antioxidative redox effect could play a role in these effects. We found that antioxidative polyphenols that can accumulate in membranes (and might possibly inhibit PKC) induced the type II response in PC3 cells [46]. BIS-1 is known to accumulate in mitochondrial membranes [47]; PKCδ was shown to translocate to mitochondrial membranes during apoptosis [48,49]; and Ro-318220 was found to induce mitochondrial apoptosis [50]. We showed that inhibitors of mitochondrial oxidative phosphorylation induced the type II response in PC3 cells [45]. Superoxide and hydrogen peroxide, which are produced by mitochondria, were found to activate PKC and this effect was reversed by antioxidants [51]. These findings suggest that the type II response could involve antioxidative effects of these agents on PKCδ and/or PKCε within mitochondria or other targets in PC3 cells.

Given that PKC activity can vary depending on cell type, stage of development and environmental inputs, these findings underline the importance of controlling these conditions when assessing NT receptor function in different systems. Since NT is an important mitogen in cancer cells, our findings suggest that the PKC-directed agents in category II, which inhibit NT receptor signaling, could be useful lead compounds for the development of new anticancer drugs. If these compounds produced their effects on NT receptor function by targeting PKCδ and/or PKCε, then these NT assays might be used to screen for substances that specifically inhibit or activate these novel PKCs. This could be useful since PKCδ and PKCε play important roles in cell cycle regulation [52], growth stimulation [53], growth inhibition [54] and apoptosis [55]. It should be noted that androgens can regulate the activity of the NT system [56, 57] as well as the expression of PKCδ in prostate LNCaP cells [58].

In conclusion, constitutive PKC activity was demonstrated in PC3 cells, which under basal conditions caused an inhibition of NT receptor function. Accordingly, low micromolar doses of PKC inhibitors released NT receptor from this inhibitory influence, enhancing NT binding and NT-induced IP formation moderately. This type I response appeared to involve inhibition of the conventional PKCs (α, βI). In addition, a second response was identified, which was induced by higher concentrations of a select group of PKC inhibitors. This type II response, characterized by a dramatic increase in NT binding and a pronounced inhibition of NT-induced IP formation, appeared to involve the novel PKCs (δ, ε), although other targets were possible. Overall, the results were consistent with the hypothesis that NT receptor function was subject to heterologous regulation by PKC and that two distinct mechanisms were involved.

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