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Neurotensin Negatively Modulates Akt Activity in Neurotensin Receptor-1-Transfected AV12 Cells

Feng Liu,[1]* Peiyi Yang,[1] Melvyn Baez,[1] and Binhui Ni[1,2,3]*

[1]Lilly Research Laboratories, Indianapolis, Indiana 46225
[2]Department of Psychiatry, Indiana University Medical School, Indianapolis, Indiana 46202
[3]Departments of Anatomy and Cell Biology, Indiana University Medical School, Indianapolis, Indiana 46202

Abstract Neurotensin (NT) regulates a variety of biological processes primarily through interaction with neurotensin receptor-1 (NTR1), a heterotrimeric G-protein-coupled receptor (GPCR). Stimulation of NTR1 has been linked to activation of multiple signaling transduction pathways via specific coupling to G_q, $G_{i/o}$, or G_s, in various cell systems. However, the function of NT/NTR1 in the regulation of the Akt pathway remains unknown. Here, we report that activation of NTR1 by NT inhibits Akt activity as determined by the dephosphorylation of Akt at both Ser473 and Thr308 in AV12 cells constitutively expressing human NTR1 (NTR1/AV12). The inactivation of Akt by NT was rapid and dose-dependent. This effect of NT was completely blocked by the specific NTR1 antagonist, (*S*)-(+)-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)-carbonylamino] cyclohexylacetic acid (SR 48527), but unaffected by the less active enantiomer ((*R*)-(−)-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)-carbonylamino] cyclohexylacetic acid (SR 49711)), indicating the stereospecificity of NTR1 in the negative regulation of Akt. In addition, NT prevented insulin- and epidermal growth factor (EGF)-mediated Akt activation. Our results provide insight into the role of NT in the modulation of Akt signaling and the potential physiological significance of Akt regulation by NT. J. Cell. Biochem. 92: 603–611, 2004.

Key words: Akt; GPCR; neurotensin; NTR1

Neurotensin (NT) is a tridecapeptide that was initially isolated from bovine hypothalamus [Carraway and Leeman, 1973] and subsequently discovered in a wide range of brain regions and different sections of the gastrointestinal tract [Carraway and Leeman, 1976]. NT functions as a neuromodulator and neurotransmitter in the central nervous system (CNS) and as a local hormone in the periphery through its specific receptors [Vincent et al., 1999]. Three neurotensin receptors (termed NTR1, NTR2, and NTR3) have been identified to date [Tanaka et al., 1990; Vita et al., 1993; Mazella et al., 1996, 1998]. Among them, the high-affinity NTR1 and the low-affinity NTR2 are members of the G-protein-coupled receptor (GPCR) superfamily with seven-transmembrane domains [Tanaka et al., 1990; Vita et al., 1993; Mazella et al., 1996], whereas NTR3 is a type I receptor with a single transmembrane domain [Mazella et al., 1998]. The majority of the known biological effects of NT in the CNS and periphery have been attributed to the interaction of NT with the high-affinity receptor, NTR1 [Tanaka et al., 1990; Vita et al., 1993]. NTR1 is highly expressed in the brain, small intestine, and other peripheral tissues [Tanaka et al., 1990; Vita et al., 1993]. Stimulation of NTR1 activates multiple signaling transduction pathways via specific coupling to G_q, $G_{i/o}$, or G_s,

Abbreviations used: CTX, cholera toxin; EGF, epidermal growth factor; NT, neurotensin; NTR, neurotensin receptor; PI 3-kinase, phosphatiylinositol 3-kinase; PTX, pertussis toxin; SPA, scintillation proximity assay; SR 48527, (*S*)-(+)-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)-pyrazol-3-yl)-carbonylamino] cyclohexylacetic acid; SR 49711, (*R*)-(−)-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)-carbonylamino] cyclohexylacetic acid.

*Correspondence to: Feng Liu and Binhui Ni, PhD, Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46225.
E-mail: liu_feng@lilly.com

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in various cell systems [Ehlers et al., 2000; Gailly et al., 2000; Zhao et al., 2001; Najimi et al., 2002]. It promotes cell growth, survival, and secretion [Sehgal et al., 1994; Barrocas et al., 1999; Somai et al., 2002]. NTR1 preferentially activates the G_q family proteins, resulting in the direct activation of phospholipase C (PLC), production of diacylglycerol and inositol (1,4,5)-trisphosphate, and subsequent mobilization of intracellular Ca^{2+}, and activation of protein kinase C (PKC) [Hermans and Maloteaux, 1998]. However, NTR1 can also functionally couple to G_s- and pertussis toxin (PTX) sensitive $G_{i/o}$-type G-proteins [Yamada et al., 1993; Gailly et al., 2000; Najimi et al., 2002] leading to modulation of a wide range of signaling transduction activities, such as increases/decreases in cyclic AMP (cAMP) levels, the activation of extracellular signal-regulated protein kinases (ERK1/2) and c-Jun N-terminal kinase (JNK) [Poinot-Chazel et al., 1996; Ehlers et al., 2000; Zhao et al., 2001].

The serine/threonine protein kinase Akt, also known as protein kinase B (PKB) is a downstream effecter of phosphatiylinositol 3-kinase (PI 3-kinase) [Burgering and Coffer, 1995; Franke et al., 1995]. Akt can be activated by insulin and growth factors such as epidermal growth factor (EGF) via PI 3-kinase-dependent membrane translocation, resulting in subsequent phosphorylation of Ser473 in the C-terminal regulatory domain and Thr308 in the activation loop of the kinase domain [Alessi et al., 1996; Kohn et al., 1996; Hill and Hemmings, 2002]. Conversely, the dephosphorylation of Akt at Ser473 and Thr308 leads to inactivation of the kinase [Meier et al., 1998; Hill and Hemmings, 2002; Stoica et al., 2003]. Therefore, the phosphorylation status of Akt at these two sites directly reflects the kinase activity. Akt has been recognized as an important mediator of the pleiotrophic effects of insulin or growth factors such as EGF on metabolic processes as well as on gene expression [Valverde et al., 2003]. Studies with overexpression of constitutively active or dominant negative forms of Akt/PKB as well as with anti-Akt/PKB antibodies indicate the involvement of Akt in the regulation of anabolic metabolism [Chavez et al., 2003]. Indeed, mice lacking the Akt2/PKB isoform develop a diabetes-like syndrome characterized by insulin resistance in both skeletal muscle and liver [Cho et al., 2001]. Therefore, it is of great interest to investigate the effect of NT on insulin or EGF mediated Akt signaling.

AV12 is an adenovirus-transformed Syrian hamster kidney cell line. When transfected with NTR1 cDNA, AV12 cells produce fully functional human NTR1. More importantly, no endogenous NTRs have been reported in these cells, therefore, it is possible to study signaling mediated by NTR1 without interference of other NTRs. In this report, we studied the effect of NTR1 activation by NT on the regulation of Akt by evaluating Akt phosphorylation at both Ser473 and Thr308 in AV12 cells constitutively expressing human NTR1. In addition, we investigated the role of NT in insulin- and EGF-induced Akt activation.

MATERIALS AND METHODS

Reagents

Human colon cDNA was purchased from Clontech (Palo Alto, CA). TOPO TA cloning kits and High Fidelity Polymerase were from Invitrogen (Carlsbad, CA). FuGENE6 transfection reagent was from Roche (Indianapolis, IN). NT was purchased from Peninsula Laboratories Europe (Merseyside, England) and was reconstituted in dH_2O containing 0.1% BSA. [^{125}I]Tyr3-NT was obtained from Perkin Elmer (Boston, MA). Wheatgerm agglutinin (WGA) scintillation proximity assay (SPA) beads were from Amersham (Little Chalfont, Buckinghamshire, UK). Fluo-3/acetoxymethyl (AM) and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). NTR1 antibody was from Alpha Diagnostic International (San Antonio, TX), anti-β-actin was from Santa Cruz (Santa Cruz, CA), and anti-phospho-Akt (Ser473), phospho-Akt (Thr308), and Akt antibodies were from Cell Signaling (Beverly, MA). EGF was from R&D Systems (Minneapolis, MN). Porcine insulin, (*S*)-(+)-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)-carbonylamino] cyclohexylacetic acid (SR 48527), and (*R*)-(−)-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)-carbonylamino] cyclohexylacetic acid (SR 49711) were from Eli Lilly & Co. (Indianapolis, IN). PTX, cholera toxin (CTX), and H89 were from Calbiochem (San Diego, CA). The protein molecular weight marker (Precision Protein Standards) was from Bio-Rad (Hercules, CA). All other common reagents and cell culture media were from Invitrogen unless specified otherwise.

Cloning and Expression of Human NTR1

A full length NTR1 clone was isolated from a human colon cDNA library using PCR primers designed from the 5′ and 3′ ends of the human NTR1 sequence. The 5′ primer was 5′-GGGGTACCGACTTCCAGCCCCGGAGGCGCCGGA-3′ and the 3′ primer was 5′-GCTCTAGATGGCCAGGCTCCTCCTGGACA-3′. Two rounds of PCR reactions were performed using the following conditions with High Fidelity Polymerase. Round one: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 52°C for 2 min, and 72°C for 2 min. Round two: 2 μl of the PCR product from round one was used as templates for round two PCR under the same condition as described above. A single 1.35 kb fragment generated from PCR was sub-cloned into TOPO TA cloning vector, sequenced using standard procedure, and sub-cloned into the mammalian expression vector pGTH (Eli Lilly & Co.). Finally, the construct was transfected into AV12 cells using FuGENE6 Transfection Reagent following the manufacturer's instructions. Cells constitutively expressing NTR1 (designated as NTR1/AV12) were selected with 350 μg/ml hygromycin B. The expression levels of NTR1 protein were assessed by Western immunoblotting using anti-NTR1 antibody.

Receptor Binding Assay

NTR1/AV12 cells were scraped from culture plates into PBS, pelleted in tubes, and resuspended using a glass homogenizer in a buffer containing 20 mM Tris, pH 7.4 and complete protease inhibitor (Roche). Saturation experiments were done in 96-well plates with NT membrane, [^{125}I]Tyr3-NT (4 nM–9.3 pM), and WGA SPA beads. Non-specific binding was defined as binding in the presence of 2 μM unlabelled ligand. The radioactivity was measured using a 1450 Microbeta Liquid Scintillation & Luminescence Counter (Perkin Elmer). Data derived from three independent experiments were analyzed using GraphPad Prism 2.01 (GraphPad Software, San Diego, CA).

Measurement of Intracellular Ca^{2+} Levels

Ca^{2+} mobilization was monitored by a Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA) using the method described previously [Sullivan et al., 1999] with minor modifications. Briefly, AV12 cells (NTR1 transfected or untransfected) were seeded in black-walled, transparent-bottomed 96-well plates (7×10^4 cells/well) (Costar, Corning, NY) and cultured for 24 h. The dye-loading medium was prepared by mixing equal volumes of fluo-3/AM and Pluronic F-127, followed by addition of Hank's balanced salt solution (HBSS) supplemented with 10% FBS such that the final concentrations of fluo-3/AM and Pluronic F-127 in the loading medium were 3.33 μM and 0.03%, respectively. Cells were then incubated with the loading medium in the dark for 2 h (room temperature). The loading of fluo-3/AM was terminated by rapid aspiration of the dye and addition of HBSS to each well. Plates were then transferred to the FLIPR machine and changes in intracellular Ca^{2+} were monitored following automatic addition of serial doses of NT (1 μM–3 pM). Data were analyzed using GraphPad Prism 2.01.

Cell Culture and Treatment

AV12 (AV12-664) cells were purchased from American Type Culture Collection (Manassas, VA) (CRL-9595) and cultured in DMEM supplemented with 5% FBS, 10 mM HEPES, 1 mM L-glutamine, 1 mM sodium pyruvate, and 50 μg/ml penicillin–streptomycin. For NTR1/AV12 cell culture, 350 μg/ml hygromycin B was included in the above medium. All cell lines were maintained at 37°C in a humidified incubator supplied with 5% CO_2 and 95% air, and utilized below passage 20. For cell preparation, NTR1/AV12 or AV12 were rinsed with DMEM and incubated with DMEM supplied with 0.1% BSA (4 h) for serum starvation prior to treatment. For NT treatment, controls were left untreated; for antagonist pretreatment, 0.1% DMSO was used as vehicle control.

Detection of Akt Activity by Western Immunoblotting

Because Akt is fully activated by phosphorylation at both Ser473 and Thr308 [Alessi et al., 1996], and is inactivated by dephosphorylation at both sites [Meier et al., 1998], the phosphorylation status of Akt denotes its kinase activity. Therefore, we measured Akt activities by evaluating phospho-Akt^{Ser473} and phospho-Akt^{Thr308} by Western analyses as reported previously [Liu et al., 2003]. Briefly, cells were plated in 24-well plate (0.5×10^6 cells/well) and allowed to grow to confluence. Cells were then serum-starved in DMEM containing 0.1% BSA for 4 h, followed by incubation with appropriate reagents. Upon the

completion of treatment, media were removed and cells were immediately lysed with 300 μl of LDS sample buffer (Invitrogen), and then transferred into microcentrifuge tubes, and boiled for 10 min. Ten microliters of each sample was analyzed using the NuPAGE Bis-Tris gel and transfer system (Invitrogen). After protein transfer, nitrocellulose blots were probed with anti-phospho-Akt (Ser473) (1:1,000 dilution), anti-phospho-Akt (Thr308) (1:250), or anti-Akt (1:1,000), followed by reactions with HRP-conjugated goat-anti-rabbit antibody (1:3,000). Finally, protein was detected by ECL Plus (enhanced chemiluminescent) reagents (Amersham), protein bands were scanned using UMAX PowerLook 2100XL scanner, and the net band intensities were quantified using Kodak Digital Science ID Image Analysis software.

RESULTS

Expression and Characterization of NTR1-Transfected AV12 Cells (NTR1/AV12)

Human NTR1 cDNA was cloned and stably expressed in AV12, an adenovirus-transformed Syrian hamster cell line [Desai et al., 1996]. The protein levels of NTR1 in NTR1/AV12 and untransfected AV12 were assessed by Western blotting using an antibody specifically recognizing the cytoplasmic, C-terminus of human NTR1. As shown in Figure 1A, robust NTR1 expression was detected in NTR1/AV12, while there was no measurable NTR1 in the untransfected cells. Consistent with this result, radioligand binding studies (SPA) revealed the specific binding of [^{125}I] NT to NTR1/AV12 with a K_d value of approximately 1.5 nM (Fig. 1B). To ensure that NTR1/AV12 responds properly to NT stimulation, intracellular Ca^{2+} mobilization was monitored by FLIPR in the absence and presence of NT. Figure 1C shows that NT triggered a robust increase in Ca^{2+} concentration in NTR1/AV12 ($EC_{50} = 0.7$ nM), whereas there was little change in untransfected AV12 following NT challenge.

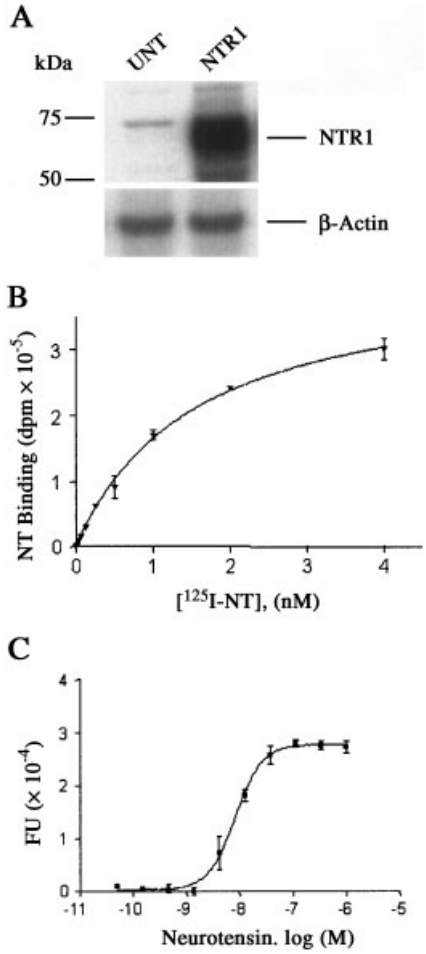

Fig. 1. Characterization of neurotensin receptor-1 (NTR1)-transfected AV12 cells (NTR1/AV12). **Panel A**: NTR1 expression. Each (10^4) of untransfected AV12 (UNT) and NTR1/AV12 (NTR1) cells were homogenized in LDS sample loading buffer and analyzed by Western blotting using anti-NTR1 antibody (**upper blot**). The blot was then probed with anti-β-actin antibody to ensure equal protein loading (**lower blot**). **Panel B**: Ligand binding property. NTR1/AV12 cell membrane (2 μg) was incubated with serial dilutions of ^{125}I-labelled [monoiodo-Tyr3] neurotensin (NT) (^{125}I-NT; 4 nM–9.3 pM) and scintillation proximity assay (SPA) beads (0.5 mg) at 20°C for 2 h. The total and unspecific radioactivities were measured and the specific binding (the difference between the total and unspecific binding) was plotted. The data presents three independent experiments performed in duplicates. **Panel C**: NT induces Ca^{2+} mobilization. NTR1/AV12 as well as AV12 cells were loaded with fluo-3/AM and then stimulated with serial doses of NT (10^{-6}–$10^{-11.2}$ M). Changes in intracellular [Ca^{2+}] was determined by FLIPR and expressed in fluorescence unit (FU). The data is a representative of three independent experiments performed in triplicates.

NT Mediates Akt Inactivation in NTR1/AV12 Cells

We first tested whether NT exerted any effect on basal Akt activities by measuring phospho-Akt^{Ser473} and phospho-Akt^{Thr308} in NTR1/AV12. As shown in Figure 2, incubation of NTR1/AV12 with NT (100 nM, 8 min) greatly reduced phospho-Akt^{Ser473} and phospho-Akt^{Thr308} as detected by Western blotting using specific antibodies against Akt phosphorylated at Ser473 and Thr308, respectively. Pretreatment of NTR1/AV12 with the NTR1 antagonist SR48527 (1 μM, 30 min), but not the enantiomer, SR49711 (1 μM, 30 min), abrogated the Akt dephosphorylation

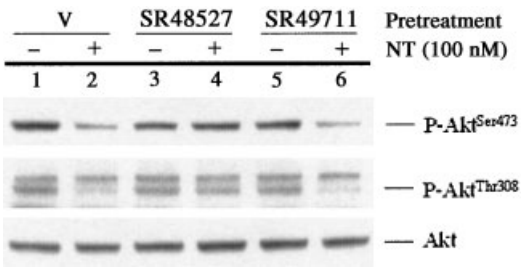

Fig. 2. NT reduces the phosphorylated Akt levels in NTR1/AV12, which was blocked by (*S*)-(+)-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)-carbonylamino] cyclohexylacetic acid (SR 48527). NTR1/AV12 cells were incubated with vehicle control (V, **lanes 1** and **2**), SR 48527 (**lanes 3** and **4**), or (*R*)-(−)-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)-pyrazol-3-yl)-carbonylamino] cyclohexylacetic acid (SR 49711) (**lanes 5** and **6**) (1 μM, 30 min), followed by treatment with NT (100 nM, 8 min; lanes 2, 4, and 6). Total cell lysates were analyzed by Western blotting. The phosphorylated Akt was detected using both phospho-Akt (Ser473) and phospho-Akt (Thr308) antibodies. The total Akt protein was measured to ensure equal sample loading. Data is a representative of three independent experiments.

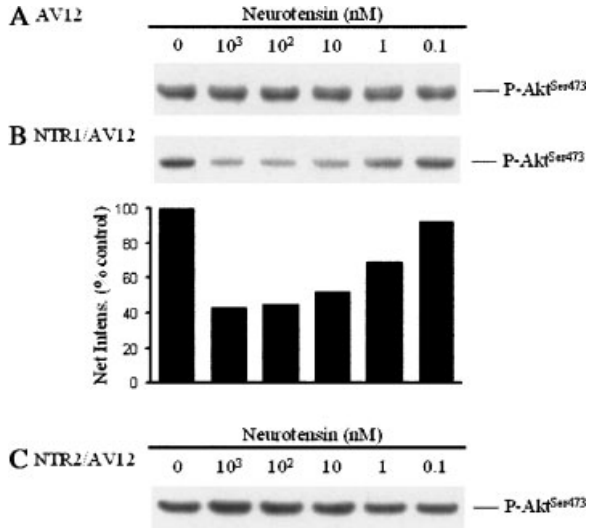

Fig. 3. NT-induced Akt dephosphorylation is dose-dependent. AV12 (**panel A**), NTR1/AV12 (**panel B**), as well as NTR2/AV12 (**panel C**) cells were either left untreated or treated with serial dilutions of NT (10^3–0.1 nM) for 8 min. Phospho-Akt^{Ser473} was detected by Western blotting. For protein samples of NTR1/AV12 cells, phospho-Akt^{Ser473} band intensities were measured and plotted as percentage of control. These data are representative of three independent experiments.

caused by NT. The total Akt protein levels were unchanged. In contrast to NT-induced Akt dephosphorylation, NT dramatically stimulated ERK1/2 phosphorylation (data not shown) similar to that reported previously in Chinese hamster ovary (CHO) cells stably transformed with human NTR1 [Poinot-Chazel et al., 1996]. Our data indicate that NT, via specific interaction with NTR1, negatively regulates the constitutive activities of Akt in NTR1/AV12.

To further characterize NTR1-mediated Akt inactivation, NTR1/AV12 as well as untransfected AV12 cells were treated for 8 min with serial dilutions of NT ranging from 10^3 to 0.1 nM. Phospho-Akt^{Ser473} was detected by Western immunoblotting and quantified by densitometry. While NT failed to influence the phosphorylation status of Akt in control AV12 cells (Fig. 3A), NT decreased Akt phosphorylation in a dose-dependent manner in NTR1/AV12 (Fig. 3B). Akt phosphorylation was reduced to about 75% of control with 1 nM NT (25% inhibition) and plateaued with 100 nM NT (approximately 45% of control or 55% inhibition). A similar inhibitory effect of NT on phospho-Akt^{Thr307} was observed (data not shown). Moreover, treatments of an AV12 cell line stably expressing NTR2 (Baez et al., unpublished data) produced no changes in Akt phosphorylation when treated with 10^3–0.1 nM NT (Fig. 3C). Together, these results show that NT inhibits Akt activity in a time- and dose-dependent manner. This effect of NT on Akt is potent, rapid, and yet transient. The data also further confirm the specificity of NTR1 in the negative modulation of Akt by NT.

NT Antagonizes EGF- and Insulin-Stimulated Akt Activation

To investigate the effect of NT on the regulation of insulin- and EGF-mediated Akt signaling, we treated NTR1/AV12 cells with NT, followed by EGF or insulin challenge. Figure 4 illustrates that NT again reduced the basal phospho-Akt^{Ser473} and phsopho-Akt^{Thr308}, and EGF (100 nM, 10 min), as well as insulin (100 nM, 10 min) increased Akt phosphorylation at both Ser473 and Thr308 (lanes 3 and 5, respectively). Pretreatment with NT (100 nM, 5 min) considerably attenuated EGF-induced phospho-Akt^{Ser473}, and completely blocked EGF-induced phospho-Akt^{Thr308} (lane 4). The inhibitory effect imposed by NT was even stronger on insulin-stimulated Akt activation. NT pretreatment not only fully prevented insulin-stimulated increases in phospho-Akt^{Ser473}, but also reduced phospho-Akt^{Thr308} to below basal level (compare lane 6 with lane 1) while the Akt protein level remained constant. These data reinforce the notion that NTR1 inactivation of Akt is mediated via G_q coupling, and suggest that activation

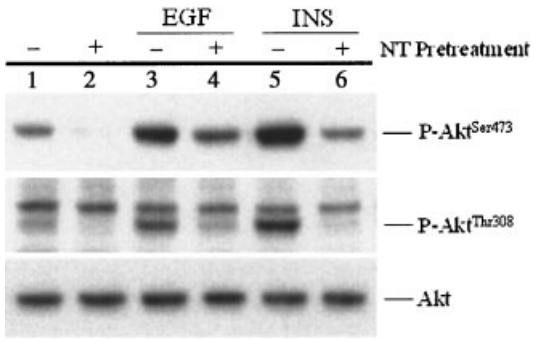

Fig. 4. NT inhibits epidermal growth factor (EGF)- and insulin-stimulated Akt activation. NTR1/AV12 cells were either left untreated (**lanes 1**, **3**, **5**) or pretreated with 100 nM NT for 5 min (**lanes 2**, **4**, **6**), followed by incubation with 100 nM EGF (lanes 3, 4) or 100 nM insulin (INS; lanes 5, 6) for 10 min. Cell homogenates were analyzed by Western blotting. Phospho-Akt^{Ser473}, phospho-Akt^{Thr308}, and total Akt were detected using the respective antibodies. The data are representative of three independent experiments.

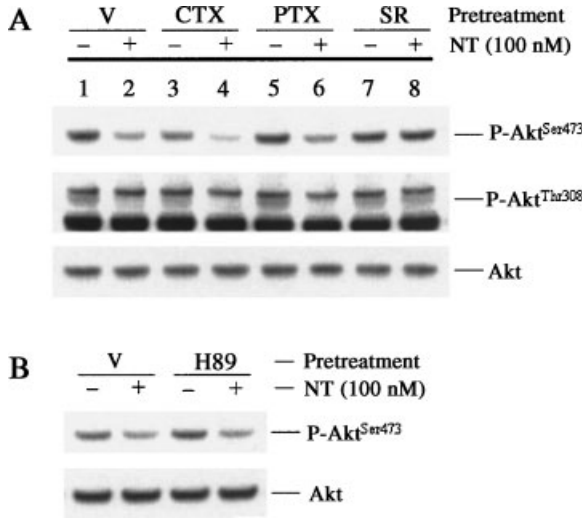

Fig. 5. Pertussis toxin (PTX), cholera toxin (CTX), and H89 had no effect on NT-mediated Akt inactivation. **Panel A**: NTR1/AV12 cells were pretreated with vehicle (V; **lanes 1**, **2**), 10 ng/ml of CTX (**lanes 3**, **4**), or 10 ng/ml of PTX (**lanes 5**, **6**) overnight, or 1 μM SR 48527 (SR; **lanes 7**, **8**) for 30 min, followed by incubation with NT (100 nM, 8 min; lanes 2, 4, 6, 8). Cell homogenates were analyzed by Western blotting. Phospho-Akt^{Ser473}, phospho-Akt^{Thr308}, and total Akt were detected using the respective antibodies. **Panel B**: NTR1/AV12 cells were pretreated either with vehicle or 10 μM H89 for 30 min, followed by incubation with NT (100 nM, 8 min). Cell homogenates were probed with phospho-Akt^{Ser473} and Akt antibodies. The data are representative of three independent experiments.

of G_q by NTR1 leads to the inhibition of PI 3-kinase with the subsequent inhibition of Akt.

NT/NTR1-Mediated Akt Inactivation Is not Affected by PTX, CTX, and H89

Previous studies have revealed that in addition to preferential coupling with G_q proteins [Gailly et al., 2000; Najimi et al., 2002], NTR1 also functionally links to G_s and G_i resulting in the modulation of adenylyl cyclase activity and the synthesis of cAMP [Oury-Donat et al., 1995; Najimi et al., 2002]. Interestingly, cAMP has been found to exert opposite effects on Akt activity depending on the relative availability of the downstream effectors of cAMP [Mei et al., 2002]. Therefore, the possibility of G_s- and/or G_i-type protein involvement in NTR1-elicited Akt inhibition required consideration. Consequently, two bacteria toxins, CTX and PTX, known to increase cAMP levels, were utilized to elucidate the role of G_s and G_i in NTR1-mediated inhibition of Akt. CTX stimulates G_s through ADP-ribosylation thereby increasing cellular cAMP, while PTX inhibits G_i through ADP-ribosylation also leading to elevated cAMP. NTR1/AV12 cells were pre-incubated with either CTX (10 ng/ml, o/n) or PTX (10 ng/ml, o/n), followed by NT (100 nM, 8 min) treatment. As shown in Figure 5A, both CTX and PTX failed to influence the decreases in Akt phosphorylation caused by NT. As a control, pre-incubation with SR 48527 abolished the NT-induced Akt dephosphorylation. In addition, inhibition of cAMP-dependent protein kinase (PKA) activities with H89 also had no effect on NT-mediated Akt dephosphorylation (Fig. 5B). The Akt protein levels were unchanged under all conditions tested. These data strongly suggest that G_i/G_s and cAMP are not involved in the NTR1-mediated negative regulation of Akt.

DISCUSSION

A growing number of studies have suggested that NT plays a significant role in cell survival, proliferation, and migration [Sehgal et al., 1994; Yano et al., 1998; Somai et al., 2002; Martin et al., 2003]. These biological processes often involve activation of Akt and ERK signaling pathways [Hill and Hemmings, 2002; Guha et al., 2003]. We investigated the effect of NT-induced NTR1 activation on Akt activity in NTR1-transfected AV12, an adenovirus-transformed Syrian hamster cell line that has been previously used for functional studies of G-protein coupled receptors [Baez et al., 1995; You et al., 2000; Cho et al., 2001]. We showed that

NT drastically reduced Akt activities, as visualized by decreases in phospho-Akt^{Ser473} and -Akt^{Thr307}, in a dose-dependent manner. The inhibitory effect of NT on Akt was completely blocked by the NTR1 antagonist SR 48527, but not by the same concentration of the stereoisomer, SR 49711, which is two-orders of magnitude less potent than SR 48527 [Labbe-Jullie et al., 1994]. Additionally, NT did not trigger alterations in Akt activity in naïve AV12 or NTR2-transfected AV12 cells. Recently, Martin et al. [2003] reported that PI 3-kinase-dependent Akt activation is involved in NT-mediated microglia migration via NTR3, a non-GPCR receptor. Our results suggest, for the first time, that NT negatively modulates Akt signaling via specific NTR1 activation.

NTR1 is known to promiscuously couple to G_s and G_i [Ehlers et al., 2000; Gailly et al., 2000; Zhao et al., 2001; Najimi et al., 2002] in addition to its preferential coupling to G_q. NT/NTR1, therefore, is capable of regulating cAMP accumulation [Yamada et al., 1993; Gailly et al., 2000; Najimi et al., 2002] which in turn positive or negatively modulates Akt activity [Mei et al., 2002], via G_i and G_s. Our data, however, do not support the involvement of G_i or G_s in NTR1-mediated Akt inhibition. Inactivation of G_i by PTX thereby relieving the inhibition of G_i on adenylyl cyclase activity and cAMP syntheses exerted no effect on the basal Akt activity and failed to attenuate NT-mediated negative regulation of Akt signaling. Similarly, stimulation of G_s by CTX did not block the NT effect on Akt activity. The same result was obtained when PKA activities were inhibited with a specific inhibitor (H89), implying that NT-mediated Akt inactivation is independent of cAMP. Taken together, our results suggest that the negative regulation of Akt by NT/NTR1 is likely coupled to G_q proteins, a notion further supported by the fact that activated NTR1 by NT abrogated the stimulatory effect of insulin or EGF on Akt, which requires G_q for PI 3-kinase/Akt activation [Imamura et al., 1999; Ballou et al., 2003]. Therefore, the inhibitory effect of NT on insulin- and EGF-stimulated Akt not only further confirms the negative regulation of Akt by NT, but also strengthens the argument for the role of G_q in the signaling cascade. In addition, several research groups have shown that activated G_q can negatively regulate Akt signaling [Folli et al., 1997; Bommakanti et al., 2000; Ballou et al., 2003]. Constitutively active $G_{\alpha q}$ mutant, GαqQ209L, for example, inhibits the kinase activity of epitope-tagged transfected Akt in HEK293 and COS-7 cells [Ballou et al., 2003]. Moreover, angiotensin II receptor and alpha1A adrenergic receptor, both G_q-coupled receptors, have been found to antagonize activations of PI 3-kinase or Akt by growth factors or insulin [Folli et al., 1997; Ballou et al., 2001]. Together, these results suggest that that G_q activation plays a critical role in the negative regulation of Akt by NT/NTR1.

In conclusion, we described a novel function of NT and its high affinity GPCR, NTR1, in Akt signal transduction. We showed that NT/NTR1, probably through the activation of G_q, inhibits Akt activities as well as insulin- and EGF-stimulated Akt activation. The revelation of the inhibitory effect of NT on Akt activity could contribute to our better understanding of NT signaling and its physiological functions, particularly in the regulation of the pleiotrophic effect of insulin or EGF, where PI 3-kinase/Akt signaling plays a pivotal role.

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