Neurotensin Phosphorylates GSK-3α/β through the Activation of PKC in Human Colon Cancer Cells

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Abstract

Neurotensin (NT), a gastrointestinal hormone, binds its receptor [neurotensin receptor (NTR)] to regulate the growth of normal and neoplastic intestinal cells; molecular mechanisms remain largely undefined. Glycogen synthase kinase-3 (GSK-3) regulates diverse cellular processes, including cell growth and apoptosis. Here, we show that NT induces the phosphorylation of GSK-3α/β in the human colon cancer cell line HT29, HCT116, or SW480, which possesses high-affinity NTR. The effect of NT was blocked by inhibitors of protein kinase C (PKC), but not by inhibitors of mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK1) or phosphatidylinositol-3 kinase/extracellular signal-regulated kinase (ERK), suggesting a role for PKC in GSK-3 phosphorylation by NT. Pretreatment with G66976 (which inhibits PKCα and PKCβ1) or downregulation of endogenous PKCα or PKCβ1 blocked NT-mediated GSK-3α phosphorylation, suggesting a predominant role for PKCα or PKCβ1 in the NT-mediated phosphorylation of GSK-3α. Moreover, a selective PKCα inhibitor, LY379196, reduced NT-mediated GSK-3α phosphorylation, suggesting a role for PKCα in the NT-mediated phosphorylation of GSK-3α and an undefined kinase in the NT-mediated phosphorylation of GSK-3α.

Introduction

Regulatory hormones, which are localized to specialized endocrine cells in the small bowel, control numerous physiological functions of the gastrointestinal (GI) tract, including secretion, motility, and mucosal growth [1]. The gut peptide neurotensin (NT), a tridecapeptide localized to enteroendocrine cells of the distal small bowel [2,3], facilitates fatty acid translocation [4], affects gut motility [5], and stimulates the growth of normal gut mucosa [6,7]. In addition to its trophic effects on normal GI tissues, NT stimulates the proliferation of certain pancreatic, colonic, and prostatic cancers bearing high-affinity neurotensin receptors (NTRs) [8]. NTR and its mRNA are expressed in many human colon cancer cell lines and primary colorectal cancers [9]. Therefore, it is important to characterize NT-mediated signal transduction pathways in human colon cancer cells, which may identify potential targets for therapeutic interventions.

Previously, we have shown that NT activates the extracellular signal-regulated kinase (ERK) pathway in human colon and pancreatic cancer cells [10,11]. In agreement with our studies, other investigators have demonstrated that NT activates ERK in colon adenocarcinoma cells [12], colonic epithelial cells [13], and pancreatic cancer cells [14]. NT-stimulated ERK activation is dependent on Ras and is involved in NT-induced IL-8 expression [13]. NT-mediated stimulation of IL-8 gene expression involves a nuclear factor κB–dependent pathway [13]. In addition to the activation of ERK, NT induces the activation of protein kinase C (PKC) and phosphatidylinositol-3 kinase (PI3-kinase)/Akt in some cells [15–17]. NT plays an important role in the stimulation of intestinal cell proliferation; however, the signal pathways mediated by NT are largely undefined.

PKC, particularly the PKCα and PKCβ1 isozymes, has been shown to phosphorylate glycogen synthase kinase (GSK) 3α and GSK-3β [18,19]. PKC is ubiquitously expressed and involved in diverse cellular functions, such as the potentiation of the proliferative response to G protein–coupled receptor agonists [20–22]. NT stimulates the formation of inositol trisphosphate [1,4,5], increases intracellular calcium [23,24], and activates PKC [25]. However, the functional effects of PKC-mediated NT signaling are not known.

GSK-3α is an evolutionarily conserved signaling molecule that plays an important role in diverse biologic processes [26]. More recent studies indicate a role for GSK-3 in the control of cell

Abbreviations: ECL, enhanced chemiluminescence; FBS, fetal bovine serum; GSK-3, glycogen synthase kinase-3; MAPK, mitogen-activated protein kinase; NTR, neurotensin receptor; PKB/Akt, protein kinase B; PKC, protein kinase C

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proliferation and survival in mammalian cells and in the identification of GSK-3 as a component of the Wnt signaling pathway, which controls development in invertebrates and vertebrates [27–29]. GSK-3 phosphorylates cyclin D1, resulting in its degradation [30,31]. Two isoforms of GSK-3, GSK-3α and GSK-3β, have been identified [32]. Both isoforms are phosphorylated at Ser-21 in GSK-3α and at Ser-9 in GSK-3β [26], resulting in the inhibition of enzymes. Although studies have focused primarily on GSK-3β, GSK-3α has similar (but not identical) biochemical properties [26,33]. Several kinases have been shown to phosphorylate these inhibitory sites in vitro [26]. Protein kinase B (PKB/Akt), a serine/threonine kinase located downstream of PI3-kinase, phosphorylates both of these sites in vitro and in vivo [34], suggesting that certain growth factors repress GSK-3 activity through the PI3-kinase–PKB/Akt signaling cascade. In addition, p90RSK, a downstream target of the MEK/ERK pathway, and certain PKC isoforms have been shown to phosphorylate and inactivate both isoforms of GSK-3 [19,35]. These findings suggest that GSK-3 represents an important convergence point that integrates signals from multiple signaling cascades.

In our current study, we show that NT stimulates GSK-3 phosphorylation in human colon cancer cells that possess the high-affinity NTR through an intracellular signaling pathway involving PKC, independent of previously identified PI3-kinase–PKB/Akt and MEK/ERK pathways. These results indicate that, depending on the stimulatory context, the activity of GSK-3 can be regulated through multiple signaling mechanisms. Moreover, the NT-stimulated induction of cell growth noted in NTR+ colon cancers may be mediated, in part, through PKC-dependent GSK-3 inhibition.

Materials and Methods

Materials

GF109203x, Ro-318220, PD98059, and G66976 were provided by Calbiochem (La Jolla, CA). LY319796 was a generous gift from Eli Lilly Co. (Indianapolis, IN). The GSK-3 inhibitor SB-216763 was purchased from Tocris (Ellisville, MO). Rabbit anti-phospho-GSK-3α/β (Ser-21 and Ser-9), rabbit anti-phospho-Akt, and rabbit anti-Akt antibodies were purchased from Cell Signaling (Beverly, MA). Mouse monoclonal anti-GSK-3 (clone 4G1E) and secondary antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Phorbol-12-myristate-13 acetate (PMA), wortmannin, NT, myelin basic protein (MBP), and rabbit anti-α-actin antibody were from Sigma (Solon, OH). Mouse anti-phospho-ERK1/2, rabbit anti-ERK1, rabbit anti-PKCα, rabbit anti-PKCβ1, and rabbit anti-cyclin D1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). [γ-32P] adenosine triphosphate (ATP) was obtained from PerkinElmer Life Sciences (Boston, MA). The enhanced chemiluminescence (ECL) system for Western immunoblot analysis and protein A-Sepharose was from Amersham Biosciences (Piscataway, NJ). The concentrated protein assay dye reagent was from Bio-Rad Laboratories (Hercules, CA). Tissue culture media and reagents were from Invitrogen (Carlsbad, CA). All other reagents were of molecular biology grade and were from Sigma.

Cell Culture

The human colon cancer cell lines HT29, HCT116, and SW480 were obtained from the American Type Culture Collection (Manassas, VA). HT29 and HCT116 cells were grown in McCoy’s 5A supplemented with 10% fetal bovine serum (FBS). SW480 cells were grown in RPMI 1640 supplemented 10% FBS. Before stimulation with NT, cells were grown to subconfluence in 60-mm dishes and starved in serum-free medium for 24 hours, unless otherwise indicated.

Western Blot Analysis

Total protein (100 μg) was resolved on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. Filters were incubated for 1 hour at room temperature in a blotting solution. Phospho-GSK-3α/β, GSK-3, cyclin D1, PKCα, PKCβ1, and actin were detected with specific antibodies following blotting with a horseradish peroxidase–conjugated secondary antibody and were visualized using an ECL detection system.

In Vitro Kinase Assays

PKCα or PKCβ1 activity was determined in cell extracts, as described previously [36]. Briefly, total PKCα or PKCβ1 was determined by measuring the incorporation of [32P] into MBP. Extracts from HT29 cells treated with or without NT were incubated with PKCα or PKCβ1 antibody overnight and with protein A beads for 3 hours at 4°C by gentle rocking. Immunocomplexed beads were washed twice with cell lysis buffer and twice with kinase buffer (25 mM Tris, pH 7.4; 2 mM dithiothreitol; 0.1 mM Na3VO4; 10 mM MgCl2; and 5 μCi of [γ-32P]ATP). Immunocomplexes were resuspended in 40 μl of kinase buffer supplemented with 10 μM ATP and 5 μg of MBP and incubated for 30 minutes at 30°C. Kinase reactions were terminated with a sodium dodecyl sulfate (SDS) sample buffer. Samples were size-fractionated by SDS polyacrylamide gel electrophoresis (PAGE), and 32P-labeled MBP was quantified autoradiographically after fixing and drying the gel.

Results

NT Induces the Phosphorylation of GSK-3α/β

GSK-3 not only functions as a regulator of metabolism but also plays a critical role in the signal transduction of many growth factors such as IGF-1, insulin, and neuronal growth factor [26]. To assess whether GSK-3 is also regulated by NT, a GI hormone that binds its receptor to regulate the proliferation of normal and neoplastic intestinal cells [8], we treated HT29 cells, a human colon cancer cell line that possesses high-affinity NTR [9], with different concentrations of NT. Phosphorylation of GSK-3 was determined by immunoblotting with a GSK-3 phospho-specific antibody that recognizes GSK-3α phosphorylated at Ser-21 and GSK-3β phosphorylated at Ser-9 (Figure 1). NT treatment induced a dosage-dependent
Figure 1. Treatment with NT induces GSK-3α/β phosphorylation in HT29 cells. HT29 cells were serum-starved for 24 hours, followed by treatment with different concentrations of NT (A) for 20 minutes or NT (100 nM) over a time course (B). Total protein was extracted from cells and resolved by SDS-PAGE, transferred to PVDF membranes, and probed with anti–phospho-GSK-3α/β antibody; membranes were stripped and reprobed with anti–GSK-3α/β antibody. Representative data from five separate experiments are shown.

**NT Induces Signaling Pathways That May Mediate the Induction of GSK-3α/β Phosphorylation**

Several serine/threonine kinases, including PI3-kinase/Akt [34], p90Rsk [37], a downstream target of MEK/ERK, and some PKC isoforms [18,19], have been shown to phosphorylate and inhibit GSK-3 in vitro. A number of studies suggest that ERK, PKC [12–14], and PI3-kinase/Akt pathways are involved in NT signaling in certain cells [15,16,23,38]. In addition, NT-induced MAP kinase activation can be attenuated by the PKC inhibitor GF109203x [12]. Therefore, we measured ERK1/2 and Akt activity by Western blot analysis using anti–phospho-ERK1/2 or anti–phospho-Akt antibody, and PKCα and PKCβ1 activity by an immunocomplex kinase assay, after treating HT29 cells with NT for various times. As shown in Figure 2A, treatment with NT increased ERK1/2 and Akt phosphorylation in a time-dependent fashion in HT29 cells, suggesting the activation of ERK1/2 and Akt by NT. In vitro kinase assay showed that PKCα and PKCβ1 activity increased at 5 minutes after NT treatment. As a positive control, PMA induced PKCβ1 activity in HT29 cells (Figure 2B). Our results demonstrate the activation of ERK1/2, Akt, PKCα, and PKCβ1 by NT; any of these pathways may mediate NT-induced GSK-3 phosphorylation in HT29 cells.

**NT Induces GSK-3β Phosphorylation through PKC Activation**

We next examined the involvement of these kinases in the NT-induced phosphorylation of GSK-3 (Figure 3). Cells were pretreated with inhibitors to MEK (PD98059), PKC (Ro-318220 and GF109203x), or PI3-kinase (wortmannin)
for 30 minutes before stimulation with NT (100 nM). GSK-3 phosphorylation was analyzed by Western blot analysis (Figure 3). As expected, treatment with NT increased the phosphorylation of both GSK-3α and GSK-3β (lane 6). Interestingly, NT-mediated phosphorylation of GSK-3β, but not GSK-3α, was completely blocked by treatment with Ro-318220 (lane 3) or GF109203x (lane 4). In contrast, treatment with PD98059 or wortmannin did not block either GSK-3α or GSK-3β phosphorylation by NT (lanes 2 and 5). Collectively, these results suggest a role for the PKC pathway, but not for MEK/ERK or PI3-kinase, in the NT-induced phosphorylation of GSK-3β.

To examine the specific isoforms of PKC involved in mediating NT signaling to GSK-3, we determined whether NT-induced GSK-3 phosphorylation is blocked by the conventional PKC inhibitor Gö6976 (which inhibits PKCα and PKCβ1). HT29 cells were pretreated with Gö6976 for 30 minutes before stimulation with NT, and GSK-3 phosphorylation was visualized by Western blot analysis. Pretreatment with Gö6976 blocked NT-mediated GSK-3β phosphorylation in a dose-dependent fashion (Figure 4A). The role of PKCα and PKCβ1 was further examined in cells treated with or without 1 μM PMA for 24 hours to downregulate these enzymes (Figure 4, B and C). Treatment with PMA (1 μM) for 24 hours significantly reduced the basal expression of PKCα and PKCβ1 (Figure 4B). To address the potential role of these proteins in the induction of GSK-3 phosphorylation by NT, HT29 cells were treated with PMA (1 μM) for 24 hours, followed by the addition of NT (100 nM) for 20 minutes. GSK-3 phosphorylation was determined by Western blot analysis (Figure 4C). In agreement with changes in PKCα and PKCβ1 levels, the phosphorylation of GSK-3β, but not GSK-3α, was abolished by chronic PMA treatment, further suggesting a role for PKCα or PKCβ1 in the NT-mediated phosphorylation of GSK-3β.

To further examine the specific isoforms of PKC involved in mediating NT signaling to GSK-3, we used LY379196, a currently available potent and selective PKCβ inhibitor with an IC50 = 30–50 nM for PKCβ1 [39,40], to examine the role of a single classic PKC isozyme, PKCβ1. Pretreatment with LY379196 completely blocked NT-mediated GSK-3β (but not GSK-3α) phosphorylation (Figure 4D). These results suggest a role for PKCβ1 in the NT-mediated phosphorylation of GSK-3β and for an undefined kinase that may be responsible for the NT-induced phosphorylation of GSK-3α.

**Figure 4. Inhibition of PKCβ1 blocked NT-induced GSK-3α/β phosphorylation in HT29 cells.** (A) HT29 cells were serum-starved for 24 hours; cells were pretreated with Gö6976 (which inhibits PKCα and PKCβ1) for 30 minutes, followed by treatment with NT (100 nM) for 20 minutes. Total protein was extracted from cells and resolved by SDS-PAGE, transferred to PVDF membranes, and probed with anti–phospho-GSK-3α/β antibody; membranes were stripped and reprobed with anti-GSK-3α/β antibody. Representative data from three separate experiments are shown.

**Cyclin D1 Expression in HT29 Cells**

We have shown that NT induces GSK-3 phosphorylation in the HT29 human colon cancer cell line. To determine whether this induction is limited to HT29 cells or occurs in other colon cancer cells expressing the high-affinity NTR, SW480 and HCT116 cells were treated with NT (100 nM) for 20 minutes. Total protein was extracted, and Western blot analysis was performed. As shown in Figure 5, NT induced GSK-3α/β phosphorylation in SW480 (Figure 5A) cells and HCT116 (Figure 5B). These results confirm our findings in HT29 cells and, moreover, suggest a general regulation of GSK-3α/β phosphorylation by NT in colon cancer cells that possess NTR.

**Treatment with NT or GSK-3α/β Inhibition Increases Cyclin D1 Expression in HT29 Cells**

Cyclin D1 protein, which plays a critical role in intestinal epithelial cell growth and carcinogenesis [41–43], is stabilized by GSK-3β inhibition [44–46]. To investigate whether
NT-mediated GSK-3 phosphorylation is related to increased cyclin D1 levels, we first determined whether NT treatment increases cyclin D1 expression in HT29 cells. As shown in Figure 6 (upper panel), cyclin D1 expression was increased at 4 hours after the addition of NT. Subsequently, we used SB216763, a potent inhibitor of GSK-3α and GSK-3β, in an ATP-competitive manner [47] to determine whether inhibition of GSK-3 leads to increased cyclin D1 expression levels in HT29 cells. As expected, treatment with SB216763 increased cyclin D1 expression in HT29 cells (Figure 6, lower panel).

Discussion

GSK-3-mediated signaling is implicated in multiple biologic processes, including embryonic development, cell differentiation, apoptosis, and insulin response, by the phosphorylation of a broad range of substrates, including several critical transcription factors (e.g., c-Myc, c-Jun, and c-Myb, and the translation factor eIF2B) [30,31]. GSK-3 phosphorylates β-catenin (at Ser-33, Ser-37, and Thr-41) and leads to its ubiquitination and proteasomal degradation, mimicking the effects of Wnt signaling activation [48]. The activation of canonical Wnt signaling stabilizes cytosolic β-catenin, resulting in its translocation to the nucleus and the stimulation of T-cell factor/lymphoid enhancer factor and target genes such as cyclin D1 [49]. Recently, we have shown that GSK-3 is involved in the regulation of TRAIL expression in human colon cancer cells [50], and inhibition of GSK-3 inhibits intestinal cell differentiation (Wang et al., unpublished data). GSK-3 is rapidly phosphorylated at Ser-21 in GSK-3α or at Ser-9 in GSK-3β, resulting in the inhibition of GSK-3 kinase activity [51,52]. Numerous stimuli lead to the inactivation of GSK-3 through S9/S21 phosphorylation, including growth factors such as epidermal growth factor and platelet-derived growth factor, which stimulate the GSK-3–inactivating kinase p90RSK through MAP kinases [37], activators of p70 ribosomal S6 kinase (p70S6K) [53], and activators of cAMP-activated protein kinase A (PKA) [54] and PKC [18,19]. Consistent with its position downstream of the PI3-kinase–PKB/Akt pathway, GSK-3 activity suppresses cell proliferation and survival [28,29]. In striking contrast, virtually nothing is known about the regulation of GSK-3 phosphorylation at Ser9 residues by GI growth hormones that stimulate intestinal growth. In the present study, we show that the GI hormone NT induces a rapid increase in GSK-3 phosphorylation. Our results also indicate that PKC activation increases the phosphorylation of GSK-3β, implying a pathway that may be involved in physiological functions mediated by NT.

NT plays an important role in the stimulation of cell proliferation through multiple signaling pathways, including the activation of the ERK1/2 [12–14], PKC, and PI3-kinase–Akt pathways in some cells [15–17]. Although we show that NT stimulated ERK1/2, Akt, and PKC, only the inhibition of PKC blocked NT-mediated GSK-3β phosphorylation. In agreement with our results, Vilimek and Duronio [55] showed that cytokine-stimulated phosphorylation of GSK-3 is dependent on PKC, but not on PI3-kinase/Akt. These data suggest that NT, similar to cytokines, acts on GSK-3 through PKC activation and is independent of the PI3-kinase/Akt pathway.

We have shown that NT activates PKC, resulting in the phosphorylation of GSK-3. Bozou et al. [24] reported that NT only weakly stimulates PKC activity in HT29 cells. In this study, PKC activation was assessed indirectly by measuring epidermal growth factor binding to HT29 cells. We showed that NT stimulated obvious PKCα and PKCβ1 activation. The differences noted in activation may be a result of the different methods used for PKC activity assays. We assessed PKC activity directly by immunoprecipitation using anti-PKCα or anti-PKCβ1 antibody followed by in vitro kinase assay using MBP as substrate.

Our results indicate that the PKC inhibitors Go6976 and LY379196 (which selectively inhibit either PKCα and PKCβ1, respectively) [56,57] or downregulation of PKCα and PKCβ1 (by prolonged PMA treatment) blocked NT-mediated GSK-3β (but not GSK-3α) phosphorylation. These findings suggest a

**Figure 5.** NT induces GSK-3α/β phosphorylation in SW480 and HCT116 cells. SW480 (A) or HCT116 (B) cells were serum-starved for 24 hours. Cells were treated with NT (100 nM) for 20 minutes. Total protein was extracted from cells and resolved by SDS-PAGE, transferred to PVDF membranes, and probed with anti-phospho-GSK-3α/β antibody; membranes were stripped and reprobed with anti-GSK-3α/β antibody. Signals from three separate experiments are quantitated densitometrically and expressed as fold changes with respect to total GSK-3α and PKCβ1, respectively.

**Figure 6.** Inhibition of GSK-3 increases cyclin D1 expression in HT29 cells. HT29 cells were serum-starved for 24 hours, followed by treatment with NT or a specific GSK-3 inhibitor, SB216763, for 4 hours. Protein was extracted, and cyclin D1 expression was assessed by Western blot analysis. Membranes were stripped and reprobed with antiactin. Representative data from three separate experiments are shown.
role for PKCβ1 in the NT-mediated phosphorylation of GSK-3β; NT-mediated GSK-3α phosphorylation likely occurs through other kinase(s). Although it was noted that PKCα and PKCβ1 phosphorylate GSK-3β and GSK-3α [19], our results are largely consistent with a previous report by Goode et al. [18]. In this study, the effect of individual PKC isoforms on GSK-3 kinase activity in vitro was assessed. PKCα and PKCβ1 were among the PKC isoforms shown to phosphorylate GSK-3β, but not GSK-3α; however, the kinase that phosphorylates GSK-3α was not identified [10]. GSK-3α and GSK-3β are conserved with high homology within their kinase domains (98% identity), but only at 36% identity in the last 76 C-terminal residues [32]. GSK-3α and GSK-3β, although structurally similar, are not functionally identical. This became obvious in the ablation of the GSK-3β isoform in mice, which resulted in an embryonic lethal phenotype due to severe liver degeneration during midgestation [33]. The inability of GSK-3α to rescue GSK-3β-null mice indicates that a degenerative liver phenotype arises specifically from the loss of the β isoform. The phenotype of mice lacking GSK-3α has not yet been reported. It is interesting to speculate on a potentially unique role of GSK-3α in NT-mediated cellular responses. Future studies will assess the kinase(s) that contributes to NT-mediated GSK-3α phosphorylation.

Several studies have shown that NT stimulates intestinal cell growth [6–8,58]; however, the molecular mechanisms by which NT promotes cell proliferation and growth are largely undefined. In the present study, we showed that NT treatment or GSK-3 inhibition induced cyclin D1 expression. GSK-3 is a well-known regulator of cyclin D1 protein [59,60]. Phosphorylation of cyclin D1 on Thr-286 by GSK-3α results in the exclusion of cyclin D1 from the nucleus to initiate its proteasomal degradation [45]. Conversely, inactivated GSK-3β results in the suppression of cyclin D1 phosphorylation at Thr-286, thereby suppressing cyclin D1 degradation. Inhibition of GSK-3β stabilizes β-catenin expression, catalyzes its localization to the nucleus, and upregulates the downstream target gene cyclin D1 [59]. Extracellular mitogens promote cellular proliferation through receptor-mediated signaling circuitry, which ultimately converges on the cell cycle machine, and D-type cyclins function as critical sensors of these signals. During the G1 phase, cyclin D1 accumulates and G1 progression to mitogenic growth factors. The active cyclin D1 holoenzyme assembles with either cyclin-dependent kinase in response to extracellular mitogens – coupled receptor signaling in human ductal pancreatic cancer cell line, MIA PaCa-2. MIP-induced activation of MAPK pathways and AP-1 binding in the human pancreatic cancer cell line, Mia PaCa-2. Biochim Biophys Acta 203, 455–471.


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