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Advances in Brief

Neutral Endopeptidase Inhibits Neuropeptide-mediated Transactivation of the Insulin-like Growth Factor Receptor-Akt Cell Survival Pathway[1]

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Abstract

G-protein coupled receptor (GPCR) agonists such as neuropeptides activate the insulin-like growth factor-1 receptor (IGF-IR) or the serine-threonine protein kinase Akt, suggesting that neuropeptides-GPCR signaling can cross-communicate with IGF-IR-Akt signaling pathways. Neutral endopeptidase 24.11 (NEP) is a cell-surface peptidase that cleaves and inactivates the neuropeptides endothelin-1 (ET-1) and bombesin, which are implicated in progression to androgen-independent prostate cancer (PC). We investigated the mechanisms of NEP regulation of neuropeptide-mediated cell survival in PC cells, including whether neuropeptide substrates of NEP induce phosphorylations of IGF-IR and Akt in PC cells. Western analyses revealed ET-1 and bombesin treatment induced phosphorylation of IGF-IRβ and Akt independent of IGF-I in TSU-Pr1, DU145, and PC-3 PC cells, which lack NEP expression, but not in NEP-expressing LNCaP cells. Recombinant NEP and induced NEP expression in TSU-Pr1 cells using a tetracycline-repressive expression system inhibited ET-1-mediated phosphorylation of IGF-IRβ and Akt, and blocked the protective effects of ET-1 against apoptosis induced by serum starvation. Incubation of TSU-Pr1 cells with specific kinase inhibitors together with ET-1 or bombesin showed that IGF-IR activation is required for neuropeptide-induced Akt phosphorylation, and that neuropeptide-induced Akt activation is predominantly mediated by Src and phosphatidylinositol 3-kinase but not by mitogen-activated protein kinase or protein kinase C. These data show that the neuropeptides ET-1 and bombesin stimulate ligand-independent activation of the IGF-IR, which results in Akt activation, and that this cross-communication between GPCR and IGF-IR signaling is inhibited by NEP.

Introduction

Neuropeptides such as ET-1,[3] bombesin, and neurotensin have been implicated in various stages of PC, including PC development, PC cell migration, and progression to hormone-independence (1–3). These neuropeptides bind to GPCRs and initiate various signal transduction pathways that stimulate cell growth (4). Furthermore, recent studies showing that ET-1 and bombesin act as an antiapoptotic factor (5, 6) suggest that neuropeptides may also modulate cell survival pathways, although the precise manner by which neuropeptides affect signaling necessary for cell survival is unknown.

Akt/protein kinase B is a serine-threonin kinase that mediates cell survival in various cell types, including PC cells (7, 8). Akt is activated constitutively and by external stimuli such as IGF-I, which binds to and activates IGF-IR, resulting in activation of PI3-K. The PI3-K substrate phosphatidylinositol-3-kinase binds Akt and targets Akt to the cell membrane, where it is activated by phosphorylation. Activated Akt phosphorylates survival-mediating targets, including the Bcl-2 family member Bad and Caspase-9, inhibiting apoptosis and promoting cell survival (9, 10). Activated Akt may contribute to PC cell survival (8) and has been implicated in progression to androgen-independent PC (11, 12).

Activation of IGF-IR occurs through ligand-binding by the α-subunits, which results in the tyrosine phosphorylation of the β-subunits and activation of the receptor tyrosine kinase. Recent studies demonstrate that GPCR agonists such as angiotensin-II, thrombin, and ET-1 also stimulate the phosphorylation of IGF-IR and/or Akt (13–16), suggesting that the neuropeptide-GPCR signaling pathway crosscommunicates with IGF-IR-Akt signaling.

NEP (CD10), which is expressed on benign prostate epithelial cells, normally functions to reduce local concentrations of neuropeptides such as ET-1 and bombesin available for receptor-binding and signal-transduction (17). In PC cells, NEP is highly expressed by LNCaP cells (18), a PC cell line which is often used as a model for androgen-sensitive PC, whereas it is not expressed in androgen-insensitive PC cell lines TSU-Pr1, DU 145, and PC-3 (18, 19). In the present study, we investigated whether NEP neuropeptide substrates contribute to Akt-mediated cell survival and progression via GPCR-mediated IGF-IR transactivation, and we assessed the role of NEP in regulating neuropeptide-induced IGF-IR phosphorylation and PC cell survival.

Materials and Methods

Cell Culture and Reagents. PC cell lines were maintained in RPMI 1640 supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 units/ml streptomycin and penicillin, and 10% FCS. TSU-GK27-NEP (WT-5) and TSU-GK27-Neo (TN-12) cells were constructed and maintained as described previously (18, 19). rNEP was obtained from Arris Pharmaceutical Corp. CGS24592, a competitive inhibitor of NEP, was supplied by Novartis Pharmaceutical (19). The Src inhibitor PP2, the PI3-K inhibitor Wortmannin, the mitogen-activated protein or Erk kinase (MEK) inhibitor PD 98059, and the PKC inhibitor GF 109203X were purchased from Calbiochem-Novabiochem Ltd. (La Jolla, CA). The IGF-R kinase-specific inhibitor Tryphostin AG 1024 was purchased from Alexis Corporation (San Diego, CA). The EGFR kinase-specific inhibitor Tryphostin AG 1478 and the PDGFR kinase-specific inhibitor Tryphostin AG 1926 were purchased from Sigma Chemical Co. (St. Louis, MO).

Immunoprecipitation and Immunoblotting. Cells were lysed, and immunoprecipitation was performed as described previously (19). For immunoprecipitation, precleared proteins (500 μg) were incubated for 1 h with 2 μg of anti-IGFRβ1 (C-20; Santa-Cruz Biotechnology, Inc., Santa Cruz, CA) and incubated for an additional 1 h with 40 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech., Piscataway, NJ) at 4°C. Immunoprecipitates were collected by centrifugation at 12,000 *g* for 1 min, washed with radioim-

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[3] The abbreviations used are: ET-1, endothelin-1; PC, prostate cancer; GPCR, G-protein coupled receptor; IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; PI3-K, phosphatidylinositol 3-kinase; NEP, neutral endopeptidase 24.11; rNEP, recombinant NEP; PKC, protein kinase C; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor;

munoprecipitation assay buffer, resuspended in 2× Laemmli sample buffer, resolved on 8% or 10% SDS-PAGE, and transferred to nitrocellulose. After incubation in blocking buffer (1% BSA) for 2 h, membranes were immunoblotted with anti-IGFRβ1 (1:200) or anti-pTyr (PY99; Santa-Cruz Biotechnology; 1:2,000) using the enhanced chemiluminescence system (Amersham Pharmacia Biotech) for detection. Relative intensities of each band were measured by NIH image and levels of phosphorylated protein relative to total protein calculated as the phosphorylation ratio. For Akt detection, cells were lysed, resolved by 8% SDS-PAGE, and transferred. Membranes were immunoblotted with anti-Akt antibody (Santa-Cruz Biotechnology, Inc.; 1:500), anti-phospho-Akt Ab (Ser 473; New England Bio Lab; 1:1,000) or anti-actin Ab (Chemicon International, Inc., Temecula, CA; 1:3,000) and detected. All experiments were performed on at least two separate occasions, using different cell lysates, with similar results.

Apoptosis Assays. Early apoptotic cells were detected using the annexin V apoptosis detection kit (Santa-Cruz Biotechnology, Inc.). Briefly, cells evenly distributed in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) were treated with various reagents for 48 h. Cells were washed twice with cold PBS, washed once with 1× Assay Buffer and 1 μg of Annexin V FITC with 500 μl of 1× Assay Buffer added. Propidium iodide (0.5 μg) was added to each well for nuclear counter stain. After incubation for 15 min at room temperature in the dark, positively stained cells were enumerated using a fluorescence microscope at ×100–400. Each data point represented the average cell number in six independent microscopic fields of a single experiment. The statistical analysis was performed using an unpaired *t* test. All assays were performed on three separate occasions with similar results.

Results

Neuropeptides Induce Phosphorylation of Both IGF-IR and Akt in NEP-negative PC Cells. Recent studies suggest that various GPCR agonists can induce phosphorylation of IGF-R (13, 14) and Akt (15, 16). We examined whether ET-1 and bombesin could stimulate IGF-R phosphorylation in androgen-sensitive, NEP-expressing LNCaP cells compared with androgen-independent, NEP-negative TSU-Pr1, DU145, and PC-3 cells. Anti-pTyr Western blotting of IGF-IRβ immunoprecipitates of all four cell lines revealed that incubation in serum-free media with 10 nM ET-1 for 10 min resulted in an increase in IGF-IRβ phosphorylation in TSU-Pr1, DU145, and PC-3 cells but not inLNCaP cells (Fig. 1*A*, *panel 1*) despite similar levels of IGF-IRβ protein in all cell lines (Fig. 1*A*, *panel 2*). Western blotting using an antibody to Ser 473-phospho-specific Akt revealed that Akt phosphorylation was also induced by ET-1 (Fig. 1*A*, *panels 3* and *4*) in TSU-Pr1, DU145, and, to a lesser degree, PC-3 cells. LNCaP cells possess a constitutively activated Akt as reported previously (8). As shown in *panel 5*, Western blot analysis confirmed high levels of NEP protein in LNCaP cells but not in the androgen-independent PC cell lines. Additional analysis of TSU-Pr1 cells under similar conditions showed that IGF-I (10 ng/ml), ET-1 (10 nM), and bombesin (10 nM) each induced IGF-IRβ phosphorylation (Fig. 1*B*, *panel 1*) and Akt phosphorylation (*panel 3*), although the degree of phosphorylation was less in cells treated with ET-1 and bombesin compared with cells treated with IGF-I. These data show that ET-1 and bombesin stimulate phosphorylation of IGF-IRβ and Akt in the absence of IGF-I in PC cells that lack NEP expression.

NEP Inhibits Neuropeptide-induced Phosphorylation of IGF-IR and Akt. NEP enzymatic activity strongly inhibits neuropeptide-mediated signal transduction (19, 20). As shown in Fig. 2*A*, incubation of LNCaP cells for 10 min in serum-free media containing 10 nM ET-1 did not alter the level of phosphorylation of IGF-IRβ (Fig. 2*A*, *panel 1*, *Lane 2*, compared with *Lane 1*) and Akt (Fig. 2*A*, *panel 3*, *Lane 2* compared with *Lane 1*). However, pretreatment with the specific NEP enzyme inhibitor CGS24592 (10 nM) for 2 h before the addition of ET-1 resulted in a marked increase in phosphorylation of IGF-IRβ (Fig. 2*A*, *panel 1*, *Lane 4*) and a slight increase in Akt phosphorylation (Fig. 2*A*, *panel 3*, *Lane 4*). Incubation in serum-free media with CGS24592 alone had little effect (Fig. 2*A*, *panels 1* and *3*, *Lane 3*). Similar experiments investigating the effect of rNEP on IGF-IRβ and Akt phosphorylation in TSU-Pr1 cells showed that rNEP inhibits ET-1 and bombesin-induced phosphorylation of IGF-IRβ and Akt (Fig. 2*B*).

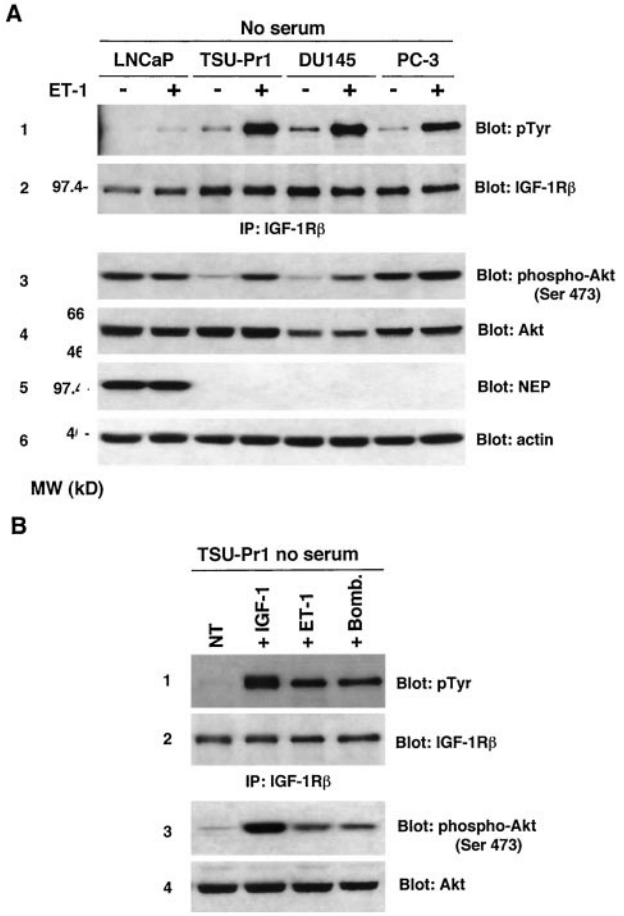

Fig. 1. Neuropeptides NEP substrates activate the phosphorylation of both IGF-IR and Akt in NEP-negative PC cells. *A*, PC cells in serum-free media treated with or without 10 nM ET-1 for 10 min. *Panel 1*, 500 μg of PC total cell lysates were immunoprecipitated with anti-IGF-IRβ antibody C-20, separated by SDS-PAGE, transferred to nitrocellulose, and Western blotted with anti-pTyr monoclonal antibody PY99. *Panel 2*, the same blot shown in *panel 1* was stripped and reprobed with Ab C-20 for IGF-IRβ. *Panel 3*, total cell lysates (20 μg) from PC cells were analyzed for Ser 473 phosphorylated Akt by Western blot as described in "Materials and Methods" using the anti-phospho-Akt Ab. *Panel 4*, the same blot shown in *panel 3* was stripped and reprobed with Ab for Akt. The same blot shown in *panel 3* was stripped and reprobed with anti-NEP mAb (NCL, *panel 5*). The blot was stripped and reprobed with anti-actin (*panel 6*) to confirm equal loading. *B*, TSU-Pr1 cells in serum-free media were treated with 10 ng/ml IGF-I, 10 nM ET-1, or 10 nM bombesin for 10 min. Cells were lysed and analyzed as described above (*A*).

Finally, studies of WT-5 cells, a derivative of TSU-Pr1 cells which express cell-surface NEP after tetracycline removal (Fig. 2*C*, *panel 1*), and control TN-12 cells (18, 19) showed that expression of NEP after tetracycline removal also blocks ET-1-induced phosphorylation of IGF-IRβ in WT-5 cells (Fig. 2*C*, *panel 2*, *Lanes 1–4*). Similar results were obtained in Akt phosphorylation (Fig. 2*C*, *panel 4*, *Lanes 1–4*). In control TN-12 cells, ET-1 treatment resulted in a marked increase in the phosphorylation of both IGF-IRβ and Akt, which were not affected by tetracycline removal (Fig. 2*C*, *panels 2* and *4*, *Lanes 5–8*). Taken together, these data show that constitutively expressed NEP in LNCaP cells, rNEP in TSU-Pr1 cells, and overexpression of cell-surface NEP in TSU-Pr1 cells inhibit phosphorylation of IGF-IR and Akt, which is induced by neuropeptide substrates of NEP.

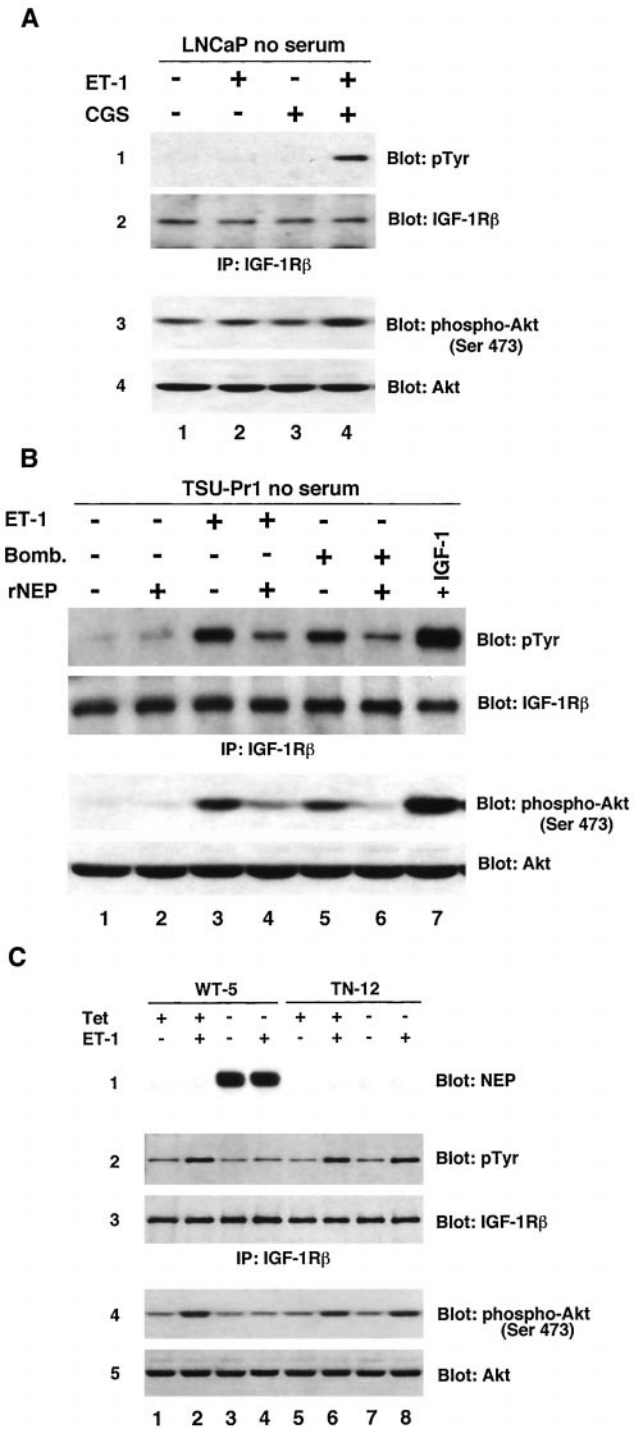

Fig. 2. NEP enzymatic activity inhibits neuropeptide-induced phosphorylation of both IGF-IR and Akt in PC cells. *A*, LNCaP cells were cultured in RPMI without serum (*Lane 1*), in 10 nM ET-1 for 10 min (*Lane 2*), in 10 nM CGS24592 for 2 h (*Lane 3*), or in 10 nM CGS24592 for 2 h and then with 10 nM ET-1 for 10 min (*Lane 4*). Cells were lysed and analyzed as described in Fig. 1*A*. *B*, TSU-Pr1 cells were cultured in RPMI without serum (*Lanes 1*); in RPMI without serum and then with the addition of 10 nM ET-1 (*Lane 3*); or in 10 nM bombesin (*Bomb., Lane 5*) for 10 min; in RPMI without serum and then with the addition of 50 μg/ml of rNEP for 2 h (*Lane 2*); rNEP and then with 10 nM ET-1 (*Lane 4*); rNEP and then with 10 nM bombesin (*Lane 6*) for 10 min; or in RPMI without serum and then with the addition of 10 ng/ml IGF-I (*Lane 7*). Cells were lysed and analyzed as described in the legend to Fig. 1*A*. *C*, TSU-Pr1-derived cell lines containing wild-type NEP (*WT-5*) or control empty vector (*TN-12*) were cultured with (+) and without (−) 1 μg tetracycline (*Tet*) for 48 h, washed with PBS three times, incubated in serum-free media with or without tetracycline for 1 h, and incubated further with or without 10 nM ET-1 for 10 min. Cells were lysed and analyzed as described in the legend to Fig. 1*A*.

Neuropeptide-induced IGF-IR Phosphorylation Is Mediated by Src Kinase. Src kinase activates the IGF-IR signaling pathway (21). Recently, we reported that neuropeptides activate Src kinase activity in PC cells (20). TSU-Pr1 cells cultured in serum-free media treated with 10 μM of the specific Src kinase inhibitor PP2 for 30 min before the addition of 10 nM ET-1 or bombesin for 10 min resulted in >90% decrease in neuropeptide-induced IGF-IRβ phosphorylation (Fig. 3*A*). The inhibitory effect of PP2 on IGF-IRβ phosphorylation induced by ET-1 was dose-dependent (Fig. 3*B*). These results suggest that neuropeptide-induced IGF-IR phosphorylation is mediated by Src kinase activity.

NEP Inhibits ET-1-induced Antiapoptotic Effect in TSU-Pr1 Cells Dependent on Src-mediated Activation of IGF-IR-Akt Pathway. PI3-K activation induced by IGF-IR activation results in phosphorylation and activation of Akt. To confirm that PI3-K was also necessary for neuropeptide-mediated Akt phosphorylation, we investigated whether the PI3-K inhibitor Wortmannin inhibited ET-1-induced Akt phosphorylation. As shown in Fig. 4*A*, TSU-Pr1 cells cultured in serum-free media treated with increasing concentrations of Wortmannin for 30 min before the addition of 10 nM ET-1 for 10 min resulted in a dose-dependent decrease in Akt phosphorylation that was similar to the inhibition observed with the Src-kinase inhibitor PP2. In contrast, the inhibition of other kinases that are activated by GPCR signaling, such as MEK and PKC, using inhibitors PD 98059 (MEK) and GF 109203X (PKC) did not alter ET-1-induced Akt phosphorylation; although MEK or PKC have been reported to be involved in PI3-K (15) or Akt activation (22, 23).

GPCR agonists transactivate other growth factor receptors in addition to IGF-IR, including EGFR and PDGFR (24, 25). Both EGFR and PDGFR also activate Akt (26–28). Therefore, next we investigated whether IGF-IR activation is required for ET-1-induced Akt phosphorylation. Treatment of TSU-Pr1 cells with the IGF-R kinase-specific inhibitor Tryphostin AG 1024 for 30 min before the addition

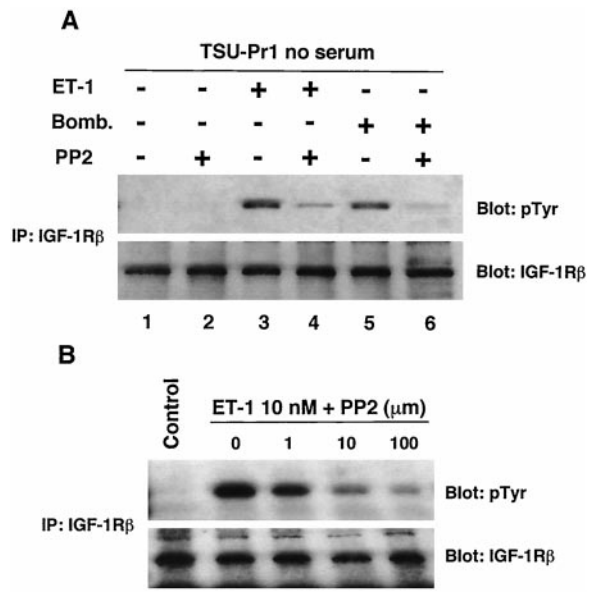

Fig. 3. Neuropeptide-induced IGF-IR phosphorylation is mediated by Src kinase. *A*, TSU-Pr1 cells were cultured in RPMI without serum (*Lanes 1*); in RPMI without serum and then with the addition of 10 nM ET-1 (*Lane 3*) or 10 nM bombesin (*Bomb., Lane 5*) for 10 min; in RPMI without serum treated with 10 μM of the specific Src kinase inhibitor PP2 for 30 min (*Lane 2*), without serum treated with 10 μM of the specific Src kinase inhibitor PP2 for 30 min before 10 nM ET-1 (*Lane 4*), or 10 nM bombesin (*Lane 6*) for 10 min. Cells were lysed and analyzed with anti-pTyr mAb or anti-IGF-IRβ Ab. *B*, TSU-Pr1 cells were cultured in RPMI without serum; or in RPMI without serum and then with the addition of various concentrations of PP2 for 30 min, and then with 10 nM ET-1 for 10 min. Cells were lysed and analyzed described above.

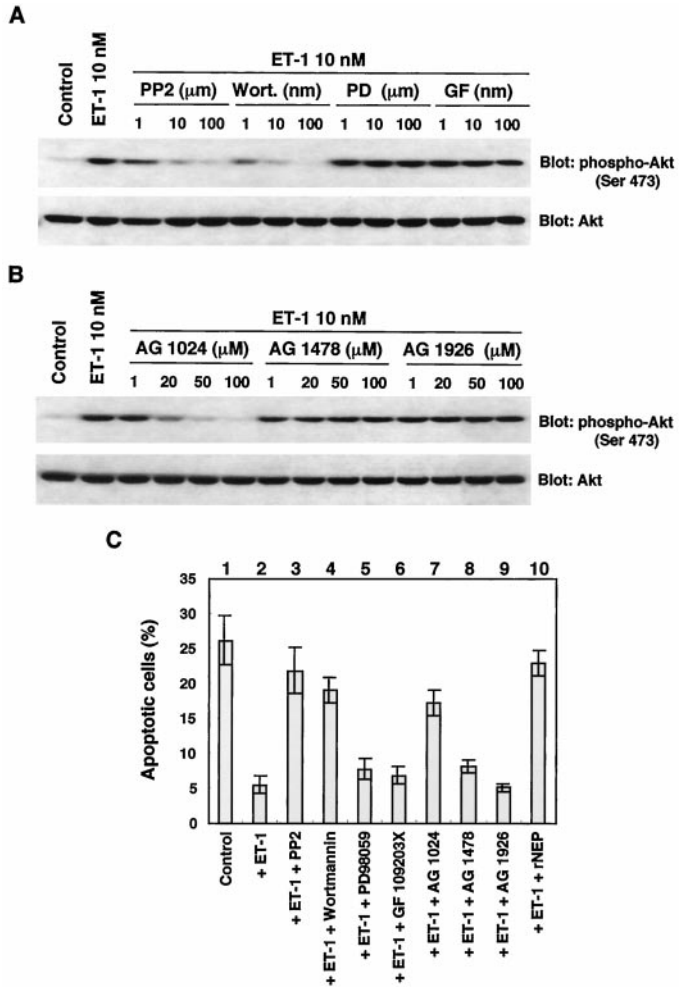

Fig. 4. NEP inhibits ET-1-induced antiapoptotic effect in TSU-Pr1 cells dependent on Src-mediated activation of the IGF-IR-Akt pathway. *A*, TSU-Pr1 cells were cultured in RPMI without serum; in RPMI without serum and then with the addition of 10 nM ET-1 for 10 min; or in RPMI without serum treated with various concentrations of PP2, Wortmannin (*Wort.*), PD 98059 (*PD*), or GF 109203X for 30 min before the addition of 10 nM ET-1 for 10 min. Cells were lysed and analyzed with anti-phospho-Akt Ab or anti-Akt Ab. *B*, TSU-Pr1 cells were cultured in RPMI without serum; in RPMI without serum and then with the addition of 10 nM ET-1 for 10 min; or in RPMI without serum treated with various concentrations of receptor kinase inhibitors Tryphostin AG 1024, AG 1478, or AG 1926 for 30 min before the addition of 10 nM ET-1 for 10 min. Cells were lysed and analyzed as described in the legend to Fig. 4*A*. *C*, TSU-Pr1 cells were cultured in RPMI without serum for 48 h (*Lane 1*); in RPMI without serum and then with the addition of 10 nM ET-1 for 48 h (*Lane 2*); or in RPMI without serum treated with 10 μM PP2 (*Lane 3*), 100 nM Wortmannin (*Lane 4*), 100 μM PD 98059 (*Lane 5*), 100 nM GF 109203X (*Lane 6*), 100 μM Tryphostin AG 1024 (*Lane 7*), 100 μM Tryphostin AG 1478 (*Lane 8*), or Tryphostin AG 1926 (*Lane 9*) for 30 min or 50 μg/ml rNEP for 2 h (*Lane 10*) before the addition of 10 nM ET-1 for 48 h. Cells were stained with Annexin V-FITC (Annexin V binds to membrane phosphatidylserine, which accumulates to the extracellular surface in apoptotic cells). Positively Annexin V-FITC-stained cells were enumerated in six independent microscopic fields. *Bars*, SD. Experiments were repeated twice with similar results.

of 10 nM ET-1 for 10 min resulted in a dose-dependent decrease in Akt phosphorylation, whereas similar experiments using the EGFR kinase-specific inhibitor Tryphostin AG 1478 or the PDGFR kinase-specific inhibitor Tryphostin AG 1926 had little effect on ET-1-induced Akt phosphorylation (Fig. 4*B*). Taken together, these data suggest that neuropeptide-induced Akt phosphorylation is dependent on Src and PI3-K, and that Src-mediated kinase activation of IGF-IR, but not EGFR or PDGFR, plays a critical role in ET-1-induced Akt phosphorylation in PC cells.

Finally, we investigated whether neuropeptide-mediated IGF-IR-Akt activation was protective against apoptosis in PC cells induced by serum starvation. Annexin V apoptosis detection assays revealed that TSU-Pr1 cells cultured in RPMI without serum treated with 10 nM ET-1 for 48 h resulted in a significant decrease in Annexin V positively stained cells compared with untreated control cells (Fig. 4*C*, *Lane 2* compared with *Lane 1*; $P < 0.001$). Pretreatment before the addition of ET-1 with 10 μM PP2 (Src, *Lane 3*), 100 nM Wortmannin (PI3-K, *Lane 4*), 100 μM Tryphostin AG 1024 (IGFR, *Lane 7*), and 50 μg/ml rNEP (*Lane 10*) significantly inhibited the cell-survival effect by ET-1 ($P < 0.01$ to *Lane 2*), whereas pretreatment with 100 μM PD 98059 (MEK, *Lane 5*), 100 nM GF 109203X (PKC, *Lane 6*), 100 μM Tryphostin AG 1478 (EGFR, *Lane 8*), or Tryphostin AG 1926 (PDGFR, *Lane 9*) had little inhibitory effect. Treatment with each inhibitor without ET-1 resulted in no significant increase in apoptotic cell number compared with untreated control (data not shown). These results suggest that ET-1 promotes cell survival predominantly through Src-mediated IGF-IR activation, which can modulate PI3-K-Akt phosphorylation.

Discussion

The current study assessed the potential role of NEP in regulating the effects of the neuropeptides ET-1 and bombesin in the context of cell survival. We show that these NEP substrates transactivate IGF-IR via Src kinase independent of IGF-1, and that activation of IGF-1R results in PI3-K mediated Akt phosphorylation. These data implicating IGF-IR signaling are similar to other studies suggesting that IGF-IR signaling plays a crucial role in the development and progression of PC cells (29–31). In contrast to those studies, however, we demonstrate that neuropeptides function to induce IGF-IR signaling in the absence of IGF-I. These results are not surprising inasmuch as other GPCR ligands such as angiotensin-II and thrombin can also stimulate phosphorylation of IGF-IR or its downstream effector, Akt (13, 14, 16, 22).

GPCR agonists can also transactivate other growth factor receptors, such as EGFR and PDGFR (24, 25), resulting in Akt phosphorylation (26–28). However, our results indicate that neuropeptide-mediated IGF-IR and Akt activation in PC cells is mainly dependent on IGF-IR signaling.

IGFs and their receptors frequently have been implicated in the development and progression of PC (29–32). However, only a few studies have suggested that neuropeptides can enhance IGF signaling. Nelson *et al.* (2) reported that ET-1 synergizes with IGF-I to promote cell proliferation in androgen-independent PC cells, and bombesin-receptor antagonists inhibit PC-3 xenografts concomitant with a decrease in serum IGF-I levels and IGF-II mRNA levels (33). Our studies indicate that neuropeptides activate IGF-IR signaling via a Src-dependent pathway. Similarly, Rao *et al.* (13) reported that thrombin stimulates IGF-IR phosphorylation through Src activation in Rat aortic smooth muscle cells, and Peterson *et al.* (21, 34) reported that Src activates IGF-IR phosphorylation on the same autophosphorylation sites as does IGF-I, suggesting that many peptides which activate Src kinase activity can activate ligand-independent IGF-IR phosphorylation and activation. We recently showed that Src kinase activity is activated by ET-1 and bombesin in PC cells (20), and that this activation plays an important role in neuropeptide-mediated cell migration (19). Thus, neuropeptides activate Src, which affects various signal transduction pathways that contribute to PC tumor cell survival and progression.

A variety of studies now implicate IGF-IR signaling and Akt activation in androgen-independent PC (11, 12, 35). We show that activation of the Akt cell survival pathway is inhibited by NEP, suggesting that the loss of NEP expression by PC cells will result in resistance to apoptosis. Together with our previous studies, the current results provide additional evidence of the importance of NEP in

regulating PC cell growth. Expression of NEP in androgen-independent PC cells inhibits cell migration (19), inhibits cell growth and tumorigenicity in the prostate (36), and induces apoptosis and cell cycle arrest (36). Furthermore, NEP inhibits neuropeptide-induced, Src-mediated PKCδ degradation, which is necessary for phorbol ester-induced apoptosis (20). Moreover, expression of the NEP gene is androgen-regulated; NEP expression decreases following androgen withdrawal (37), and methylation of the NEP promoter can also result in diminished NEP expression (38). Together, these data suggest a model in which decreased NEP expression contributes to the development and progression of androgen-independent PC. The current study provides insight into one of the consequences of NEP loss in PC cells and demonstrates how NEP substrates induce IGF-IR activation in the presence or absence of IGF-I and lead to the activation of the Akt cell survival pathway.

Acknowledgments

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References

1. Sehgal, I., Powers, S., Huntley, B., Powis, G., Pittelkow, M., and Maihle, N. J. Neurotensin is an autocrine trophic factor stimulated by androgen withdrawal in human prostate cancer. Proc. Natl. Acad. Sci. USA, *91:* 4673–4677, 1994.
2. Nelson, J. B., Chan-Tack, K., Hedican, S. P., Magnuson, S. R., Opgenorth, T. J., Bova, G. S., and Simons, J. W. Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. Cancer Res., *56:* 663–668, 1996.
3. Markwalder, R., and Reubi, J. C. Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. Cancer Res., *59:* 1152–1159, 1999.
4. Rozengurt, E. Signal transduction pathways in the mitogenic response to G protein-coupled neuropeptide receptor agonists. J. Cell. Physiol., *177:* 507–517, 1998.
5. Tallett, A., Chilvers, E. R., Hannah, S., Dransfield, I., Lawson, M. F., Haslett, C., and Sethi, T. Inhibition of neuropeptide-stimulated tyrosine phosphorylation and tyrosine kinase activity stimulates apoptosis in small cell lung cancer cells. Cancer Res., *56:* 4255–4263, 1996.
6. Eberl, L. P., Valdenaire, O., Saintgiorgio, V., Jeannin, J. F., and Juillerat-Jeanneret, L. Endothelin receptor blockade potentiates FasL-induced apoptosis in rat colon carcinoma cells. Int. J. Cancer, *86:* 182–187, 2000.
7. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell, *95:* 29–39, 1998.
8. Carson, J. P., Kulik, G., and Weber, M. J. Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3′-kinase and Akt/protein kinase B. Cancer Res., *59:* 1449–1453, 1999.
9. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. Akt phosphorylation of BAD couples survival signals to the cell- intrinsic death machinery. Cell, *91:* 231–241, 1997.
10. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. Regulation of cell death protease caspase-9 by phosphorylation. Science (Wash DC), *282:* 1318–1321, 1998.
11. Miyake, H., Nelson, C., Rennie, P. S., and Gleave, M. E. Overexpression of insulin-like growth factor binding protein-5 helps accelerate progression to androgen-independence in the human prostate LNCaP tumor model through activation of phosphatidylinositol 3′-kinase pathway. Endocrinology, *141:* 2257–2265, 2000.
12. Graff, J. R., Konicek, B. W., McNulty, A. M., Wang, Z., Houck, K., Allen, S., Paul, J. D., Hbaiu, A., Goode, R. G., Sandusky, G. E., Vessella, R. L., and Neubauer, B. L. Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. J. Biol. Chem., *275:* 24500–24505, 2000.
13. Rao, G. N., Delafontaine, P., and Runge, M. S. Thrombin stimulates phosphorylation of insulin-like growth factor-1 receptor, insulin receptor substrate-1, and phospholipase C-γ 1 in rat aortic smooth muscle cells. J. Biol. Chem., *270:* 27871–27875, 1995.
14. Du, J., Sperling, L. S., Marrero, M. B., Phillips, L., and Delafontaine, P. G-protein and tyrosine kinase receptor cross-talk in rat aortic smooth muscle cells: thrombin- and angiotensin II-induced tyrosine phosphorylation of insulin receptor substrate-1 and insulin-like growth factor 1 receptor. Biochem. Biophys. Res. Commun., *218:* 934–939, 1996.
15. Foschi, M., Chari, S., Dunn, M. J., and Sorokin, A. Biphasic activation of p21ras by endothelin-1 sequentially activates the ERK cascade and phosphatidylinositol 3-kinase. EMBO J., *16:* 6439–6451, 1997.
16. Sasaki, T., Irie-Sasaki, J., Jones, R. G., Oliveira-dos-Santos, A. J., Stanford, W. L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., Joza, N., Mak, T. W., Ohashi, P. S., Suzuki, A., and Penninger, J. M. Function of PI3Kγ in thymocyte development, T cell activation, and neutrophil migration. Science (Wash DC), *287:* 1040–1046, 2000.
17. Shipp, M. A., Tarr, G. E., Chen, C. Y., Switzer, S. N., Hersh, L. B., Stein, H., Sunday, M. E., and Reinherz, E. L. CD10/neutral endopeptidase 24.11 hydrolyzes bombesin-like peptides and regulates the growth of small cell carcinomas of the lung. Proc. Natl. Acad. Sci. USA, *88:* 10662–10666, 1991.
18. Papandreou, C. N., Usmani, B., Geng, Y., Bogenrieder, T., Freeman, R., Wilk, S., Finstad, C. L., Reuter, V. E., Powell, C. T., Scheinberg, D., Magill, C., Scher, H. I., Albino, A. P., and Nanus, D. M. Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. Nat. Med., *4:* 50–57, 1998.
19. Sumitomo, M., Shen, R., Walburg, M., Dai, J., Geng, Y., Navarro, D., Boileau, G., Papandreou, C. N., Giancotti, F. G., Knudsen, B., and Nanus, D. M. Neutral endopeptidase inhibits prostate cancer cell migration by blocking focal adhesion kinase signaling. J. Clin. Investig., *106:* 1399–1407, 2000.
20. Sumitomo, M., Shen, R., Goldberg, J. S., Dai, J., Navarro, D., and Nanus, D. M. Neutral endopeptidase promotes phorbol ester-induced apoptosis in prostate cancer cells by inhibiting neuropeptide-induced protein kinase C δ degradation. Cancer Res., *60:* 6590–6596, 2000.
21. Peterson, J. E., Kulik, G., Jelinek, T., Reuter, C. W., Shannon, J. A., and Weber, M. J. Src phosphorylates the insulin-like growth factor type I receptor on the autophosphorylation sites. Requirement for transformation by src. J. Biol. Chem., *271:* 31562–31571, 1996.
22. Clerk, A., and Sugden, P. H. Activation of protein kinase cascades in the heart by hypertrophic G protein-coupled receptor agonists. Am. J. Cardiol., *83:* 64H–69H, 1999.
23. Zheng, W. H., Kar, S., and Quirion, R. Stimulation of protein kinase C modulates insulin-like growth factor-1- induced akt activation in PC12 cells. J. Biol. Chem., *275:* 13377–13385, 2000.
24. Daub, H., Weiss, F. U., Wallasch, C., and Ullrich, A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature (Lond.), *379:* 557–560, 1996.
25. Linseman, D. A., Benjamin, C. W., and Jones, D. A. Convergence of angiotensin II and platelet-derived growth factor receptor signaling cascades in vascular smooth muscle cells. J. Biol. Chem., *270:* 12563–12568, 1995.
26. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell, *81:* 727–736, 1995.
27. Burgering, B. M., and Coffer, P. J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature (Lond.), *376:* 599–602, 1995.
28. Sibilia, M., Fleischmann, A., Behrens, A., Stingl, L., Carroll, J., Watt, F. M., Schlessinger, J., and Wagner, E. F. The EGF receptor provides an essential survival signal for SOS-dependent skin tumor development. Cell, *102:* 211–220, 2000.
29. Chan, J. M., Stampfer, M. J., Giovannucci, E., Gann, P. H., Ma, J., Wilkinson, P., Hennekens, C. H., and Pollak, M. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science (Wash DC), *279:* 563–566, 1998.
30. DiGiovanni, J., Kiguchi, K., Frijhoff, A., Wilker, E., Bol, D. K., Beltran, L., Moats, S., Ramirez, A., Jorcano, J., and Conti, C. Deregulated expression of insulin-like growth factor 1 in prostate epithelium leads to neoplasia in transgenic mice. Proc. Natl. Acad. Sci. USA, *97:* 3455–3460, 2000.
31. Shaneyfelt, T., Husein, R., Bubley, G., and Mantzoros, C. S. Hormonal predictors of prostate cancer: a meta-analysis. J. Clin. Oncol., *18:* 847–853, 2000.
32. Pollak, M., Beamer, W., and Zhang, J. C. Insulin-like growth factors and prostate cancer. Cancer Metastasis Rev., *17:* 383–390, 1998.
33. Plonowski, A., Schally, A. V., Varga, J. L., Rekasi, Z., Hebert, F., Halmos, G., and Groot, K. Potentiation of the inhibitory effect of growth hormone-releasing hormone antagonists on PC-3 human prostate cancer by bombesin antagonists indicative of interference with both IGF and EGF pathways. Prostate, *44:* 172–180, 2000.
34. Peterson, J. E., Jelinek, T., Kaleko, M., Siddle, K., and Weber, M. J. c phosphorylation and activation of the IGF-I receptor in src-transformed cells. J. Biol. Chem., *269:* 27315–27321, 1994.
35. Wen, Y., Hu, M. C., Makino, K., Spohn, B., Bartholomeusz, G., Yan, D. H., and Hung, M. C. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. [In Process Citation]. Cancer Res. *60:* 6841–6845, 2000.
36. Dai, J., Shen, R., Sumitomo, M., Walburg, M., Geng, Y., Navarro, D., Boileau, G., Papandreou, C. N., Giancotti, F. G., Knudsen, B., and Nanus, D. M. Tumor suppressive effects of neutral endopeptidase in androgen-independent prostate cancer cells. Clin. Cancer Res., in press, 2001.
37. Shen, R., Sumitomo, M., Dai, J., Hardy, D. O., Papandreou, C. N., Shipp, M. A., Freedman, L. P., and Nanus, D. M. Characterization of two androgen-responsive regions in the human neutral endopeptidase gene. Mol. Cell. Endocrinol., *170:* 131–142, 2000.
38. Usmani, B. A., Shen, R., Janeczko, M., Papandreou, C. N., Lee, W. H., Nelson, W. G., Nelson, J. B., and Nanus, D. M. Methylation of the neutral endopeptidase gene promoter in human prostate cancers. Clin. Cancer Res., *6:* 1664–1670, 2000.

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