Phosphorylation of Mitogen-activated Protein Kinase is Inhibited by Calcitonin in DU145 Prostate Cancer Cells

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ABSTRACT

One of the causes of insensitivity to androgen ablation therapy in prostate cancer is thought to be attributable to elevated neuropeptides secreted by neuroendocrine cells in the tumor mass. Calcitonin (CT), one of these neuropeptides, is reported to be associated with the growth of prostate cancer. There is an increase in mitogen-activated protein (MAP) kinase activation as prostate cancer progresses to a more advanced and androgen-independent disease. We examined the effect of CT on signal transduction and the relation between CT and early-response genes in the human androgen-insensitive prostate cancer cell line, DU145. The basal phosphorylation level of extracellular signal-regulated kinase 1/2, which is a key kinase in the mediation of growth factor-induced mitogenesis in prostate cancer cells, was constitutively up-regulated. N-[2-(4-bromocinnamnamyl)aminoethyl]-5-isoquinoline-sulfonamide (H89), a specific inhibitor of protein kinase A, potentiated the effects of more increased phosphorylation of extracellular signal-regulated kinase 1/2. CT induced the inhibition of this MAP kinase phosphorylation, and this effect was completely abolished by pretreatment with H89. Our findings demonstrate that CT caused the inhibition of constitutive MAP kinase phosphorylation in a protein kinase A-dependent manner in DU145. The transient increase of c-fos expression was detected after CT treatment, whereas expression of c-jun RNA was down-regulated after CT treatment. These results suggest that CT may regulate early-response genes, c-fos and c-jun, via a MAP kinase cascade. In conclusion, these findings suggest that DU145 might be a useful model as a therapeutic approach of neuropeptides in androgen-independent prostate carcinoma.

INTRODUCTION

The early stages of prostate cancer growth are typically androgen-dependent and are therefore usually responsive to androgen ablation therapy. However, following such therapy, the disease almost invariably progresses to an androgen-independent state, rendering androgen ablation therapy ineffective. This may be caused by altered specificity of the androgen receptor, by ligand-independent stimulation of the androgen receptor, by paracrine and autocrine growth modulation of androgen receptor, by ligand-independent stimulation of the androgen receptor, by paracrine and autocrine growth modulation of the androgen receptor, by ligand-independent stimulation of the androgen receptor, by paracrine and autocrine growth modulation of the androgen receptor, by ligand-independent stimulation of the androgen receptor, by paracrine and autocrine growth modulation of the androgen receptor, by ligand-independent stimulation of the androgen receptor, by paracrine and autocrine growth modulation of the androgen receptor, by ligand-independent stimulation of the androgen receptor.

As prostate cancer progresses to a more advanced and androgen-independent disease, there is an increase in the activation of MAP kinase.

The aim of the present study was to investigate the effect of CT upon signal transduction in the human prostate cancer cell line DU145, which is androgen-insensitive.

MATERIALS AND METHODS

Cell Lines and Cell Cultures. The three human prostate cancer cell lines DU145, PC3, and LNCaP were used. LNCaP, derived from a lymph node metastasis of a prostate cancer patient, was a gift from Dr. Kazuo Gohji (Osaka Medical College, Osaka, Japan) and was cultured in DMEM with 10% FBS (HyClone, Logan, UT). PC3, derived from a bone metastasis of a prostate cancer patient, was purchased from the Health Science Research Resources Bank and cultured in F12K medium supplemented with 7% FBS. DU145, derived from a brain metastasis of a prostate cancer patient, was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen Department of Human and Animal Cell Cultures (Braunschweig, Germany) and cultured in RPMI 1640 with 10% FBS. All cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

Antibodies and Other Reagents. Salmon CT was purchased from Peninsula Laboratories, Inc. (Belmont, CA). The antibodies against total ERK1/2 were purchased from BioLegend, Inc. (San Diego, CA).

RNA Extraction and RT-PCR. Total RNA was isolated by RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), following the manufacturer’s instructions. The cDNA was synthesized from 20 μg of total RNA using reagents from SuperScript (Life Technologies, Inc., Gaithersburg, MD). Oligonucleotides used to amplify DNA of CT receptor mRNA were CTR-P1 (forward; 5′-CCCTCTCAACAGAACATGTTTCG-3′) and CTR-P2 (reverse; 5′-CTCTAGGATGTTGGGATTC-3′). Forty cycles of reactions were performed under the conditions of denaturation for 30 s at 95°C, annealing was performed for 30 s at 58°C, and extension was performed for 30 s at 72°C. RT-PCR of β-actin was performed as a control for the quality of extract RNA.

Western Blot Analysis. Western blotting was performed as previously described except for sonication (11). Briefly, 70—80% confluent cell cultures were washed twice with PBS and then cultured in 0.5% serum medium for 24 h with or without CT. DU145 prostate cancer cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. PC3 cells were grown in RPMI 1640 medium supplemented with 10% FBS. LNCaP cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. The human prostate cancer cell line DU145, PC3, and LNCaP were used. LNCaP, derived from a lymph node metastasis of a prostate cancer patient, was a gift from Dr. Kazuo Gohji (Osaka Medical College, Osaka, Japan) and was cultured in DMEM with 10% FBS (HyClone, Logan, UT). PC3, derived from a bone metastasis of a prostate cancer patient, was purchased from the Health Science Research Resources Bank and cultured in F12K medium supplemented with 7% FBS. DU145, derived from a brain metastasis of a prostate cancer patient, was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen Department of Human and Animal Cell Cultures (Braunschweig, Germany) and cultured in RPMI 1640 with 10% FBS. All cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.
18 h. Quiescent cell cultures were treated with various concentrations of CT or other reagents. At the end of these treatments, the medium was aspirated and cells quickly washed twice with cold PBS. After 1 min in lysis buffer at 4°C, the cell lysate was scraped and protein concentration was determined. Twenty μg of protein were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were incubated with primary antibody overnight at 4°C. The secondary antibody was antirabbit IgG conjugated to horseradish peroxidase (DAKO, Carpinteria, CA). After washing, immunoreactive protein complexes were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and then exposed to X-ray films. Signals were quantified by computer densitometry (Atto, Co., Tokyo, Japan).

Northern Blot Analysis and cDNA Probe Synthesis. The c-fos probe was a 2.1-kb EcoRI fragment from the plasmid pSPT-fos cDNA purchased from Japan Health Sciences Foundation (Osaka, Japan). To prepare DNA probes, the EcoRI fragment was labeled with [32P]α ATP using the Hotprime DNA labeling kit (Gene Hunter, Nashville, TN). The c-jun probe was a 763 bp PCR product amplified by the PCR primers 5'-GACCTTATGGCTACAGTA-ACC-3' (forward) and 5'-GGGATCTCTCCAGCCTCC-3' (reverse) using human leukocyte cDNA. Total RNA was extracted from the cells according to the manufacturer’s protocol (RNeasy Mini Qiagen Inc.). Northern blot analysis was carried out as described previously (12).

RESULTS

Prostate Cancer Cell Lines Express CT Receptor mRNA. To examine the expression of CT receptor mRNA, the three prostate cancer cell lines DU145, LNCaP, and PC3 were subjected to RT-PCR using CTR-P1 and CTR-P2 primers. All cell lines yielded a 476-bp product (data not shown), which is the expected length from human CTR cDNA, thereby confirming expression of CTR mRNA.

Basal Phosphorylation of ERK1/2 via MEK 1 Cascade Is Detectable in DU145 Cells but not in LNCaP and PC3 Cells. The phosphorylation status of ERK1/2 was determined by Western blotting of the cell lysate with phosphospecific antibodies. DU145 cells displayed elevated activity of phosphorylated ERK1/2 compared with LNCaP and PC3 cells (Fig. 1). To determine whether the increased levels of ERK1/2 phosphorylation were dependent upon the MEK 1 cascade, we examined the effect of the MEK1-specific inhibitor PD98059 on ERK phosphorylation. Treatment of cells with 50 μM PD98059 for 30 min almost completely abolished phosphorylation of ERK1/2, with the maximum response occurring at a concentration of 10−8 M (Fig. 3A). Inhibition of ERK1/2 phosphorylation by 10−8 M was maximum at 60 min, at which time ERK1/2 phosphorylation was 10% of control levels (Fig. 3B). Therefore, CT inhibits phosphorylation of ERK transiently in DU145 cells.

Inhibition of ERK1/2 Phosphorylation by CT Is Blocked by PKA-Inhibitor. To examine the effect of H89 upon the CT-mediated suppression of ERK phosphorylation, DU145 cells were serum-deprived, exposed to 10−5 M H89, and then stimulated with 10−8 M CT. As shown in Fig. 4, H89 decreased CT-mediated suppression of ERK phosphorylation, suggesting that activation of PKA might be required for the action of CT. These results indicated that the cAMP/PKA pathway contributes significantly to the inhibition of ERK1/2 phosphorylation induced by CT in DU145 cells.

CT Regulates Immediate Early Gene Expression in DU145 Cells. We used Northern blot analysis to investigate the relationship between CT and immediate early gene expression. A transient increase in c-fos mRNA expression was detected at 30 min after CT treatment, whereas expression of c-jun mRNA was down-regulated up to 2 h after CT treatment (Fig. 5).

DISCUSSION

The effects of CT are mediated by the CT receptor, which has seven transmembrane domains coupled to the G protein-coupled receptor. Several isoforms of the hCTR have been identified as alternatively spliced products from a single gene (13), and these isoforms have different signaling properties. Both of the two isoforms with (hCTR-1) or without (hCTR-2) the 16-amino acid insert in the first intracellular domain can activate adenylate cyclase leading to cAMP formation, but only the hCTR-2 receptor can activate phospholipase C...
to generate inositol phosphate second messengers (14). cAMP/PKA is a major biochemical pathway for CTR signaling (15). Wu et al. (16) have reported that the hCTR-2 receptor is present in the prostate NE cells secreting CT. We speculated that CT exerted an autocrine and paracrine function upon prostatic glandular epithelial cells as well as NE cells. In this study, the results of RT-PCR demonstrated that the three prostate cancer cell lines DU145, LNCaP, and PC3 express the CT receptor mRNA. We were unable to identify the isoform of CT receptor because the primers for PCR coded at the third transmembrane domain.

In the present study, it was clear that constitutive phosphorylation of ERK1/2 occurred in androgen-insensitive DU145 cells in serum-free conditions but was not present in androgen-insensitive PC3 cells or androgen-sensitive LNCaP cells. These results are consistent with a previous report (15). Putz et al. (15) suggested that this may be attributable to increased expression of EGF and TGF-α together with higher levels of EGF receptors in DU145 cells compared with LNCaP cells. The role of the PKA pathway in the regulation of ERK has been investigated in prostate cancer cells. In various cell lines, second messenger pathways provide links to the Ras/Raf/ERK cascade, resulting in a subsequent reduction of MAP kinase activity (2, 17). The influence of PKA on Ras/Raf interaction is regarded as a key regulatory step in the integration of cAMP and growth factor signals into the ERK cascade. Differential regulation of Raf isoforms by cAMP has been reported (17, 18). This mechanism is cell-type specific and dependent on isotype expression of the signaling molecules involved.

In PC12 pheochromocytoma cells, elevation of cAMP activates MAPK by a Rap1- and B-Raf-dependent pathway (19). Elevated cAMP activates PKA, which then activates B-Raf resulting in inhibition of the Ras/c-Raf-1 pathway. According to this hypothesis, constitutive up-regulated phospho-ERK was elevated by further H89 (Fig. 2B), suggesting that the Ras/c-Raf-1 pathway was strongly inhibited by PKA rather than that the Rap1/B-Raf pathway was activated by PKA in DU145 cells.

In this study, we have demonstrated that CT inhibited phosphorylation of ERK1/2 in DU145 cells. In addition, constitutive basal levels of phosphorylation of ERK1/2 were partially inhibited by the activity of the PKA pathway. Furthermore, CT-mediated inhibition of ERK1/2

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**Fig. 3.** The effect of CT on the phosphorylation of ERK1/2 in DU145 cells. A, effects of concentrations of CT on the phosphorylation of ERK1/2. Subconfluent DU145 cells were incubated in RPMI 1640 containing 0.5% FBS for 18 h and subsequently treated with the indicated concentrations of CT for 30 min. A dose-response relationship was obtained for the effect of CT on DU145 cells, with maximal response occurring at $10^{-8}$ M CT. B, effects of duration of treatment with CT on the phosphorylation of ERK1/2. Subconfluent cells were incubated in medium containing 0.5% FBS for 18 h and treated with $10^{-8}$ M CT for the indicated times. CT transiently inhibited ERK1/2 phosphorylation. Bottom graph, the percentage of activity of the control by densitometric analysis. The data represent average values of three independent experiments.

**Fig. 4.** The effect of H89 on ERK1/2 phosphorylation by CT in DU145 cells. Cells were starved and then treated with $10^{-8}$ M CT alone, or $10^{-8}$ M H89 combined with $10^{-8}$ M CT for the indicated length of time. Results indicate that the inhibition of ERK1/2 phosphorylation induced by CT can be blocked by H89. Graph, the percentage activity of the indicated time by densitometric analysis; the density of Lane 30 min (CT) was considered as 100%.
phosphorylation via the CTR was PKA dependent. Although a previous report showed that treatment with CT significantly increased intracellular cAMP levels only in DU145 cells (20), the nature of the intracellular signal transduction pathways involved was not clear. This cross-talk of the CT receptors to the ERK signaling pathway has not been demonstrated previously in DU145 prostate cancer cells, and, to the best of our knowledge, this is the first study to address the nature of the signaling pathways stimulated by CT in DU145 cells.

Among the targets of MAP kinases are other kinases and nuclear transcriptional factors including the oncogenes myc, fos, and jun. The activator protein-1, a heterodimer of c-Jun and c-Fos, activates transcription of the target gene by binding to specific promoter elements. In addition, the interaction between CTR signal transduction and transactivation of transcriptional factors has not been reported. Our results showed that the blocking of MAP kinase phosphorylation by CT results in phosphorylation of transcriptional factors, which in turn induces a rapid increase in c-fos expression and down-regulation of c-jun expression. The induction of c-fos gene expression was transient, whereas c-jun mRNA expression was down-regulated.

Fig. 5. Effects of CT on c-fos and c-jun mRNA levels in DU145 cells. Cells were treated with medium containing 10^-9 M CT for the time indicated. Twenty μg of total mRNA isolated from untreated control and CT-treated cells were hybridized with 32P-labeled c-fos or c-jun DNA probes as described in “Materials and Methods.” The increase in c-fos mRNA expression was transient, whereas c-jun mRNA expression was down-regulated.

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