The Two Functions of Calcitonin: Cell Type-specific Up- and Down-regulation of ERK Signaling and Urokinase-type Plasminogen Activator Expression

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Abstract
Calcitonin (CT) is a 32 amino-acid polypeptide hormone secreted by C-cells of the thyroid. Clinically, CT is administered to treat humoral hypercalcemia of malignancy and osteoporosis. Recently, an association between CT use and cancer incidence has been suggested. Extracellular signal regulated kinases 1 and 2 (Erk1/2) are ubiquitous protein kinases that are involved in functions including the regulation of cell proliferation, adhesion, and migration. Urokinase-type plasminogen activator (uPA) modulates cell adherence to the extracellular matrix and is involved in both normal tissue repair and malignancy. We previously reported that CT can either increase or decrease the phosphorylation of Erk1/2 and the expression of uPA, depending on the cell type. In the human breast cancer cell line MDA-MB-231, CT inactivates c-Raf via the protein kinase A pathway, thereby inhibiting Erk1/2 phosphorylation and decreasing uPA expression. However, in LLC-PK1 porcine renal cells, CT induces Erk1/2 phosphorylation through the protein kinase C pathway, and thus increasing the uPA expression. Furthermore, CT has different effects on tumor progression and metastasis in cancer cells. Thus, CT selectively regulates the Erk1/2 pathway in a cell type-specific manner, and these modes of regulation may play an important role in the actions of CT on various other types of cells. We focus on the mechanism of the opposite effects of CT on Erk1/2 phosphorylation and uPA expression, and review the differential regulation of CT depending on the tumor type.

Keywords: Calcitonin, cancer cell, Erk signaling, protein kinase A, protein kinase C, urokinase-type plasminogen activator

Introduction
Calcitonin (CT) is a peptide hormone comprising 32 amino-acid secreted by C-cells of the thyroid gland (1). The main physiological function of CT is the regulation of calcium metabolism through inhibition of bone resorption by osteoclasts, and enhancement of Ca²⁺ excretion by kidney (2). Therefore, CT is administered to treat diseases characterized by high bone turnover, including Paget’s disease of the bone, humoral hypercalcemia of malignancy, and osteoporosis (3). Intranasal and injectable salmon CT are used worldwide to treat postmenopausal osteoporosis. Recently, salmon CT has been shown to be an effective drug for treating cherubism or disc degeneration (4, 5). Many reports have shown that proCT (PCT), the precursor of CT, is increased in the serum of septic patients and those with systemic inflammation, particularly in bacterial infections. The serum PCT levels are correlated with the mortality and severity of infection and persist for a relatively long period of time (6-8). Hence, PCT is an important marker of sepsis (9, 10). The CT receptor (CTR) is expressed in many types of tissues and cells (11-13), suggesting that CT/CTR may have other functions as well. There are many reports that CT is involved in the regulation of cell proliferation, adhesion, and migration (12-18). In cancer cells, their proliferation, tumorigenesis and metastasis are regulated by CT (19-26).

CTR belongs to the G protein-coupled seven transmembrane domain receptor (GPCR) superfamily, one of the largest families of membrane receptors. This superfamily includes the receptors for parathyroid hormone (PTH), luteinizing hormone (LH), and follicle stimulating hormone (FSH) (27). These receptors regulate the intracellular signaling cascades. The GPCRs interact with membrane-associated heterotrimeric GTP-binding proteins (G proteins) isoforms composed of α, β and γ subunits. It has been reported that CTR couples to Gs and Gq, resulting activation of adenylate cyclase (AC) and phospholipase C (Fig. 1) (28-34).

Fig. 1. Signaling pathways of CTR (28, 31-34, 85, 90).

GPCRs also control the activity of several members of the mitogen-activated protein kinase (MAPK) superfamily. Among these kinases are extracellular signal-regulated kinases 1 (Erk1) and 2 (Erk2) (or p44 MAPK and p42 MAPK), c-Jun NH2-terminal kinases (JNKs), p38 MAPK, and Erk5 (or BMK) (35). The Erks are ubiquitous protein kinases that are related with many cellular responses, including cell proliferation, adhesion, and...
migration (36). GPCRs may signal to Erks via numerous signaling pathways that differ dramatically in different cell types or cell lines. Thus, GPCRs activate complex pathways that ultimately interconnect events at the plasma membrane to those in the nucleus, such as modulation of gene transcription via phosphorylation of transcription factors.

Previously, we performed cDNA subtractive hybridization experiments to study CT-induced changes in gene expression of the porcine kidney cell line LLC-PK1 (37). We identified urokinase-type plasminogen activator (uPA) as one of the genes whose expression was induced by CT stimulation, and that this up-regulation was mediated by phosphorylation of Erk1/2. Similarly, CT increases phosphorylation of Erk1/2 in human embryonic kidney cells (HEK293) transfected with rabbit CTR as well as in human nucleus pulposus (5, 38). Interestingly, we have found that CT decreases uPA expression by suppressing constitutive phosphorylation of Erk1/2 in breast cancer cells, MDA-MB-231 (39). These studies suggest that CT has opposite actions on Erk1/2 phosphorylation in different cells. In this review, we focus on the mechanism of the two functions of CT on Erk1/2 phosphorylation, and uPA expression in LLC-PK1 cells and MDA-MB-231 cells in particular, and introduce an idea that this differential regulation of CT depends on the tumor type.

**CT induces transient activation of Erk1/2 through the protein kinase C (PKC) pathway in LLC-PK1 cells**

LLC-PK1 cells were established from the porcine kidney and are used as an in vitro model to study renal proximal tubule function (40, 41). The LLCPK1 cells have been used in many studies on CT/CTR function because the CTR was first cloned from the cells (42). We have demonstrated that CT induces Erk1/2 phosphorylation in LLCPK1 cells (37, 43). Time-course studies have shown that 10^9 M of CT maximally stimulates the phosphorylation of Erk1/2 after 30 min of treatment (Fig. 2A). H89, a protein kinase A (PKA) inhibitor, has no apparent effects on basal or CT-stimulated Erk1/2 phosphorylation. However, the PKC inhibitor, Calphostin C (CC), partially blocks the CT-induced Erk1/2 phosphorylation. These results suggest that CT-induced Erk1/2 phosphorylation is contributed by the PKC pathway, but not by the PKA pathway in LLC-PK1 cells. These results are consistent with those obtained from CTR-transfected HEK293 cells (38).

**PKA contributes significantly to CT-mediated attenuation of constitutive Erk1/2 phosphorylation in MDA-MB-231 cells**

The MAPK pathway plays a vital role in regulating the cell proliferation and differentiation (36). Constitutive phosphorylation of MAPK or elevated level of MAPK mRNA has been reported in a variety of tumors and to be associated with carcinogenesis (44-51). When the MAPK pathways are constitutively activated, normal mammalian cells are transformed into a cancerous phenotype (52-54), whereas blockade of MAPK activity inhibits the proliferation of Ras-transformed cells in vitro (55, 56). Thus, constitutive activation of MAPK promotes cell transformation, suggesting that the MAPK pathway is a therapeutic target for cancer (57-60).

Our previous studies have shown that CT inhibits phosphorylation of Erk1/2 in the cell lines of breast cancer (MDA-MB-231, MDA-MB-435, and T24), renal cell carcinoma (VIMC-RCW), rectal carcinoma (CaR-1), and prostate cancer (DU145), while these lines exhibit constitutive phosphorylation of Erk1/2. In these cell lines of breast cancer (MCF-7, MRK-M1), thyroid cancer (TT, FTC-133), pancreatic carcinoma (KP-1NL, KP-3), and colon cancer (CCK81) without constitutive Erk1/2 activation, CT has no effects on the Erk1/2 phosphorylation (61). MDA-MB-231 cells are hormone-insensitive, highly invasive, and a representative of late-stage breast cancer. In our study, constitutive activation of JNK or p38 MAPK was not detected in MDA-MB-231 cells, and CT did not induce the Erk1/2 phosphorylation (61). As mentioned above, CT suppresses Erk1/2 phosphorylation in MDA-MB-231 cells (Fig. 2B). Because the CTR is capable of activating PKA and PKC (31, 33), we investigated whether these pathways were involved in attenuation of Erk1/2 phosphorylation by CT. When MDA-MB-231 cells were treated with a PKA inhibitor, H89, or a PKC inhibitor, CC, in the presence or absence of CT, H89 diminished Erk1/2 phosphorylation induced by CT, but CC had no effects (61). These results suggest that the PKA pathway, but not the PKC pathway, may contribute to the attenuation of Erk1/2 phosphorylation by CT in MDA-MB-231 cells, which is consistent with our previous studies on the prostate cancer cell line, DU145 (62).

**Activation of Ser259 of c-Raf is related to the CT-induced down regulation of constitutive Erk1/2 phosphorylation in a PKA-mediated manner in MDA-MB-231 cells**

MAPK are regulated by intracellular phosphorylation cascades. In these cascades, Erk1/2 is activated by MAPK/ERK kinase 1, MEK1 and MAPK/ERK kinase 2, MEK2 (63). These MEKs are phosphorylated by MEKKs composed of A-Raf, B-Raf, and c-Raf (or Raf-1) (64). Essentially, c-Raf activation occurs by phosphorylation of Ser338 and Tyl341in the N-Raf (64). When 14-3-3 binds to Ser259 antagonizes c-Raf. On the contrary, binding of Ser259 to c-Raf activates Raf (64). When 14-3-3 bound to Ser259 or Ser621 in c-Raf (64). When 14-3-3 bound to Ser621 of c-Raf, it appears to activate of c-Raf. On the contrary, binding to Ser259 of c-Raf appears to inhibit of its activity. Therefore binding of 14-3-3 to Ser259 antagonizes c-Raf activity (65, 66). Dumaz et al. reported that activated PKA block c-Raf activation by stimulating 14-3-3 binding and preventing c-Raf interaction with Ras (67). We studied the contribution of c-Raf to
the regulation of Erk1/2 phosphorylation by CT in MDA-MB-231 cells was investigated (61). The results showed that CT enhanced phosphorylation of Ser259 of c-Raf. However, H89 inhibits this effect of CT, producing a statistically significant reduction in c-Raf phosphorylation. As noted above, H89 also attenuates the inhibition of Erk1/2 phosphorylation by CT in these cells. These results suggest that activation of Ser259 of c-Raf contributes to PKA-dependent attenuation of constitutive Erk1/2 phosphorylation by CT in MDA-MB-231 cells.

CT increases cAMP levels in both LLC-PK1 and MDA-MB-231 cells

To confirm that CTR is related to the cAMP pathway in MDA-MB-231 and LLC-PK1 cells, we measured the intracellular cAMP concentrations following CT treatment. The result showed that CT substantially increased cAMP level in both cell lines: 3.6-fold higher in LLC-PK1 cells, and 2-fold higher in MDA-MB-231 cells (Fig. 2C and D). However, increased cAMP level is contributed by the inhibition of Erk1/2 phosphorylation in MDA-MB-231 cells, but not in LLC-PK1 cells (37, 61).

The effects of AC and PKC activators on Erk1/2 phosphorylation in LLC-PK1 cells are similar to those in MDA-MB-231 cells, but the CT signaling is different in the two cell lines

The effects of the AC activator, forskolin, and the PKC activator, phorbol 12-myristate (PMA), on the Erk1/2 phosphorylation have been studied in LLC-PK1 cells and MDA-MB-231 cells (37, 61). In LLC-PK1 cells, PMA but not forskolin increases Erk1/2 phosphorylation. Furthermore, PMA augments the CT stimulation to Erk1/2 phosphorylation, while forskolin partially suppresses it. In MDA-MB-231 cells, PMA increases the basal Erk1/2 phosphorylation and blocks the suppression induced by CT, whereas forskolin eliminates the Erk1/2 phosphorylation in both CT-treated and untreated cells. These results suggest that the inhibitory effect of forskolin and the stimulatory effect of PMA on the Erk1/2 phosphorylation are similar in the two cell lines, but in the LLC-PK1 cells, PMA mimics or augments the effects of CT, whereas forskolin antagonizes these effects. The reverse is true in MDA-MB-231 cells: forskolin mimics or augments the effects of CT, whereas PMA antagonizes these effects.

The effects of CT on uPA expression in LLC-PK1 cells and MDA-MB-231 cells

We have found that CT induces expression of many genes including uPA, connective tissue growth factor (CTGF), NF-xB, NF-IL-3-A, and interleukin-8 in LLC-PK1 cells. Of these genes, uPA and CTGF are induced by CT via the Erk1/2 pathway in LLC-PK1 cells (37, 43). The uPA system consists of uPA, uPA receptor, and uPA inhibiting plasminogen activator inhibition types 1 and 2. Upon binding to uPA receptor, uPA breaks down various components of extracellular matrix, including collagen and fibronectin, through generation of plasmin converted from plasminogen (68). Thereby uPA participates in cellular invasion and migration (69-71). Ma et al. reported high levels of uPA expression through constitutive Erk1/2 phosphorylation in MDA-MB-231 cells (72). As shown in Figure 3, we previously reported that exogenous CT increased uPA expression about 18-fold in LLC-PK1 cells, but decreased it about 4-fold in MDA-MB-231 cells (37, 40). Consistent with its effects on uPA mRNA in MDA-MB-231 cells. CT decreased the uPA protein concentration in the medium to 67% of control levels. In the presence of the MEK1 inhibitor PD98509, CT-induced uPA expression was significantly decreased in LLC-PK1 cells, and constitutive uPA expression was also decreased in MDA-MB-231 cells. Our study has also shown that CT administration for 2 weeks to nude mice injected with MDA-MB-231 cells inhibits the expression of uPA mRNA in primary tumors by 25% compared to control (40). These results indicate that CT has opposite effects on uPA expression in these cell lines, which may be due to its opposite effects on Erk1/2 phosphorylation as shown the model of these system in Figure 4.

Fig. 3. CT has opposite effects on uPA expression in LLC-PK1 and MDA-MB-231 cells (37, 39). Serum-starved LLC-PK1 and MDA-MB-231 cells were incubated with 10^7 M CT for the indicated times, and then assayed for uPA mRNA using Northern blotting (top panels). The expression level of the uPA gene was normalized to the level of 18S rRNA (middle panels). To compare the expression levels among samples after 3 h incubation with CT, the expression level of uPA gene in the control cells was set to 100% (bottom panels). *P<0.05 compared with the control.

Fig. 4. A model of differential regulation by CT on Erk1/2 signaling and its downstream effectors in LLC-PK1 and MDA-MB-231 cells. In MDA-MB-231 cells, constitutive activation of Erk1/2 and constitutive uPA expression are present. CT inhibits c-Raf activation by phosphorylation of c-Ras at Ser259 via PKA, thus suppressing Erk1/2 activation and ultimately decreasing uPA expression. In LLC-PK1 cells, neither Erk1/2 activation nor uPA expression occurs constitutively. CT activates Erk1/2 via the PKC pathway, and thus induces uPA expression.

CT has opposite effects on biological functions

Consistent with the results from CTR-transfected HEK293 cells (39), our results suggest that CT induces Erk1/2 activation in LLC-PK1 cells by a PKC-dependent mechanism. Figure 3 depicts the differential regulation of Erk1/2 activity and uPA expression by CT in LLC-PK1 and MDA-MB-231 cells. In the MDA-MB-231
cells, constitutive activation of the Erk1/2 is suppressed by CT through the PKA pathway, while in the LLC-PK1 cells, activation of the Erk1/2 is induced by CT through the PKC pathway. We speculate that the PKA-mediated suppression of Erk1/2 phosphorylation by CT as observed in MDA-MB-231 and DU145 cells depends on the constitutive activation of Erk1/2 in these cells. Such contradictory effects have been reported for other peptide hormones. Adrenomedullin (ADM) is a 52 amino-acid peptide belonging to the CT gene superfamily (73). ADM is a tumor progression factor that acts in controlling angiogenesis (74). Conflicting results regarding ADM in vascular smooth muscle cells (VSMCs) have been reported. ADM has been reported to inhibit MAPK activity that is stimulated by epidermal platelet-derived growth factor, growth factor and endothelin in cultured rat glomerular mesangial cells and cultured rat VSMCs (75, 76). In contrast, ADM has also been reported to increase MAPK activity in serum-deprived rat VSMCs (77).

PTH, a polypeptide secreted by parathyroid glands, is a well-known mediator of bone remodeling and regulator of calcium homeostasis (78). PTH has catabolic effects, causing bone resorption by indirect activation of osteoclasts. PTH also has anabolic effects, leading to bone formation (79). PTH/PTH-related peptide (PTHrP) receptors comprise a distinct family of seven-transmembrane GPCRs to which CTR also belongs. Similar to the findings with CT, both stimulation (78, 80-81) and inhibition (82) of the MAPK pathway by PTH or PTHrP have been reported in osteoblasts. Verheijen et al. have demonstrated that PTH inhibits growth factor-induced Erk2 phosphorylation in osteosarcoma cells via the PKA pathway (83). On the other hand, this group has also reported that PTH induces transient activation of Erk1/2 in both Chinese hamster ovary R15 cells stably expressing high levels of rat PTH/PTHrP and parietal yolk sac carcinoma cells that endogenously express the receptor (83). They have concluded that PTH regulates MAPK activity in a cell type-specific manner. Chen et al. proposed that these opposite results might be due to the different cell systems used, e.g., osteosarcoma versus normal osteoblasts, and the various differentiation states of the cells (84).

Why does signaling by CT, like ADM and PTH signaling, vary in different cells? As noted above, CTR couples to Gs and Gq, resulting activation of AC and phospholipase C, respectively (31-34). Moreover, CTR can couple to Gi, but the inhibition of AC by Gqi is negatively regulated by PKC (38, 85) (Fig. 1). A dominant negative mutant Gnos containing three separate mutations that affect distinct functions has been characteristic in HEK293 cells. The results have shown that these triple mutants prevent CTR from activating AC and block activation of proteinase inhibitor turnover by CTR (85, 86). These results suggest that the different abilities of the G proteins to compete with each other for binding to CTR may influence differential signal transduction by CT, depending on the cell types. However, other factors must also play an important role, because CT increases cAMP in both LLC-PK1 and MDA-MB-231 cells. Other possibilities are that CT may bind to different, unknown receptors and that CTR may interact with different receptors in LLC-PK1 and MDA-MB-231 cells.

The effects of CT on tumor progression and invasiveness

We have found that CT suppresses the tumor proliferation in vivo by inhibiting the constitutive Erk1/2 phosphorylation (60). Nude mice were treated with CT or saline every day after injection with MDA-MB-231 cells with constitutively phosphorylated Erk1/2 or MCF-7 cells without constitutively phosphorylated Erk1/2. In MDA-MB-231 cells, CT significantly reduced the tumor volume compared with saline. However, CT had no significant effects on tumor growth in MCF-7 cells. Furthermore, CT also inhibited the invasiveness of MDA-MB-231 cells by 37% in a matrigel invasion assay (39). These results suggest that CT suppresses cancer proliferation in vivo and invasiveness in vitro.

On the other hand, some reports have shown that CT increases tumorigenicity and metastatic potential in the prostate cancer cell lines PC-3, PC-3M and LNCaP (23, 86-91) and tumor tissues (24-26). None of these cell lines shows constitutive activation of Erk1/2, as seen in MCF-7 cells (62, 92, 93). In PC-3 and LNCaP cells, uPA expression induced by CT was reported (94). PC-3M cells were derived from a liver metastasis of PC-3 cells following intraspleen injection, and are androgen-refractory and have highly metastatic potential (95). LNCaP cells were established from lymph node metastasis in a prostate cancer patient, and are androgen-sensitive and have low metastatic potential (96). PC-3 cells, which were established from bone metastasis of a prostate cancer patient, are androgen refractory and moderately metastatic (97). DU145 cells, which were established from brain metastasis of a prostate cancer patient, are androgen refractory and have moderately metastatic potential (98). Shah et al. demonstrated that CT may induce the loss of cell-cell adhesion, an increase in the surface activity of or integrin’s, and in the secretion of matrix metalloproteinases 2 and 9 and uPA (23). Therefore, CT may increase the tumorigenicity and invasiveness of prostate cancer cells thorough these molecular events. Regarding CT and CTR, LNCaP cells do not express CT, PC-3 cells do not express endogenous CTR, and PC-3M cells co-express CT and CTR (99). Overexpression of CT in LNCaP cells and CTR in PC-3 cells by transfection method increases their tumorigenicity and metastatic potential. On the contrary, silencing of CT expression in PC-3M cells does not only reduce their tumorigenicity, but also their metastatic potential (23).

Our results demonstrate that CT through its different effects on Erk1/2 phosphorylation depending on cell types performs correspondingly opposite effects on the gene expressions that influence biological processes including tumorigenesis, metastasis, and others. We propose that the clinical efficacy of CT will be useful in cancer that expresses the CTR and shows constitutive Erk1/2 phosphorylation. On the other hand, CT may induce malignancy in cancer cells that lack phosphorylated Erk1/2 signaling.

Conclusion

We introduced that the effects of CT on Erk1/2 phosphorylation and uPA expression are specific for cell types and are opposite in different cell types. These findings will assist in solving the problem of the variable functions of CT via CTR in many organs, tissues, and cells throughout the body. Furthermore, clarification of the detailed mechanisms of the action of CT is needed for its use as a drug in the future.

References


