Corticotropin-releasing hormone receptors mediate apoptosis via cytosolic calcium-dependent phospholipase A<sub>2</sub> and migration in prostate cancer cell RM-1

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Abstract

Peripheral corticotropin-releasing hormone receptors (CRHRs) are G protein-coupled receptors that play different roles depending on tissue types. Previously, we discovered the mechanism of CRHR-mediated apoptosis of mouse prostate cancer cell line (RM-1) to be a change of Bcl-2:Bax ratio, and CRH was found to inhibit transforming growth factor β migration of breast cancer cells via CRHRs. In the present study, we investigated cytosolic calcium-dependent phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) bridging CRHR activations and Bcl-2:Bax ratio and the effect of CRHR activation on cell migration. Silencing of cPLA<sub>2</sub> attenuated a CRHR1 agonist, CRH-induced apoptosis, and the decrease of the Bcl-2:Bax ratio, whereas silencing of cPLA<sub>2</sub> aggravated CRHR2 agonist, Urocortin 2 (Ucn2)-inhibited apoptosis, and the increase of the Bcl-2:Bax ratio. CRH in a time- and concentration-dependent manner increased cPLA<sub>2</sub> expression mainly through interleukin 1β (IL1β) upregulation. Ucn2 decreased cPLA<sub>2</sub> expression through neither tumor necrosis factor α nor IL1β. CRH-suppressed decay of cPLA<sub>2</sub> mRNA and Ucn2 merely suppressed its production. Overexpression of CRHR1 or CRHR2 in HEK293 cells correspondingly upregulated or down-regulated cPLA<sub>2</sub> expression after CRH or Ucn2 stimulation respectively. In addition, both CRH and Ucn2 induced migration of RM-1 cells. Our observation not only established a relationship between CRHRs and cell migration but also for the first time, to our knowledge, demonstrated that cPLA<sub>2</sub> participates in CRHR1-induced apoptosis and CRHR2-inhibited apoptosis.

Key Words
- CRHR1
- CRHR2
- cPLA<sub>2</sub>
- apoptosis
- migration

Introduction

The corticotropin-releasing hormone (CRH) family is composed of CRH, Urocortin 1 (Ucn1), Ucn2, and Ucn3, which can bind to two known receptors, CRH receptor type 1 (CRHR1) and CRHR2 (Lewis et al. 2001), which share 69% amino acid homology but have different tissue distributions and pharmacological properties (Perrin et al. 1995). CRH has a tenfold higher affinity for CRHR1 than for CRHR2, whereas Ucn1 has equal affinity for both receptors, although Ucn2 and Ucn3 are natural ligands for CRHR2 (Fekete & Zorrilla 2007). Recently, increased interest has been developing in the expression and biological role of the CRH system in malignant
tumors. Several lines of evidence have indicated that CRH inhibited human endometrial adenocarcinoma cell growth (Graziani et al. 2002) and that Ucn1 inhibited hepatocellular carcinoma growth and angiogenesis (Bale et al. 2002, Wang et al. 2008). Rassouli et al. (2011) suggested that dermal fibroblasts, deficient in CRH (CRH−/−), had higher proliferation and migration rates compared with WT (CRH+/+) cells.

In the prostate, cellular growth and differentiation are precisely regulated by autocrine and paracrine regulatory factors (Cunha et al. 1987). Ectopic CRH, an endocrine regulator, is associated with a growing list of tumors. It has been reported that a few patients with prostate cancer presented CRH secretion (Carey et al. 1984, Saeger et al. 1993, Rickman et al. 2001), and urocortin, one of the CRH family peptides, was expressed in prostate and prostate adenocarcinoma (Arcuri et al. 2002). However, the molecular basis of the role of CRH in prostate cancer is not clearly presented. We previously found that CRH activated CRHR1 to induce apoptosis through reducing ratio of Bcl-2:Bax expression, lowering mitochondrial membrane potential, and activating caspase-9. In addition, CRHR2 activated by Ucn2 had the opposite effects to those of CRHR1 in mouse prostate cancer cell line RM-1 (Jin et al. 2011). We found that CRH induced apoptosis through androgen receptor (Jin et al. 2012) and inhibited transforming growth factor β-induced epithelial–mesenchymal transition via CRHRs in breast cancer cells (our unpublished data). However, what intermediates are responsible for CRH-family-mediated apoptosis and whether the family and its receptors are involved in migration remain to be determined.

Cytosolic calcium-dependent phospholipase A2 (cPLA2), an 85-kDa protein, is a rate-limiting enzyme catalyzing the release of arachidonic acid (AA) from membrane phospholipids for eicosanoid production in response to a wide variety of extracellular stimuli (Hirabayashi & Shimizu 2000). AA is a precursor in the biosynthesis of prostaglandins, thromboxanes, and leukotrienes, which play important roles in smooth muscle stimulation, platelet aggregation control, and the release of histamine during anaphylactic shock and other allergic reactions. Activated cPLA2 is aberrant in many types of cancers (Dannenberg et al. 2001) including prostate cancer (Edwards et al. 2004, Patel et al. 2008). It has been reported that cPLA2 is present in all prostate cancer cell lines and that eicosanoids contribute to cancer progression (Patel et al. 2008). Gao et al. (2008) demonstrated that CRH and Ucn1 increased mRNA and protein expression of cPLA2 via CRHR1 but not CRHR2 in human placental trophoblasts. The expression of cPLA2 at the protein and mRNA levels is regulated by a number of different substances including interleukin 1β (IL1β; Dolan-O’Keefe et al. 2000), tumor necrosis factor α (TNFα; Wu et al. 1994, Gezginci-Oktayoglu & Bolkent 2012), dexamethasone (Tay et al. 1994), and platelet-derived growth factor (Lin et al. 1992). At present, there is no clear consensus as to the precise effect of cPLA2 in apoptosis, as strong evidence both in favor and against apoptosis has been presented (Huber et al. 2007, Lei et al. 2008). Our previous data of distinct effects of active CRHRs in apoptosis of mouse RM-1 prostate cancer cells (Jin et al. 2011) prompted us to explore whether cPLA2 is involved in CRHR-mediated apoptosis and its precise role in apoptosis in RM-1 cell.

In this study, we propose a significant relationship between cPLA2 and the CRH system in apoptosis of RM-1 cells. cPLA2 expression was increased in CRHR1-mediated apoptosis, in which delayed mRNA decay and upregulated IL1β expression were involved. While cPLA2 expression was suppressed by Ucn2 via CRHR2, we also found a significant promotion of migration of RM-1 cells under CRH or Ucn2 treatment.

Materials and methods

Cells and reagents

Mouse RM-1 prostate cancer cells (Baley et al. 1995) and HEK293 cells were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, People’s Republic of China). RM-1 and HEK293 cells were cultured in RPMI-1640 and DMEM respectively with 10% fetal bovine serum and 1% penicillin–streptomycin. The cells were incubated at 37°C in a humidified environment of 95% air and 5% CO2. Cells from passages 15–30 were used for experiments.

CRH, Ucn2 (mouse), CRHR1 antagonist antalarmin (Anta), CRHR2 antagonist antipsauvagine-30 (Anti-30), thapsigargin (TG), actinomycin D (ActD), and mitomycin C were purchased from Sigma. An effect of the two antagonists alone was not found in our previous studies. cPLA2 antibody was obtained from Santa Cruz Biotechnology. CRHR1 and CRHR2 antibodies were from Bioworld (Jiangsu, China). TNFα, IL1β, and phospho- cPLA2 antibodies were obtained from Cell Signaling Technologies (Boston, MA, USA). Specific antibodies to Bcl-2 and Bax were provided by Abcam (Cambridge, UK). siRNA kit was from GenePharma (Shanghai, China) and the Annexin V–FITC Apoptosis Detection Kit was from Calbiochem (Darmstadt, Germany). Lipofectamine 2000 transfection reagent and RNA isolation kit (TRIzol) were...
from Invitrogen and SYBR Green PCR Supermix ROX was obtained from Bio-Rad. The 24-well transwell chamber was provided by Corning (Tewksbury, MA, USA).

RNA interference, plasmid constructs, and transfection

An siRNA kit was used for gene knockdown. There were three cPLA2-specific sequences: cPLA2-mus-286 (siRNA1), 5'-AGAUCUUAGUUGGAACUTT-3' (sense) and 5'-AA-GUUCACAUAAAGAUCUTT-3' (antisense); cPLA2-mus-379 (siRNA2), 5'-GACCUCUUGAGUUCUUGT-3' (sense) and 5'-CCAUAUGACUAAAGGUCTT-3' (antisense); cPLA2-mus-1196 (siRNA3), 5'-GGUGGAAUUAGUCCAUATT-3' (sense) and 5'-AUUGUCAUUAAUCCACCTT-3' (antisense). There were three TNFα-specific sequences: IL1b-mus-480 (si-IL1b1), 5'-GGGACCCAUGAGCUUAAATT-3' (sense) and 5'-UUUCAGCUCAUAGGGUCCTT-3' (antisense); IL1b-mus-444 (si-IL1b2), 5'-GCUCGAGAUGAACAAACATT-3' (sense) and 5'-UUGUUGUUAUCUGGAGCTT-3' (antisense); IL1b-mus-164 (si-IL1b3), 5'-GCUGCUUCAAACUUUGATT-3' (sense) and 5'-UCAAAGGUUUAGACGAGCTT-3' (antisense). There were three TNFα-specific sequences: TNFα-mus-689 (si-TNFα1), 5'-CCUGGUAUGAACCAUATT-3' (sense) and 5'-UUUAUGGCUCUAUACCGCTT-3' (antisense); TNFα-mus-367 (si-TNFα2), 5'-CUUCUCUCUCAUGUUCUATT-3' (sense) and 5'-UAGAACUGAGAGGGAGCTT-3' (antisense); TNFα-mus-344 (si-TNFα3), 5'-GGG-AUGAAGUGUUCACAAATT-3' (sense) and 5'-UUUGGGAACCUUACUCCCTT-3' (antisense). The full-length cDNAs for human CRHR1x and CRHR2β were provided by the BGI Company (Beijing, China) and inserted into pE-N1 (HindIII-BamHI, Promega) vector in frame. HEK293 cells were maintained at 37 °C in a 5% CO2 atmosphere in DMEM medium supplemented with 10% fetal bovine serum. In general, HEK293 cells were seeded in six-well plates overnight and transfected with CRHR1/CRHR2 using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Briefly, 2 µg plasmid DNA/well was mixed with Superfect reagent, incubated at room temperature for 20 min, and added into the culture medium. After 4 h, HEK293 cells were transferred to fresh DMEM for subsequent experiments.

Immunoblotting

Immunoblotting was carried out as previously described (Jin et al. 2011). Briefly, total cell lysate was collected, and the amount of protein was determined by the Bradford method. The protein lysate was revoked on SDS-polyacrylamide and then electrically transferred to PVDF membranes (Roche). Then the membranes were blocked and incubated with primary anti-Bcl-2 (1:200), anti-Bax (1:1000), anti-CRHR1 (1:1000), anti-CRHR2 (1:1000), anti-cPLA2 (1:1000), anti-phospho-cPLA2 (1:1000), anti-glyceraldehyde phosphate dehydrogenase (GAPDH; 1:5000), anti-TNFα (1:1000), and anti-IL1b (1:1000) antibodies overnight at 4 °C and incubated with secondary HRP-conjugated IgG for 1 h. Finally, the protein bands were visualized using chemiluminescence gel imaging system (Syngene, Cambridge, UK).

Apoptosis assay by Annexin V–FITC/propidium iodide staining

Annexin V–FITC/propidium iodide (PI) double staining was carried out according to a procedure described previously (Jin et al. 2011). Briefly, RM-1 cells were transfected with siRNA against cPLA2 and then treated with CRH/Ucn2 to detect apoptosis. In order to observe a significant antiapoptotic effect, TG (apoptosis inducer) was added to cells of the Ucn2 group to induce apoptosis in advance. After 24 h, the cells were harvested, washed, and resuspended in 0.5 ml PBS. Then cells were incubated for 15 min at room temperature in the dark in 10 µl Media Binding Reagent containing 1.25 µl Annexin V–FITC and 10 µl PI. Afterward, apoptosis was analyzed by fluorescence microscopy (Olympus) and flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Real-time PCR assay

Total RNA was isolated using TRIzol (Invitrogen), 2 µg of RNA was subjected to reverse transcription and the cDNA was amplified by SYBR Green PCR mix. The amplified products were detected using an ABI 7300 Real-Time PCR System (Applied Biosystems) and the changes in cPLA2 gene expression were determined by the comparative CT (ΔΔCT) method. The primers used were as follows: cPLA2,
forward (5'-CTGGCCAACATCAACTTCCAG-3') and reverse (5'-GCCAGTCTCTTCTGATGAT-3'); GAPDH forward (5'-CCATGGAGAAGGCTGGG-3') and reverse (5'-CAAAA-GTTGTCATGGATGACC-3') (Kiabei et al. 2005).

Analysis of mRNA stability by ActD chase
RM-1 cells were incubated with or without CRH/Ucn2 for 2 h followed by addition of ActD (5 μg/ml) and harvested at subsequent time intervals (0, 2, 4, 6, and 8 h). cPLA2 mRNA levels were measured using real-time PCR as described earlier and GAPDH was used as a normalization control (Jin et al. 2012).

Migration and wound-healing assay
The migration assay was carried out in 24-well transwell chamber (Corning) with 8 μm polycarbonate Nuclepore filters. Briefly, RM-1 cells wereseeded at a density of 10⁵ cells into the upper chambers and 5% BSA was added to the lower chambers to induce cell migration. After 48 h, the noninvasive cells in the chambers were wiped away using cotton swab and the migrated cells were fixed with 100% methanol and stained with 0.5% crystal violet. Then the cells were visualized with fluorescence microscope (Olympus) and the numbers of the cell migrating into the lower side were counted in ten random visual fields.

Cells were seeded in six-well plates at 10⁶ cells/well. After 24 h, the cells were treated with 2 μg/ml mitomycin C to inhibit cell proliferation then scraped with a 200 μl pipette tip, washed twice with PBS to remove debris, and treated with or without CRH/Ucn2 and the corresponding antagonists for 24 or 48 h (Ma et al. 2012). The wound was observed under microscope (Olympus) and analyzed with Image J 2.0 Software (Bethesda, MD, USA).

Statistical analysis
The results are expressed as means ± S.E.M. Data were analyzed using GraphPad Prim 5.0 Software (La Jolla, CA, USA) by One-way ANOVA with Tukey's multiple comparison tests. \( P < 0.05 \) was considered to represent statistical significance. Each experiment was repeated at least three times.

Results
Knockdown of cPLA2 attenuated CRH-induced apoptosis but aggravated Ucn2-inhibited apoptosis in RM-1 cells. To determine whether cPLA2 expression is involved in CRHR-mediated apoptosis, we examined the apoptosis of RM-1 cells stimulated by CRH or Ucn2 using Annexin V–FITC/PI double staining, after cPLA2 mRNA was silenced with siRNA. First of all, we transfected RM-1 cells with three different siRNA sequences against cPLA2 to identify the most efficient sequence. Real-time PCR (Fig. 1a, left) and western blot (Fig. 1a, middle and right) results showed that cPLA2-mus-1196 (siRNA3) reduced cPLA2 expression at the level of ~50% in RNA and ~70% in protein compared with control cells transfected with negative siRNA (Vector). RM-1 cells were treated with siRNA3 for 48 h and CRH (10⁻⁷ M) and Ucn2 (10⁻⁷ M) and TG (0.5 μM) for another 24 h. Then the apoptotic RM-1 cells were stained and detected. Figure 1b shows that CRH activated CRHR1 to induce apoptosis, which was attenuated by siRNA3 transfection (Fig. 1b, upper left) and Ucn2 activated CRHR2 to inhibit TG-induced apoptosis, which was strengthened by siRNA3 transfection (Fig. 1b, upper right). Flow cytometry assay (Fig. 1b, lower) showed that siRNA3 transfection attenuated CRH-induced apoptosis (5.305 vs 9.7625%) and strengthened inhibition by Ucn2 of apoptosis induced by TG (4.5925 vs 8.18%), indicating that cPLA2 was an apoptotic inducer in CRH/Ucn2 treatment. The presence of several cells showing apple-green fluorescence indicated that CRH family peptides affected apoptosis at an early time, consistent with our previous research (Jin et al. 2011).

Our previous study demonstrated that CRHR mediated apoptosis through Bcl-2/Bax regulations (Jin et al. 2011). To further confirm the proapoptotic role of cPLA2 in CRHR-mediated apoptosis, the expressions of Bcl-2 and Bax were detected by western blotting after transfection and CRH/Ucn2 treatment. Figure 1c shows that RM-1 cells treated with CRH and siRNA3 transfection had higher expression of Bcl-2 but lower expression of Bax compared with cells treated with CRH alone. In contrast, siRNA3 transfection aggravated Ucn2-induced Bcl-2 upregulation and Bax downregulation. These results further confirmed that cPLA2 played an important role in CRHR-mediated apoptosis.

CRH increased but Ucn2 decreased cPLA2 expression
In order to explore whether CRHR activation is involved in cPLA2 regulation, the expression of cPLA2 at both the mRNA and protein levels was detected using real-time PCR and immunoblotting. RM-1 cells were treated with CRH/Ucn2 at different concentrations ranging from 10⁻⁷ to 10⁻¹¹ M or for different time periods. As shown in Fig. 2, CRH increased cPLA2 mRNA (Fig. 2a) and protein...
CRH receptors mediate apoptosis and migration

(a) 

(b) 

(c) 

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DOI: 10.1530/JME-13-0270
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(Fig. 2b) expression in a concentration-dependent manner, with the most significant effect observed at $10^{-7}$ M, consistent with the optimal concentration of Ucn2. Unlike CRH, Ucn2 decreased cPLA$_2$ expression (Fig. 2a and b). Figure 2 shows that CRH time-dependently increased cPLA$_2$ expression, peaking at 12 h for mRNA and 24 h for protein. Ucn2 treatment resulted in significant reduction in cPLA$_2$ expression at 12 h for mRNA and 24 h for protein. Finally, we treated RM-1 cells with CRH/Ucn2 alone or along with the corresponding antagonists, Anta/Anti-30, for 24 h to provide further evidence of the effect of CRHR on cPLA$_2$ expression (Fig. 2c). There was no effect of Anta/Anti-30 alone on apoptosis (data not shown).

**CRH phosphorylated cPLA$_2$ via CRHR1**

cPLA$_2$ was activated by CRH in 1 h. As shown in Fig. 2d, CRH stimulated time-dependent cPLA$_2$ phosphorylation at intervals ranging between 10 and 30 min, and the change of total protein amount of cPLA$_2$ was undetectable at 1 h. Then the cells were treated with CRH alone or along with Anta for 30 min (Fig. 2e). CRH-induced cPLA$_2$ activation was inhibited by Anta. These results indicate that CRH not only increased the expression of cPLA$_2$ but also enhanced the activity of it.

**CRH enhanced but Ucn2 reduced cPLA$_2$ protein expression via CRHR1 and CRHR2 in HEK293 cells**

We selected HEK293 cells for CRHR1/CRHR2 transfection to further validate the role of each receptor for cPLA$_2$ expression because of the opposing effects of CRHR1 and CRHR2. To ensure that both CRHR1 and CRHR2 constructs (CRHR1-N1 and CRHR2-N1) were effectively transfected into HEK293 cells, their expressions were checked by western blotting. Figure 3a shows the bands of CRHR1 and CRHR2 overexpression respectively in HEK293 cells, illustrating the success of CRHR1 and CRHR2 transfections. After transfection, the HEK293 cells were treated with CRH/Ucn2 for 12 or 24 h and then harvested and detected by real-time PCR or western blotting. As shown in Fig. 3b, the expression of cPLA$_2$ in cells treated with CRHR1-N1 and CRH underwent an 8.8-fold increase for RNA and a twofold increase for protein compared with cells treated with empty vector. The cells treated with CRHR2-N1 and Ucn2 showed around a 70% reduction in both RNA and protein expression of cPLA$_2$ compared with cells treated with empty vector.

**CRH increased cPLA$_2$ expression through IL1β upregulation**

It has been reported that various proinflammatory mediators including TNFα and IL1β are natural stimulators of cPLA$_2$ gene expression (Dolan-O’Keefe et al. 2000). CRH family peptides are closely associated with inflammation (Webster et al. 1998). In order to investigate whether CRH family peptides adjust cPLA$_2$ expression through inflammation, the expression of TNFα and IL1β in RM-1 cells treated with CRH/Ucn2 for different times was determined using western blotting. In addition, cPLA$_2$ expressions were examined after IL1β or TNFα interference in CRH/Ucn2-treated cells. Figure 4b (left) shows the results for interference efficiency, and the most efficient interfering RNA sequences were chosen for the subsequent experiments. Figure 4a showed that CRH treatment resulted in a prominent increase in IL1β at the time points from 8 h onwards. Furthermore, transfection against IL1β but not TNFα inhibited CRH-induced cPLA$_2$ upregulation (Fig. 4b, upper right), indicating that IL1β may participate in CRH-induced cPLA$_2$ expression. Interestingly, both TNFα and IL1β expression dramatically increased under Ucn2 treatment, and silencing of IL1β or TNFα could not block the effect of Ucn2 treatment (Fig. 4b, lower right), which is inconsistent with our speculation. The mechanism of action of Ucn2 in cPLA$_2$ regulation needs more studies.

**Figure 1**

Knockdown of cPLA$_2$ inhibited apoptosis with CRH or Ucn2 treatment. (a) Detection of transfection efficiency on three sequences of siRNA against cPLA$_2$ by real-time PCR (left) and western blotting (middle and right). Vector; negative control; siRNA1, cPLA$_2$-mus-1186; siRNA2, cPLA$_2$-mus-379; siRNA3, cPLA$_2$-mus-296. (b) Annexin V–FITC/PI staining of apoptotic cells by fluorescence microscopy at ×100 magnification (upper) and flow cytometry (lower). Early apoptotic cells showed apple green fluorescence, while necrotic and late apoptotic cells had yellow–red cytoplasm and red nuclear staining.

(c) Western blotting analysis showed that cPLA$_2$ inhibition increased the Bcl-2:Bax ratio. si-cPLA$_2$-cPLA$_2$-mus-296. Western blotting results were quantified and counted at the right sides. Experiments were performed three times and results of a representative experiment are shown. Data were expressed as mean±S.E.M. of three independent experiments. *$P<0.05$ vs control; **$P<0.01$ vs TG; ***$P<0.05$ vs CRH; ****$P<0.01$ vs CRH; $$$P<0.05$ vs Ucn2; and $\&\&\&\&P<0.01$ vs Ucn2+TG.
CRH upregulated and activated cPLA₂ but Ucn2 decreased cPLA₂ expressions. Concentration- and time-dependent increases in cPLA₂ mRNA (a, left two panels) and protein (b, top row) expression induced by CRH. cPLA₂ mRNA (a, right two panels) and protein (b, bottom row) were decreased by Ucn2 in a concentration- and time-dependent manner. (c) Cells were stimulated with 10⁻⁶ M Anta or Anti-30 followed by incubation with 10⁻⁷ M CRH or Ucn2 for 30 min. After 24 h, expression of cPLA₂ was evaluated by western blotting. (d) Time-dependent cPLA₂ phosphorylation of cells exposed to CRH assessed by western blotting. (e) CRH antagonist, Anta, inhibited CRH-induced phosphorylation of cPLA₂. Experiments were performed five times and results of a representative experiment are shown. Data were expressed as mean ± S.E.M. of five independent experiments.

*P < 0.05 vs control/0 min; **P < 0.01 vs control/0 min; ***P < 0.001 vs 0 min; *P < 0.05 vs CRH; **P < 0.01 vs CRH; and $P < 0.05 vs Ucn2. Anta, antalarmin; Anti-30, antisauvagine-30.
CRH activated CRHR1 to increase mRNA stability of cPLA2

Figure 2a illustrates a time-dependent induction of mRNA levels in RM-1 cells in response to CRH treatment. To determine whether the posttranscriptional regulation mechanism is involved in cPLA2 gene expression, RM-1 cells were treated with ActD alone or along with CRH/Ucn2. As shown in Fig. 4c, the mRNA stability of cPLA2 was increased when cells were treated with CRH, and there was no obvious difference between control and Ucn2-treated cells, which indicated that active CRHR1 played an important role in cPLA2 posttranscriptional regulation.

CRH and Ucn2 induced migration of RM-1 cells via CRHR1 and CRHR2

To further investigate the other roles of locally expressed CRHRs in RM-1 prostate cancer cells, we detected the effects of CRH/Ucn2 on migration using transwell chamber and wound-healing assays. The transwell chamber assay showed that CRH and Ucn2 treatments increased the number of migrated cells by 55.2 and 58.1% respectively. These effects were abolished by Anta and Anti-30 (Fig. 5a). CRH and Ucn2 treatments led to more rapid wound closure by RM-1 cells, which were blocked by corresponding antagonists (Fig. 5b). These results indicate that CRH family peptides could induce migration of prostate cancer cells in vitro.

Discussion

Previously, we reported that CRH induced- but Ucn2 inhibited apoptosis of murine RM-1 prostate cancer cells via CRHR1 and CRHR2 respectively (Jin et al. 2011). Here we found that cPLA2 played a proapoptotic role in CRHR-mediated apoptosis. This conclusion was based on the following: i) cPLA2 is an apoptosis inducer as knockdown of cPLA2 attenuated CRH-induced apoptosis and increased the Bcl-2:Bax ratio. Silencing of cPLA2 aggravated Ucn2-inhibited apoptosis and increased the Bcl-2:Bax ratio. ii) CRH binding to CRHR1 increased- but Ucn2 binding to CRHR2 decreased cPLA2 gene and protein expression. In addition, CRH reduced the degradation rate of cPLA2 mRNA. iii) CRH upregulated the expression of IL1β, a transcription factor of cPLA2, while the downregulatory effect of Ucn2 on cPLA2 might be mediated through other unknown pathways. Finally, we also evaluated the effect of CRH and Ucn2 on migration of RM-1 cells and found both CRH and Ucn2 to be migration inducers.

cPLA2 is a rate-limiting key enzyme of AA release from the sn-2 position of membrane phospholipids (Li et al. 2011a). Accumulation of AA is strikingly associated with apoptosis, accompanied by loss of cell viability, caspase activation, and DNA fragmentation (Taketo & Sonoshita 2002). cPLA2 has been reported to be involved in NO- and TNF-induced apoptosis (Wissing et al. 1997, Pilane & LaBelle 2002). Recent data reported that cPLA2 induced
Figure 4

CRH increased IL1β protein expression and cPLA2 mRNA stability, but Ucn2 increased both TNFα and IL1β protein expression. (a) Western blotting analysis of TNFα and IL1β under CRH (upper) or Ucn2 (lower) treatment for times ranging from 0 to 48 h. (b) The cells were treated with siRNA against IL1β or TNFα for 24 h and CRH/Ucn2 for another 24 h. Interference efficiency (left) and cPLA2 expression (right) by transfection and CRH/Ucn2 treatments were examined by western blot. (c) Degradation curve of cPLA2 mRNA with CRH or Ucn2. Actinomycin D was used to suppress mRNA production. Experiments were performed five times and results for a representative experiment are shown. Data were expressed as mean ± S.E.M. of five independent experiments. *P<0.05 vs 0 h/control (Vector) and **P<0.01 vs 0 h.
endoplasmic reticulum stress by the complement membrane attack complex (Ren et al. 2010). However, there is no clear consensus as to the precise effect of cPLA2 in apoptosis, which may be attributed to certain instances where particular stimuli and/or cell types are involved (Balsinde et al. 2006). The results of the current study demonstrated that in prostate cancer cells, silencing cPLA2 inhibited CRH-induced apoptosis but aggravated Ucn2-inhibited apoptosis, indicating that cPLA2 is an apoptosis promotor involved in the apoptosis induced by CRH family peptides.

Ca\(^{2+}\)-independent PLA2 (iPLA2), another member of the PLA2 group, is proposed to participate in signal transduction, cell proliferation, inflammation, and apoptosis (Ali et al. 2013). It has been reported that urocortin inhibited elevation of the iPLA2 expression caused by ischemia in the myocytes (Takatani-Nakase & Takahashi 2010). Inhibition of iPLA2 expression by urocortin provides critical insights into the effect of CRH family peptides on expressions of PLA2 groups. Gao et al. (2008) reported that both CRH and Ucn1 increased mRNA and protein expression of cPLA2 via CRHR1 in human placental trophoblasts, which is in part consistent with our present data on prostate cancer cells. However, the role of CRHR2 in cPLA2 inhibition was also detected in our experiment. cPLA2 expression has been shown to be responsive to a number of stimuli including cytokines (Hulkower et al. 1994), growth factors (Nakamura et al. 1992), ATP (Qiu et al. 1998), and glucocorticoid (Dolan-O’Keefe & Nick 1999). A transcriptional component has been attributed to the TNF\(\alpha\)- and IL1\(\beta\)-dependent induction of cPLA2 and the glucocorticoid-dependent repression of this gene (Dolan-O’Keefe et al. 2000). In our study, the immunoblotting results revealed that CRH upregulated IL1\(\beta\) expression ahead of the time of cPLA2 increase. Furthermore, knockdown of IL1\(\beta\), but not TNF\(\alpha\), blocked CRH-increased cPLA2 expression. These results led us to postulate that CRH increases cPLA2 expression through IL1\(\beta\) upregulation. However, the observation that Ucn2 increased TNF\(\alpha\) and IL1\(\beta\) expression could not explain the Ucn2-induced cPLA2 decrease. There are two glucocorticoid-responsive elements (GREs) that have been shown to be involved in

Figure 5

CRH and Ucn2 induced migration of RM-1 cells via CRHR1 and CRHR2.
(a) Transwell chamber assay of stimulatory effect of CRH and Ucn2 on cell migration. Cells were seeded in transwell chambers and then stimulated with CRH/Ucn2 (10\(^{-7}\) M) alone or along with Anta/Anti-30 (10\(^{-6}\) M) for 48 h and the number of migrated cells, which were observed using a microscope at \(\times\)200 magnification, was determined. (b) Cells were subjected to a wound-healing assay in the absence or presence of CRH/Ucn2 alone or along with Anta/Anti-30. Cultures were photographed at \(\times\)100 magnification, right after the scratch and 24 and 48 h later. Wound healing was measured using Image J Software for percentage of wound closure (right). Experiments were performed three times and results of a representative experiment are shown. Data were expressed as mean \(\pm\) S.E.M. of three independent experiments. *P<0.05 vs control; **P<0.05 vs CRH; ***P<0.01 vs CRH; and $P<0.05 vs Ucn2. Anta, antalarmin; Anti-30, antisauvagine-30.
the cPLA₂ inhibition by glucocorticoids and there may be multiple putative GREs in the cPLA₂ promoter (Guo et al. 2010). Although glucocorticoids are regulated by CRH family peptides through the hypothalamic–pituitary axis in vivo, CRH or Ucn2 do not influence glucocorticoids in single cells. Hence, the GRE pathway for regulation of cPLA₂ by Ucn2 through glucocorticoids is ruled out. We speculate that there may be some ‘Ucn2 responsive element’ involved in the Ucn2-induced cPLA₂ decrease. In addition, we found that CRH activated cPLA₂ at Ser505 via CRHR1, indicating that CRH also increases the activity of cPLA₂. CRHRs, G protein-coupled receptors, are generally initiators of protein kinase A (PKA), PKC, MAPKs, and so on (Masliah et al. 1996). It has been reported that CRH activated PKA or PKC through CRHR1 in the central system (Van Kolen et al. 2010, Ji et al. 2013). Taken together, it is reasonable to believe that active CRHRs are crucial to cPLA₂ phosphorylation.

Our present and previous studies showed that cPLA₂ inhibition increased the Bcl-2:Bax ratio under CRH or Ucn2 treatment, indicating that cPLA₂ might be involved in regulations of Bcl-2 and Bax. As we know, cAMP response element-binding (CREB) protein and tumor suppressor protein p53 are positive transcription factors for Bcl-2 and Bax respectively (Park et al. 2013, Song et al. 2013). cPLA₂ has been demonstrated to act upstream of p53 and CREB (Ilic et al. 1998, Ho et al. 2009, Hadad et al. 2011). In addition, our previous work showed that the CRH system was associated with active CREB (Jin et al. 2011). Thus, it is very likely that CRH or Ucn2 affects the ratio of Bcl-2:Bax through the cPLA₂-CREB/p53 pathway.

Over the past few decades, there has been an argument about the precise role of CRH family peptides in migration. Some evidence both in favor (Arranz et al. 2009, Im et al. 2010, Jo et al. 2011) and against (Im et al. 2010, Rassouli et al. 2011) the involvement of CRH family peptides in migration has been presented. Our previous studies showed that CRH inhibited migration of breast cancer cells mainly via CRHR1. However, in this study, we demonstrated that both CRH and Ucn2 enhanced migration of murine prostate cancer cells, indicating that CRH family peptides may play different roles in migration depending on particular stimuli and/or cell types. cPLA₂ and corresponding downstream products such as AA are reported to promote cell motility (Li et al. 2011b, Lin et al. 2012). However, whether cPLA₂ is involved in CRH/Ucn2-mediated migration remains unresolved. The role of cPLA₂ in CRH family peptides-mediated migration is complicated and needs more investigation.

In conclusion, results of the present study demonstrated that CRH, by binding to CRHR1, induced the expression and activation of cPLA₂, resulting in an increased ratio of Bcl-2:Bax, which in turn led to apoptosis. Ucn2 inhibited apoptosis through downregulation of cPLA₂ and, which resulted in an increased ratio of Bcl-2: Bax via CRHR2. In addition, we demonstrated that CRH family peptides enhanced migration of RM-1 cells. To our knowledge, our results are the first to show that cPLA₂ is involved in CRHR-mediated apoptosis and provide more evidence about apoptosis and migration that will improve understanding of biological and pathological effects of CRH family peptides on cancer.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported by the Natural Science Foundation of China (grant numbers 81072668 and 91129711) and Jiangsu provincial funds (10KJA350031), also funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, Science and Technology Development Fund of Nanjing Medical University (KY1010831121612221), and Supporting research and innovation funding (X10131801145).

References


Received in final form 14 February 2014
Accepted 18 February 2014