Corticotropin-releasing hormone receptors mediate apoptosis via cytosolic calcium-dependent phospholipase A₂ 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 A₂ (cPLA₂) bridging CRHR activations and Bcl-2:Bax ratio and the effect of CRHR activation on cell migration. Silencing of cPLA₂ attenuated a CRHR1 agonist, CRH-induced apoptosis, and the decrease of the Bcl-2:Bax ratio, whereas silencing of cPLA₂ 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 cPLA₂ expression mainly through interleukin 1 β (IL1 β) upregulation. Ucn2 decreased cPLA₂ expression through neither tumor necrosis factor α nor IL1 β . CRH-suppressed decay of cPLA₂ mRNA and Ucn2 merely suppressed its production. Overexpression of CRHR1 or CRHR2 in HEK293 cells correspondingly upregulated or down-regulated cPLA₂ 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 cPLA₂ participates in CRHR1-induced apoptosis and CRHR2-inhibited apoptosis.

Key Words

- CRHR1
- CRHR2
- cPLA₂
- apoptosis
- migration

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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 1 β (IL1 β ; Dolan-O'Keefe *et al.* 2000), tumor necrosis factor α (TNF α ; 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 IL1 β 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 antisauvagine-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). TNF α , IL1 β , 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'-AGAUCUUUAUGUGGAACUUTT-3' (sense) and 5'-AA-GUCCACAUAAGGAUCUTT-3' (antisense); cPLA₂-mus-379 (siRNA2), 5'-GACCUUUGAGUUCAUUUUGTT-3' (sense) and 5'-CAAAAUGAACUCAAGGUUCTT-3' (antisense); cPLA₂-mus-1196 (siRNA3), 5'-GGUGGAAUUUA-GUCCAUAUTT-3' (sense) and 5'-AUAUGGACUAAAU-CCACCTT-3' (antisense). There were three IL1 β -specific sequences: IL1 β -mus-480 (si-IL1 β 1), 5'-GGACCCAU AUG-AGCUGAAATT-3' (sense) and 5'-UUUCAGCUCAU AU-GGGUCCTT-3' (antisense); IL1 β -mus-444 (si-IL1 β 2), 5'-GCUCCGAGAUGAACAACAATT-3' (sense) and 5'-UU-GUUGUUAUCUCGGAGCTT-3' (antisense); and IL1 β -mus-164 (si-IL1 β 3), 5'-GCUGCUUCCAAACCUUUGATT-3' (sense) and 5'-UCAAGGUUUGGAAGCAGCTT-3' (antisense). There were three TNF α -specific sequences: TNF α -mus-689 (si-TNF α 1), 5'-CCUGGUAUGAGCCCAUAUATT-3' (sense) and 5'-UAUAUGGGCUCAUACCAGGTT-3' (antisense); TNF α -mus-367 (si-TNF α 2), 5'-CUCCCUCUCAUCA-GUUCUATT-3' (sense) and 5'-UAGAACUGAUGAGAGG-GAGTT-3' (antisense); TNF α -mus-344 (si-TNF α 3), 5'-GGG-AUGAGAAGUCCCAAATT-3' (sense) and 5'-UUUGG-GAACUUCUCAUCCCTT-3' (antisense). The sequences for the negative control (Vector) were sense: 5'-UUCUCC-GAACGUGUCACGUTT-3' and antisense: 5'-ACGUGA-CACGUUCGGAGAATT-3' (GenePharma). Vector siRNA and siRNA against cPLA₂/IL1 β /TNF α were added to each dish at a final concentration of 100 nM using lipofectamine 2000 transfection reagent. Real-time PCR and protein immunoblot assays at 24 and 48 h posttransfections confirmed the transfection efficiency and one of the sequences against cPLA₂/IL1 β /TNF α was chosen for the experiment.

The full-length cDNAs for human CRHR1 α and CRHR2 β 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 μ g 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 revolved 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-TNF α (1:1000), and anti-IL1 β (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 μ l Media Binding Reagent containing 1.25 μ l Annexin V-FITC and 10 μ l 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 μ g 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 ($\Delta\Delta$ CT) method. The primers used were as follows: cPLA₂,

forward (5'-CTGGCCAACATCAACTTCAG-3') and reverse (5'-GCCAGTCTCTTCTGCATAGT-3'); GAPDH, forward (5'-CCATGGAGAAGGCTGGG-3') and reverse (5'-CAAAA-GTTGTCATGGATGACC-3') (Kiaei *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 were seeded 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) or 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

(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 cPLA₂ expression (Fig. 2a and b). Figure 2 shows that CRH time-dependently increased cPLA₂ expression, peaking at 12 h for mRNA and 24 h for protein. Ucn2 treatment resulted in significant reduction in cPLA₂ 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 cPLA₂ expression (Fig. 2c). There was no effect of Anta/Anti-30 alone on apoptosis (data not shown).

CRH phosphorylated cPLA₂ via CRHR1

cPLA₂ was activated by CRH in 1 h. As shown in Fig. 2d, CRH stimulated time-dependent cPLA₂ phosphorylation at intervals ranging between 10 and 30 min, and the change of total protein amount of cPLA₂ was undetectable at 1 h. Then the cells were treated with CRH alone or along with Anta for 30 min (Fig. 2e). CRH-induced cPLA₂ activation was inhibited by Anta. These results indicate that CRH not only increased the expression of cPLA₂ but also enhanced the activity of it.

CRH enhanced but Ucn2 reduced cPLA₂ 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 cPLA₂ 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 cPLA₂ 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 cPLA₂ compared with cells treated with empty vector.

CRH increased cPLA₂ expression through IL1 β upregulation

It has been reported that various proinflammatory mediators including TNF α and IL1 β are natural stimulators of cPLA₂ 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 cPLA₂ expression through inflammation, the expression of TNF α and IL1 β in RM-1 cells treated with CRH/Ucn2 for different times was determined using western blotting. In addition, cPLA₂ expressions were examined after IL1 β or TNF α 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 IL1 β at the time points from 8 h onwards. Furthermore, transfection against IL1 β but not TNF α inhibited CRH-induced cPLA₂ upregulation (Fig. 4b, upper right), indicating that IL1 β may participate in CRH-induced cPLA₂ expression. Interestingly, both TNF α and IL1 β expression dramatically increased under Ucn2 treatment, and silencing of IL1 β or TNF α 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 cPLA₂ regulation needs more studies.

Figure 1

Knockdown of cPLA₂ inhibited apoptosis with CRH or Ucn2 treatment. (a) Detection of transfection efficiency on three sequences of siRNA against cPLA₂ by real-time PCR (left) and western blotting (middle and right). Vector, negative control; siRNA1, cPLA₂-mus-1186; siRNA2, cPLA₂-mus-379; siRNA3, cPLA₂-mus-296. (b) Annexin V-FITC/PI staining of apoptotic cells by fluorescence microscopy at $\times 100$ 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 cPLA₂ inhibition increased the Bcl-2:Bax ratio. si-cPLA₂, cPLA₂-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 $\pm$ 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.

Figure 2

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 $\pm$ 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.

Figure 3

cPLA₂ expression was induced by CRH but suppressed by Ucn2 in HEK293 cells displaying CRHR1 or CRHR2 overexpression. (a) Detection of transfection efficiency by western blotting assay. (b) Effects of CRHR1 or CRHR2 on RNA (left) and protein (middle and right) expression of cPLA₂. 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 < 0.01 vs control; *** P < 0.001 vs control; & P < 0.05 vs Vector; && P < 0.01 vs Vector; * P < 0.05 vs CRH; and & P < 0.05 vs Ucn2. R1, CRHR1-N1; R2, CRHR2-N1; and Vector, empty vector.

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 IL1 β , 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 IL1 β protein expression and cPLA₂ mRNA stability, but Ucn2 increased both TNF α and IL1 β protein expression. (a) Western blotting analysis of TNF α and IL1 β under CRH (upper) or Ucn2 (lower) treatment for times ranging from 0 to 48 h. (b) The cells were treated with siRNA against IL1 β or TNF α 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 $\pm$ s.e.m. of five independent experiments. * P < 0.05 vs 0 h/control (Vector) and ** P < 0.01 vs 0 h.

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.

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.

Ca²⁺-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 α - and IL1 β -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 β expression ahead of the time of cPLA₂ increase. Furthermore, knockdown of IL1 β , but not TNF α , blocked CRH-increased cPLA₂ expression. These results led us to postulate that CRH increases cPLA₂ expression through IL1 β upregulation. However, the observation that Ucn2 increased TNF α and IL1 β expression could not explain the Ucn2-induced cPLA₂ inhibition. We silenced TNF α or IL1 β using siRNA and found no changes in cPLA₂ expression after Ucn2 treatments, indicating that Ucn2-induced TNF α and IL1 β were not involved in the cPLA₂ decrease. There are two glucocorticoid-responsive elements (GRES) that have been shown to be involved in

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 (Masliyah *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.* 2010, 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.

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