Urocortin increased endothelial ICAM1 by cPLA2-dependent NF- κ B and PKA pathways in HUVECs

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Abstract

Urocortin (Ucn1), a member of the corticotrophin-releasing hormone (CRH) family, has been reported to participate in inflammation. The increased expression of intercellular adhesion molecule 1 (ICAM1) plays important roles in inflammation and immune responses. Our previous results demonstrated that Ucn1 significantly enhanced the expression of ICAM1. However, the underlying mechanisms are still unknown. The purpose of this study is to investigate the detailed mechanisms of Ucn1-induced upregulation of ICAM1. Here, we characterized the mechanisms of Ucn1 usage to regulate ICAM1 expression in human umbilical vein endothelial cells (HUVECs). Our data revealed that Ucn1 increased ICAM1 and cyclooxygenase 2 (COX2) expressions in a time-dependent manner via CRH receptor 2 (CRHR2). In addition, COX2 was involved in ICAM1 upregulation. Furthermore, Ucn1 could increase the expression and phosphorylation of cytosolic phospholipases A2 (cPLA2) in a time-dependent manner via CRHR2 and CRHR1. Moreover, ablation of cPLA2 by the inhibitor pyrrophenone or siRNA attenuated the ICAM1 increase induced by Ucn1. In addition, nuclear factor κ B (NF- κ B) was activated, indicated by the increase in nuclear p65NF- κ B expression and phosphorylation of p65NF- κ B, depending on cPLA2 and CRHR2 activation. Pyrrolidine-dithiocarbamic acid, an inhibitor of NF- κ B, abolished the elevation of ICAM1 but not COX2. Also, Ucn1 increased the production of prostaglandin E₂ (PGE₂) which further activated protein kinase A (PKA)–CREB pathways dependent of cPLA2 via CRHR2. Moreover, the increase in NF- κ B phosphorylation was not affected by the selective COX2 inhibitor NS-398 or the PKA inhibitor H89. In conclusion, these data indicate that Ucn1 increase the ICAM1 expression via cPLA2–NF- κ B and cPLA2–COX2–PGE₂–PKA–CREB pathways by means of CRHR2.

Key Words

- urocortin
- ICAM1
- cPLA2
- CREB
- NF- κ B
- COX2

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Introduction

Cardiovascular diseases are the leading fatal diseases. Increased evidence shows that vascular inflammation plays a key role in the initiation and progression of many diseases, including atherosclerosis (Ricciotti & FitzGerald 2011), ischemia/reperfusion damage (Stallion

et al. 2005), and hypertension (Schiffrin & Touyz 2004, Savoia & Schiffrin 2006). Expression of adhesion molecules by endothelial cells and adhesion of leukocytes to the vessel wall are essential steps in vascular inflammation (Pasceri *et al.* 2000). The intercellular

adhesion molecule 1 (ICAM1) is an Ig-like cell adhesion molecule expressed in both leukocytes and endothelial cells (van de Stolpe & van der Saag 1996). Reportedly, inflammatory mediators, such as IL1 β , TNF- α , and lipopolysaccharide (LPS) induced the expression of ICAM1 in the endothelial cells (Rothlein *et al.* 1988, Haraldsen *et al.* 1996). Moreover, anti-ICAM1 MAB inhibited neutrophil migration during inflammation (Barton *et al.* 1989). Cosimi *et al.* (1990) found that anti-ICAM1 antibody inhibited T cell-mediated injury *in vivo*, and that ICAM1 was a critical molecule in the pathogenesis of allograft rejection. Thus, the regulated expression of ICAM1 plays a significant role in inflammatory process and immune responses.

Urocortin (Ucn1), a member of corticotrophin-releasing hormone (CRH) peptide family (Vaughan *et al.* 1995), acts through two G-protein-coupled transmembrane receptors, CRH receptor 1 and 2 (CRHR1 and CRHR2; Chen *et al.* 1993). Centrally produced Ucn1 exerts immunosuppressive effects by regulating the hypothalamic–pituitary–adrenal axis (Chrousos 1995). In contrast, peripheral Ucn1 has direct effects on inflammatory processes, acting as an autocrine or paracrine proinflammatory cytokine (Karalis *et al.* 1991). Recently, Ucn1 has been reported to participate in vascular inflammation. Our previous work showed that Ucn1 could promote the development of rat vasculitis induced by sodium laurate (Xu *et al.* 2009) and that the sex difference in Ucn1 production was contributory to the disparity between the sexes in this vasculitis model (Wan *et al.* 2013b). It has been demonstrated that local secretion of Ucn1 may directly promote microvascular permeability during LPS-induced inflammation (Cureton *et al.* 2009), which was consistent with our data (Wan *et al.* 2013a). Most importantly, our previous work indicated that Ucn1 regulated endothelial ICAM1 expression during vascular inflammation *in vivo* and *in vitro* (Xu *et al.* 2009, Zhang *et al.* 2009). However, little is known about the mechanisms of Ucn1 action on ICAM1 expression.

Phospholipase A2 (PLA2) enzymes are a family of proteins which are defined by their ability to specifically catalyze the hydrolysis of sn-2 ester bonds of glycerol-phospholipids (Dennis 1994, 1997). PLA2 can be divided into four classes on the basis of their nucleotide and amino acid sequence, i) cytosolic PLA2 (cPLA2 or Group IV PLA2), ii) Ca²⁺-independent intracellular PLA2 (iPLA2 or Group VI PLA2), iii) Ca²⁺-dependent secretory PLA2 (sPLA2 or Group II PLA2), and iv) platelet-activating factor acetylhydrolase (Niknami *et al.* 2009). Considerable attention has been focused on the function of cPLA2,

which exhibits a specific preference for arachidonic acid (AA; Clark *et al.* 1990). Subsequently, AA could be oxidized to prostaglandins, such as prostaglandin E₂ (PGE₂) by the cyclooxygenase enzymes cyclooxygenase 1 (COX1) and COX2 (Alberghina 2010). Zhao *et al.* (2008) found that aspirin, a nonselective COX2 inhibitor, significantly suppressed COX2 and ICAM1 expression. Moreover, our previously work showed that NS-398, the selective COX2 inhibitor, could reduce sICAM1 expression. Notably, cPLA2-dependent COX2–PGE₂ pathways have been found to play a central role in ICAM1 overexpression during inflammation both *in vivo* and *in vitro* (Hadam *et al.* 2011).

In this study, we found that Ucn1 increased ICAM1 expression. Both expression and activity of cPLA2 were increased after stimulation of Ucn1. Moreover, cPLA2-dependent nuclear factor κ B (NF- κ B) and COX2–PGE₂–protein kinase A (PKA)–CREB pathways were found to be involved in the regulation of ICAM1 expression induced by Ucn1.

Materials and methods

Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were obtained from the Institute of Biochemistry (Shanghai, China) and grown in RPMI 1640 (Gibco), supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, at 37 °C in a 95%/5% CO₂ incubator.

Ucn1 (10^{−9} M), selective CRHR1 blocker antalarmin (10^{−8} M), selective CRHR2 blocker antisauvagine-30 (10^{−8} M), nonselective COX2 inhibitor indomethacin (1 μ M), selective COX2 inhibitor NS-398 (1 μ M), PKA inhibitor H89 (20 μ M), NF- κ B inhibitor pyrrolidine-dithiocarbamic acid (PDTTC, 100 μ M), iPLA2 inhibitor bromoenollactone (BEL, 1 nM), sPLA2 inhibitor dithiothreitol (DTT, 1 mM), and PGE₂ (1 μ M) were obtained from Sigma–Aldrich. Pyrrophenone (PYR, 1 μ M) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Dibutyryl cAMP (dbcAMP, 1 μ M), a PKA activator was purchased from Santa Cruz Biotechnology. ICAM1 and COX2 antibodies were purchased from Bioworld Technology (Louis Park, MN, USA). Specific antibodies to cPLA2, p-cPLA2 (Ser505), CREB, and p-CREB (Ser133) were provided by Cell Signaling Technologies (Danvers, MA, USA). Antibodies to p65 NF- κ B and p-p65 NF- κ B (Ser536) were obtained from Abcam (Cambridge, UK). The Cytoplasmic and Nuclear Protein Extraction Kit was from Fermentas (Burlington, ON, Canada).

Isolation of nuclear and cytoplasmic proteins

Nuclear and cytoplasm proteins of HUVECs were isolated using the Cytoplasmic and Nuclear Protein Extraction Kit according to the manufacture's protocol.

RNA silencing

Specific siRNAs and scrambled siRNA as control were obtained from GenePharma (Shanghai, China) and resuspended in nuclease-free water to yield a stock concentration of 20 μ M as described previously (Wan *et al.* 2013a). The specific siRNA sequences were as follows: 5'-CCCAGACCUACGAUUUAGUTT-3' (cPLA21 sense), 5'-ACUAAAUCGUAGGUCUGGGTT-3' (cPLA21 antisense); 5'-GGCCAGAGGAGAUUAAUGATT-3' (cPLA22 sense), 5'-UCAUUAUUCUCCUCUGGCCTT-3' (cPLA22 antisense). The scrambled siRNA sequences were as follows: 5'-UUCUCCGAACGUGUCACGUTT-3' (sense), 5'-ACGU-GACACGUUCGGAGAATT-3' (antisense). HUVECs were transfected with 10 ng siRNA using lipofectamine 2000 (Invitrogen) and the efficiency of knockdown was assessed by real-time PCR for 24 h after transfection.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted with TRIzol (Invitrogen) and cDNA was synthesized using Moloney murine leukaemia virus (MMLV, Invitrogen) according to the manufacturer's protocol, as described previously (Wan *et al.* 2013a). Quantitative real-time PCR was performed using SYBR Green PCR mix (Applied Biosystems) with an ABI 7300 system (Applied Biosystems) under standard reaction conditions: one cycle at 95 $^{\circ}$ C for 5 min, 40 cycles at 95 $^{\circ}$ C for 2 min, 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 40 s. Primers for cPLA2 and β -actin were as follows: cPLA2, sense, 5'-GTGATGTGCTGTGGTAG-3', cPLA2, antisense, 5'-GGTGAGAATACAAGGTTGAC-3'; β -actin, sense, 5'-ATGGGTCAGAAGGATTCCTATGT-3', β -actin, antisense, 5'-AAGGTCTCAAACAACATGATCTGGG-3'. The expression levels of samples were determined based on the $2^{-\Delta\Delta CT}$ methods and β -actin was set as the internal control.

Western blot analysis

The proteins of HUVECs were extracted and western blot was performed as described before (Wan *et al.* 2013a). Primary antibodies against ICAM1 (1:1000), COX2 (1:1000), cPLA2 (1:1000), p-cPLA2 (1:1000), NF- κ B (1:1000), p-NF- κ B (1:1000), CREB (1:1000), p-CREB (1:1000), and GAPDH (1:5000) were used.

PGE₂ ELISA

Culture supernatants were analyzed for secreted PGE₂ with a PGE₂ ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.

Statistical analysis

Data were expressed as means $\pm$ S.E.M. and analyzed with GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) by one-way ANOVA followed by Student-Newman-Keuls *post hoc* test. *P* values <0.05 were considered statistically significant.

Results

Ucn1 increased ICAM1 expression via CRHR2

The effect of Ucn1 on ICAM1 expression was determined by western blot. As shown in Fig. 1A, Ucn1 increased the ICAM1 expression in a time-dependent manner, which reached its peak at 8 h. To explore the involvement of CRHR, the selective CRHR1 inhibitor antalarmin or the selective CRHR2 inhibitor antisauvagine-30 was added 30 min before Ucn1 stimulation. As shown in Fig. 1B, antalarmin failed to inhibit the increase in ICAM1 induced by Ucn1. Unlike antalarmin, antisauvagine-30 significantly reversed the increase in ICAM1 expression induced by Ucn1. These data indicate that Ucn1 could increase ICAM1 expression via CRHR2.

Figure 1

Ucn1 increased ICAM1 expression via CRHR2. (A) The effects of Ucn1 on the expression of ICAM1 in HUVECs were determined by western blotting. (B) HUVECs were pretreated with the selective CRHR1 inhibitor antalarmin or the selective CRHR2 inhibitor antisauvagine-30 for 30 min and then Ucn1 was added. After incubation for 8 h, the expression of ICAM1 was examined by western blotting. **P*<0.05, ***P*<0.01. All the data given are the means $\pm$ S.E.M. of values obtained from more than three independent cultures, and representative blots are shown.

Ucn1 increased COX2 expression via CRHR2

Our own and others' data indicated that aspirin, a nonselective COX2 inhibitor, significantly suppressed COX2 and ICAM1 expression (Zhao *et al.* 2008). Moreover, NS-398, a selective COX2 inhibitor, could reduce sICAM1 expression. To confirm that COX2 was involved in the process of Ucn1-induced ICAM1 upregulation, we first determined whether Ucn1 could regulate COX2 expression. As shown in Fig. 2A, Ucn1 induced COX2 expression in a time-dependent manner, which reached a maximum at 12 h. The effect of Ucn1 on COX2 expression was abolished when cells were preincubated with antisauvagine-30 for 30 min before Ucn1 stimulation (Fig. 2B). The data indicated that Ucn1 increased COX2 expression via CRHR2.

Ucn1-regulated ICAM1 expression was dependent on COX-2

Subsequently, we used the nonselective COX2 inhibitor indomethacin and the selective COX2 inhibitor NS-398 to explore the involvement of COX2 in Ucn1-regulated ICAM1 expression. As shown in Fig. 2C, both

indomethacin and NS-398 could reverse ICAM1 upregulation stimulated by Ucn1. Interestingly, the effect of indomethacin was greater than that of NS-398, which indicated the involvement of COX1.

PGE₂ is the main metabolite of COX2, which has been reported to play an important role in the signal transduction cascade events during induction of gene transcription (Danesch *et al.* 1994, Chen & Hughes-Fulford 2000). Thus, we detected the concentration of PGE₂ in culture supernatants. We found that Ucn1 could significantly elevate the concentration of PGE₂, which was inhibited by antisauvagine-30 (Fig. 2D).

To further ensure the involvement of COX2-PGE₂ signaling activation, HUVECs were pretreated with indomethacin and NS-398, and then PGE₂ and Ucn1 were added in turn. The expression of ICAM1 was examined. As shown in Fig. 2E, indomethacin and NS-398 could reverse Ucn1-induced ICAM1 upregulation. Moreover, PGE₂ supplement abolished the effects of both indomethacin and NS-398. These data indicate that Ucn1 increase ICAM1 expression depending on COX2 and its main metabolite PGE₂.

Figure 2

COX2 was involved in the process of Ucn1-regulated ICAM1 expression. (A) The effects of Ucn1 on endothelial cell COX2 expression were examined. (B) HUVECs were treated with antalarmin and antisauvagine-30 for 30 min before addition of Ucn1. The expression of ICAM1 was determined by western blot. (C) HUVECs were treated with Ucn1 in the presence of indomethacin or NS-398, the expression of ICAM1 was examined by western blot. (D) The concentration of PGE₂ in cell culture supernatants

were examined by ELISA. (E) HUVECs were treated with or without PGE₂ in the presence of indomethacin/NS-398, before addition of Ucn1. The expression of ICAM1 was measured by western blot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All the data given are the means $\pm$ S.E.M. of values obtained from more than three independent cultures, and a representative bolt/experiment is shown.

cPLA2 mediated Ucn1-induced ICAM1 and COX2 upregulation in HUVECs

Reportedly, members of the PLA2 family, mainly cPLA2, contribute to the production of prostaglandins (Balsinde *et al.* 1998, Anthonen *et al.* 2001, Farooqui & Horrocks 2004). Data shown above demonstrated that Ucn1 could increase ICAM1 expression via COX2-PGE₂ pathways. To explore whether PLA2 was involved in Ucn1-induced ICAM1 upregulation, we examined the effects of selective inhibitors of cPLA2, iPLA2, and sPLA2 on Ucn1-induced ICAM1 and COX2 expressions. As shown in Fig. 3A and B the cPLA2 inhibitor PYR significantly reversed the increase in Ucn1-stimulated ICAM1 and COX2 expressions. However, the iPLA2 inhibitor BEL and the sPLA2 inhibitor DTT had no notable effects. These data indicate that cPLA2 regulates COX2 protein synthesis, which further regulates ICAM1 protein expression.

As a cPLA2 inhibitor was indicated to affect the process of ICAM1 regulation, we then detected the effects of Ucn1 on cPLA2 expression and activation. As shown in Fig. 3C, Ucn1 increased cPLA2 expression in a time-dependent manner, and this reached its peak at 2 h. Moreover, the effects of Ucn1 on increased cPLA2 expression were abolished by the CRHR2 inhibitor antisauvagine-30 and partially by the CRHR1 inhibitor antalarmin (Fig. 3D).

It has been demonstrated that the activation of cPLA2 could be regulated by both Ca²⁺ and phosphorylation (Lin *et al.* 1992, Kudo & Murakami 2002). Moreover, p42 or p38 MAPK-induced phosphorylation at Ser505 of cPLA2 resulted in a twofold to threefold increase in catalytic activity (Lin *et al.* 1993, Nemenoff *et al.* 1993). Thus, we next examined the phosphorylation of cPLA2 Ser505 stimulated by Ucn1 which indicated the

Figure 3

cPLA2 mediated Ucn1-induced ICAM1 and COX2 upregulation in HUVECs. (A) HUVECs were treated with Ucn1 in the presence of PYR (cPLA2 inhibitor), DTT (sPLA2 inhibitor), or BEL (iPLA2 inhibitor), and western blotting was performed to determine the expression of ICAM1. (B) COX2 expression was examined when cells were treated with Ucn1 in the presence of PYR, DTT, BEL in HUVECs. (C) HUVECs were treated with Ucn1 for the indicated times, and the expression of cPLA2 was determined. (D) HUVECs were treated with Ucn1 in the presence or absence of

antalarmin/antisauvagine-30 for 2 h. The expression of cPLA2 was examined by western blotting. (E) The phosphorylation levels of cPLA2 induced by Ucn1 were measured. (F) The phosphorylation levels of cPLA2 induced by Ucn1 in the presence or absence of antalarmin/antisauvagine-30 were determined. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All the data given are the means $\pm$ S.E.M. of values obtained from more than three independent cultures, and representative blots/results are shown.

activity of cPLA2. As shown in Fig. 3E, Ucn1 increased the phosphorylation of cPLA2 Ser505 in a time-dependent manner. There was a significant increased expression of phosphorylated cPLA2 within 10 min, which reached a peak within 15 min and was sustained for 60 min. In accordance with the results shown in Fig. 3D, both antalarmin and antisauvagine-30 could reduce the Ucn1-induced increase in cPLA2 phosphorylation (Fig. 3F). The data indicates that the effects of Ucn1 on ICAM1 and COX2 expressions were mediated by cPLA2.

PKA-CREB participated in the signaling events leading to induction of ICAM1

The results described above revealed that Ucn1 could increase PGE₂ production, which was necessary for the process of ICAM1 regulation. Because activation of EP has

been found to activate PKA, we used H89, a PKA inhibitor to study the involvement of PKA in the signaling events leading to the induction of ICAM1. H89 significantly reduced the increase in ICAM1 expression stimulated by Ucn1 (Fig. 4A). Furthermore, the reduction in ICAM1 expression in the presence of indomethacin in cells treated with Ucn1 was reversed by the addition of dbcAMP, a cAMP analog that directly activates PKA (Fig. 4B).

It is well-known that PKA can phosphorylate CREB for its binding to cAMP response element (CRE), which regulates gene transcription (Sands & Palmer 2008). Moreover, a single nuclear CREB site was found in the ICAM1 gene promoter (Wawryk *et al.* 1991). Therefore, we speculated that CREB may participate in Ucn1-stimulated induction of ICAM1. Indeed, we found that Ucn1 increased the phosphorylation of CREB in a time-dependent manner (Fig. 4C). Furthermore, the

Figure 4

PKA-CREB participated in the signaling events leading to induction of ICAM1. (A) HUVECs were incubated with Ucn1 in the presence or absence of H89, ICAM1 expression was examined by western blotting. (B) HUVECs were treated with vehicle, Ucn1, Indo + Ucn1, or Indo + dbcAMP + Ucn1, and the expression of ICAM1 was measured. (C) The effects of Ucn1 on CREB phosphorylation were determined at the indicated times. (D) HUVECs were treated with Ucn1 with/without antisauvagine-30/antalarmin for 60 min, and the phosphorylation levels of CREB were examined. (E) HUVECs

were treated with PYR, si-cPLA2, indomethacin, NS-398, and then Ucn1 was added. The expression of phosphorylated CREB was measured. (F) HUVECs were treated with vehicle, Ucn1, Indo + Ucn1, Indo + PGE₂ + Ucn1, and the levels of CREB phosphorylation were determined. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. All the data given are the means ± S.E.M. of values obtained from more than three independent cultures, and representative results are shown.

CRHR2 inhibitor antisauvagine-30 abolished the increase in CREB phosphorylation stimulated by Ucn1 (Fig. 4D).

These data indicated that Ucn1 regulated ICAM1 expression via cPLA2-COX2-PGE₂ pathways. To better understand the involvement of cPLA2, RNA silencing of cPLA2 was used, and the efficiency of cPLA2 knockdown was determined by real-time PCR 48 h after transfection (Supplementary Figure S1, see section on supplementary data given at the end of this article). The efficiency of cPLA2 was 83% which was sufficient for our purposes. To study the role of cPLA2 and COX2 in CREB phosphorylation events, cells were pretreated with PYR, si-cPLA2, indomethacin, or NS-398 for 30 min before Ucn1 addition. As shown in Fig. 4E, PYR, si-cPLA2, indomethacin, and NS-398 significantly decreased the levels of CREB phosphorylation. Moreover, H89 reduced the phosphorylation levels of CREB, and PGE₂ partly restored it (Fig. 4F). Collectively, these results further support the suggestion that cPLA2-dependent COX2-PGE₂-PKA-CREB participated in Ucn1-regulated endothelial ICAM1 protein expression.

NF- κ B was involved in Ucn1-induced ICAM1 expression, but not COX2 expression

NF- κ B is well known to participate in inflammatory processes (Ghosh & Hayden 2008) and regulate ICAM1 expressions (Kim *et al.* 2001). ICAM1 gene promoters contain several NF- κ B response elements (Stade *et al.* 1990). Most importantly, PKA has been reported to phosphorylate the p65 subunit of NF- κ B, which contributed to the binding of NF- κ B to the ICAM1 gene promoter (Arun *et al.* 2009). To address the question whether NF- κ B participated in Ucn1-induced ICAM1 regulation, we used an NF- κ B inhibitor, PDTC. Results presented in Figure 5A showed that Ucn1 increased ICAM1 expression and that PDTC reversed the effects of Ucn1. These data indicated that Ucn1 increased ICAM1 expression via NF- κ B. However, in this study, the phosphorylation level of NF- κ B was not influenced by the PKA inhibitor, H89 (Supplementary Figure S2, see section on supplementary data given at the end of this article).

Figure 5

NF- κ B was involved in Ucn1-induced ICAM1 expression, but not COX2 expression. (A) HUVECs were treated with Ucn1 in the absence or presence of PDTC, and the expression of ICAM1 was determined by western blotting. (B) HUVECs were treated with Ucn1 in the absence or presence of PDTC, and the expression of COX2 was determined by western blotting. (C) The effects of Ucn1 on p65 NF- κ B phosphorylation levels were determined at the indicated times. (D) HUVECs were treated with vehicle, Ucn1, Ucn1 + antisauvagine-30,

Ucn1 + antalarmin, and the level of p65 NF- κ B phosphorylation was examined by western blotting. (E) HUVECs were treated with vehicle, Ucn1, PYR + Ucn1, si-cPLA2 + Ucn1, and the expression of NF- κ B phosphorylation was determined. (F) The cytoplasm and nuclear NF- κ B expressions were determined by western blotting. * P < 0.05, ** P < 0.01, *** P < 0.001. All the data given are the means $\pm$ S.E.M. of values obtained from more than three independent cultures, and representative results are shown.

Results shown in Fig. 2 indicated that Ucn1-regulated ICAM1 expression was dependent on COX2. Moreover, Kim *et al.* (2005) found that the induction of COX2 in mouse skin is regulated, at least in part, by an eukaryotic transcription factor NF- κ B. Therefore, we examined the effects of PDTC on COX2 expression. Surprisingly, we found that PDTC failed to reverse the effects of Ucn1 on COX2 expression (Fig. 5B). On the other hand, COX2 expression was found to be upregulated by directly binding CREB to a COX2 promoter (Diaz-Munoz *et al.* 2012), which indicates that NF- κ B may be the downstream molecule of the COX2 pathway and regulate ICAM1 expression only. Thus, selective COX2 inhibitor NS-398 was used and the phosphorylation level of NF- κ B was determined. However, NS-398 did not change the phosphorylation of NF- κ B (Supplementary Figure S3, see section on supplementary data given at the end of this article). Taken together, in this study, we found that NF- κ B regulates ICAM1 expression independently from COX2.

Based on the above data, the effects of Ucn1 on NF- κ B p65 phosphorylation were determined. As shown in Fig. 5C, Ucn1 increased the phosphorylation of NF- κ B in a time-dependent manner. The selective CRHR inhibitors antisauvagine-30, and antalarmin were used to detect the involvement of CRHR. Consistent with the results shown in Fig. 5C, Ucn1 increased the NF- κ B phosphorylation. Furthermore, antisauvagine-30 abolished the effect of Ucn1 while antalarmin did not (Fig. 5D).

Previous results indicated that cPLA2 was involved in regulation of Ucn1-induced COX2 and ICAM1 expression. Thus, we next examined the relationship between cPLA2 and NF- κ B. We found that the phosphorylation and translocation of NF- κ B was mediated by cPLA2. As shown in Fig. 5E, Ucn1 increased the phosphorylation level of NF- κ B, which is abolished in the presence of si-cPLA2 or PYR. Similarly, Ucn1 increased the translocation of NF- κ B from cytoplasm to nucleus, and si-cPLA2 and PYR inhibited it (Fig. 5F). These results further revealed that NF- κ B was involved in the regulation of ICAM1.

Discussion and conclusions

There are several significant novel findings from this study. Two interlinked pathways were found in Ucn1-induced expression of ICAM1 in HUVECs (Fig. 6). One pathway was cPLA2-dependent NF- κ B pathway. The other pathway involved cPLA2 and COX2 generation of PGE₂ and EP receptor-mediated PKA-CREB. This study gives important insights into the downstream signaling mechanisms of Ucn1 on endothelial ICAM1 expression.

Figure 6

Model for Ucn1-mediated pathway in endothelial ICAM1 expression.

A full colour version of this figure is available via <http://dx.doi.org/10.1530/JME-13-0182>.

It was found that Ucn1 could increase both the expression and activity of cPLA2, which is the key signaling molecule in ICAM1 regulation. Ucn1 elevated cPLA2 expression in a time-dependent manner, and this reached its peak at 2 h. It is well-known that there are three serine residues (Ser505, Ser515, Ser727) of cPLA2 that can be phosphorylated (Burke & Dennis 2009). Replacement of Ser505 abolished AA release (Pavicevic *et al.* 2008). Thus, we detected the effects of Ucn1 on cPLA2 phosphorylation at site Ser505 and found that Ucn1 increased the phosphorylation of cPLA2 on Ser505, which started at 10 min and was maintained for 60 min. Notably, we found that both CRHR1 and CRHR2 were involved in Ucn1-regulated cPLA2 expression and phosphorylation in endothelial cells. Inversely, in cultured human placental trophoblasts, antalarmin reduced cPLA2 expression, whereas CRHR2 antagonist astressin-2b did not alter cPLA2 expression (Gao *et al.* 2008). Moreover, data from our laboratory demonstrated that Ucn1 increased cPLA2 expression via CRHR1-dependent STAT3 in HepG2 cells (R Wan, Y Liu, L Li, C Zhu, L Jin, S Li, unpublished observations). The differences regarding the involvement of CRHR in cPLA2 expression between ours and others' reports may be due to the predominance of different CRHR in different cells used in the experiments and need more attention.

Knowledge about a possible interaction between PLA2 and Ucn1 is limited. The study by Lawrence *et al.* (2003, 2004) which found that Ucn1 could protect cardiac myocytes from ischemia/reperfusion injury by attenuating iPLA2 gene expression generated the first data, to our knowledge, on the interaction of Ucn1 and PLA2. Subsequently, a new mechanism of vasodilatation by Ucn1 was found which involved the regulation of iPLA2 in

rat coronary artery (Smani *et al.* 2007). Consistent with the results of Lawrence *et al.*, Takatani-Nakase & Takahashi (2010) found that Ucn1 could significantly suppress the expression and activity of iPLA2, which results in prevention of the ischemia-induced cell death. In this study, we observed the involvement of cPLA2, but not iPLA2, in Ucn1-related endothelial actions. Ours is the first report, to our knowledge, of cPLA2 participating in Ucn1-induced ICAM1 expression in HUVECs. Interestingly, cPLA2 was activated via both CRHRs, but Ucn1 increased cPLA2-dependent ICAM1 expression just via CRHR2 in this study. The effects of cPLA2 activated by Ucn1 via CRHR1 were not clear. To better understand the interaction between the Ucn1 and PLA2 families, intensive research work is needed.

Previous work by ourselves and others demonstrated that COX2 may be a significant molecule in ICAM1 regulation (Zhao *et al.* 2008, Zhang *et al.* 2009). In accordance with this in this study, we observed that COX2 was indeed a crucial factor in ICAM1 regulation. Attenuating COX2 using nonselective and selective COX2 inhibitors was found to significantly reverse the upregulation of ICAM1 induced by Ucn1. Interestingly, the effect of indomethacin, a nonselective COX2 inhibitor, was a little stronger than that of NS-398, a selective COX2 inhibitor. This result may indicate the involvement of COX1 in Ucn1-regulated ICAM1 expression. COX1 is expressed constitutively in most tissues of body and acts to maintain homeostatic processes. Unlike COX1, COX2, mainly an inducible enzyme, is reported to be involved in the regulation of inflammation (Smith *et al.* 1994). Our data is consistent with this report, i.e., although COX1 was indicated to participate in ICAM1 regulation, COX2 was the main molecule involved. Furthermore, we determined the concentration of PGE₂ in culture supernatants. The results showed that Ucn1 could increase the production of PGE₂, which is the main metabolite of COX2. Indeed, indomethacin and NS-398 reversed Ucn1-induced ICAM1 upregulation and PGE₂ addition restored it. These data strongly support the idea that COX2 is involved in the process of endothelial ICAM1 regulation by Ucn1.

Additionally, we demonstrated that NF- κ B was a contributor to Ucn1-induced ICAM1 upregulation, which is in accordance with results from another study showing that ICAM1 gene promoters contain several NF- κ B response elements (Stade *et al.* 1990). In this study, we tested the phosphorylation levels of p65 NF- κ B and found that Ucn1 induced an increase in p65 NF- κ B phosphorylation in a time-dependent manner and reached the peak at 2 h. Furthermore, we examined the

translocation of NF- κ B into the nuclei. Ucn1 treatment significantly increased nuclear NF- κ B expression, which indicated that Ucn1 could increase the translocation of NF- κ B. A previous study has revealed the interaction of NF- κ B and COX2 (Wang *et al.* 2012). However, we found that the NF- κ B inhibitor, PDTC, failed to reverse the increase in COX2 induced by Ucn1. The inconsistency between our study and others needs more attention.

Because cPLA2 is found to regulate the expression of COX2 while NF- κ B fails to change the expression of COX2, it is reasonable to conclude that cPLA2 is upstream of NF- κ B instead of vice versa. As we expected, attenuating cPLA2 by RNA silencing or with the inhibitor PYR markedly decreased the phosphorylation and translocation of NF- κ B. Consistent with our study, in human-derived keratinocytes, selective inhibitors against cPLA2 inhibited activation of NF- κ B (Thommesen *et al.* 1998, Anthonsen *et al.* 2001). Nevertheless, the cPLA2 promoter contains NF- κ B binding sites, which are regulated by cytokines through various signaling mechanisms (Barnes & Karin 1997). In canine tracheal smooth muscle cells, IL1 β -induced cPLA2 expression was mediated through activation of NF- κ B pathways (Luo *et al.* 2008). Addressing this controversy requires more research.

In summary, our studies provide a detailed characterization of Ucn1-mediated regulation of ICAM1 expression in human endothelial cells. It was demonstrated that Ucn1 could increase cPLA2 expression and activation, which links COX2-mediated PGE₂ production and subsequently PKA-CREB activation and NF- κ B activation that play central roles in the regulation of ICAM1.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-13-0182>.

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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Author contribution statement

Y Liu and L Li contributed equally to this work.

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