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Differential regulation of urocortins1–3 mRNA in human umbilical vein endothelial cells

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ABSTRACT

Urocortins (Ucns) are members of the corticotropin-releasing factor (CRF) family of peptides. Ucns would have potent effects on the cardiovascular system via the CRF receptor type 2 (CRF_2 receptor) in the cardiovascular system. However, an endogenous role and regulation of each Ucn have not been determined in the system. In the present study, we extended observations on stress or hormone-induced changes in Ucn gene expression in the cardiovascular system. Human umbilical vein endothelial cells (HUVECs) express Ucn1, Ucn2, and Ucn3 mRNAs, but not CRF mRNA, and the receptor, $CRF_{2\alpha}$ receptor mRNA. Lipopolysaccharides decreased Ucn1 mRNA levels, while it increased Ucn2 and Ucn3 mRNA levels in HUVECs. Interleukin-1β decreased Ucn1 and Ucn2 mRNA levels, while it increased Ucn3 mRNA levels in HUVECs. Forskolin increased Ucn1 mRNA levels, while it decreased Ucn2 and Ucn3 mRNA levels. Ucns1–3 mRNA levels are differentially regulated in HUVECs. Differential regulation of Ucns1–3 mRNA may suggest differential roles of those in HUVECs.

1. Introduction

Corticotropin-releasing factor (CRF) plays a central role in controlling the hypothalamic–pituitary–adrenal (HPA) axis during stress [1]. Urocortins (Ucns) are also members of the CRF family of peptides. Three Ucns have been found in mammals. Ucn1 is a 40 amino acid peptide cloned from the Edinger–Westphal nucleus [2], and Ucn2 and Ucn3 are identified in the human genome data base and in mouse genomic DNA, respectively [3–5]. Ucn1 and/or Ucn2 is expressed in the heart, vascular, and peripheral blood cells [3–8], while the expression of Ucn3 also has been reported in the cardiovascular system [9].

The actions of the CRF family peptides are mediated by at least two distinct G protein-coupled receptors, namely the CRF receptor type 1 (CRF_1 receptor) [10–12] and CRF receptor type 2 (CRF_2 receptor) [13–15]. These two receptors share 69% amino acid homology [16], but have different tissue distributions and pharmacological properties with respect to ligands [17]. CRF_1 receptor is the major subtype responsible for regulating synthesis and secretion of ACTH in the pituitary corticotrophs [18], whereas CRF_2 receptor with splice variants is found in the brain and in peripheral sites including the cardiac myocytes, and vascular smooth muscles [19,20].

Ucn1 binds for both CRF_1 receptor and CRF_2 receptor, while Ucn2 and Ucn3 are highly selective for CRF_2 receptor, with either little or no affinity for CRF_1 receptor. Three Ucns have considerably higher affinities for CRF_2 receptor than CRF. A recent study shows the potent effects of Ucns on the cardiovascular system [21]. Ucn1 produces vasodilation via the adenylate cyclase and protein kinase A (PKA) pathway [22]. Furthermore, Ucn1 and Ucn2 have more potent vasodilatory and cardiac inotropic effects than CRF, with a greater potential to increase coronary blood flow and reduce overall blood pressure [5,23–25]. Taken together with the expression of Ucns and the receptor, these results suggest that endogenous Ucns have a physiological role in the cardiovascular system [26]. In addition, the recent studies by Rademaker et al. suggest that Ucn2 or Ucn3 may have therapeutic potential in patients with heart failure [27,28]. Therefore, Ucns would have potent effects on the cardiovascular system via the receptor, and they serve as natural ligands for CRF_2 receptor in the cardiovascular system. However, an endogenous role and regulation of each Ucn have not been determined in the system.

In the present study, we extended observations on stress or hormone-induced changes in Ucn gene expression in the cardiovascular system. In order to assess potential factors responsible for the changes in Ucn levels, we determined the effects of lipopolysaccharides (LPSs) and forskolin (Fsk) on Ucn mRNA levels in the human umbilical vein endothelial cells (HUVECs). We then confirmed the hypothesis that Ucn mRNA levels are differently regulated via cyclic AMP (cAMP)- or cytokines-dependent pathways.

2. Materials and methods

2.1. Materials

LPS was purchased from Sigma (St. Louis, MO, USA), recombinant human interleukin (IL)-1β from PeproTech (Rocky Hill, NJ, USA), and Fsk from Calbiochem (San Diego, CA, USA).

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2.2. Cell cultures

The HUVECs were obtained from Cambrex Bio Science (Walkersville, MD, USA), and incubated in EBM-2 culture medium supplemented with 2% FBS, and Endothelial Growth Media (Cambrex Bio Science) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were plated at 10^4 cells/cm^2 7 days before each experiment, with the medium changed every 48 h. On the seventh day, cells were incubated in medium containing LPS, IL-1β, Fsk, or the vehicle. At the end of incubation, total cellular RNA was collected and stored at −80 °C until assay was performed. All treatments were performed in triplicate and repeated three times.

2.3. RNA extraction

Cellular total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Then, cDNAs were synthesized from total RNA (0.5 μg) using random hexamers as primers with the SuperScript First-Strand Synthesis System for reverse transcriptase–polymerase chain reaction (RT–PCR) Kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Reverse transcriptase–polymerase chain reaction (RT–PCR)

The RT production (0.4 μg cDNA samples for Ucns1–3, $CRF_{2\alpha}$ receptor, and GAPDH; 1.0 μg cDNA samples for CRF, and CRF_1 and $CRF_{2\beta}$ receptors) was used for the following polymerase chain reaction (PCR) reaction. PCR was carried out in a programmable thermal controller (Bio-Rad, Hercules, CA, USA) with the following oligonucleotide primers: CRF-F (5′-AAGGAAGACAACCTCCAGAGAAAGC-3′); CRF-R (5′-TCCATGAGTTTCCTGTTGCTGTGAG-3′) [29]. Ucn1-F (5′-GTTCCCCAAGGCGTCTTCA-3′); Ucn1-R (5′-CTTGCCCACCGAGTCGAAT-3′) [29]. Ucn2-F (5′-GTGTCGGCCACTGCTGAGCCTGAGAGA-3′); Ucn2-R (5′-ATCTGATATGACCTGCATGACAGTGGCT-3′) [30]. Ucn3-F (5′-TGCTGCTCCTGCTGCTGCTC-3′); Ucn3-R (5′-GTGTCCTGGCGTGGCTTTCCC-3′) [30]. CRF_1 receptor-F (5′-CAAACAATGGCTACCGGGAG-3′); CRF_1 receptor-R (5′-ACACCCCAGCCAATGCAGA-3′) [29]. $CRF_{2\alpha}$ receptor-F (5′-GACGCGGCACTGCTCCACAG-3′); $CRF_{2\alpha}$ receptor-R (5-GCATTCCGGGTCGTGTTGT-3′) [29]. $CRF_{2\beta}$ receptor-F (5′-CCCTCACCAACCTCTCAGGTCC-3′); $CRF_{2\beta}$ receptor-R (5′-CAGGTCATACTTCCTCTGCTTGTC-3′) [29]. GAPDH-F (5′-GGTCGGAGTCAACGGATTTG-3′); GAPDH-R (5′-ATGAGGTCCACCACCCTGTT-3′) [29]. Conditions for the Ucn1, and $CRF_{2\alpha}$ receptor were 1×(96 °C, 2 min), 35×(96 °C, 15 s; 64 °C, 30 s; 72 °C, 1 min) and 1×(72 °C, 5 min). Conditions for the CRF, and CRF_1 and $CRF_{2\beta}$ receptors were 1×(96 °C, 2 min), 38×(96 °C, 15 s; 64 °C, 30 s; 72 °C, 1 min) and 1×(72 °C, 5 min). Conditions for the Ucn2 were 36×(94 °C, 40 s; 55 °C, 40 s; 72 °C, 40 s) and 1×(72 °C, 10 min). Conditions for the Ucn3 were 37×(94 °C, 1 min; 63 °C, 1 min; 72 °C, 1 min) and 1×(72 °C, 10 min). Conditions for the GAPDH were 1×(96 °C, 2 min), 22×(96 °C, 15 s; 60 °C, 30 s; 72 °C, 1 min) and 1×(72 °C, 5 min). Products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide. The expected sizes of PCR products for CRF and Ucn1 were 678 bp and 468 bp, respectively. The expected sizes of PCR products for Ucn2 and Ucn3 were 195 bp and 310 bp, respectively. The expected size of PCR products for CRF_1 receptor was 475 bp, and for $CRF_{2\alpha}$ and $CRF_{2\beta}$ receptors were 233 bp and 248 bp, respectively. The expected size of PCR products for GAPDH receptor was 969 bp.

2.5. Real-time reverse transcriptase–polymerase chain reaction (RT–PCR)

Cellular total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNAs were synthesized from total RNA (0.5 μg) with random hexamer as the primer using the SuperScript First-Strand Synthesis System for RT–PCR kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions.

The resulting cDNAs were then subjected to real-time PCR as follows. The expression levels of human Ucn mRNA were evaluated using quantitative real-time PCR based on specific sets of primers and probes (Assays-on-Demand Gene Expression Products, Applied Biosystems, Foster City, CA, USA). GAPDH was used as a housekeeping gene to standardize the values, because the GAPDH mRNA levels were not changed in any treatments of these studies. Each reaction consisted of 1xTaqMan universal PCR Master Mix (Applied Biosystems), 1xAssays-on-Demand Gene Expression Products (Hs00175020_m1 for human Ucn1, Hs00264218_s1 for human Ucn2, Hs00846499_s1 for human Ucn3, and Hs99999905_m1 for human GAPDH) and 2 μl cDNA in a total volume of 50 μl using the following parameters on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems): 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The above assays involve specific sets of primers and TaqMan probe spanning exon/exon junction and should not therefore have been influenced by DNA contamination. Data were collected and recorded by ABI PRISM 7000 SDS Software

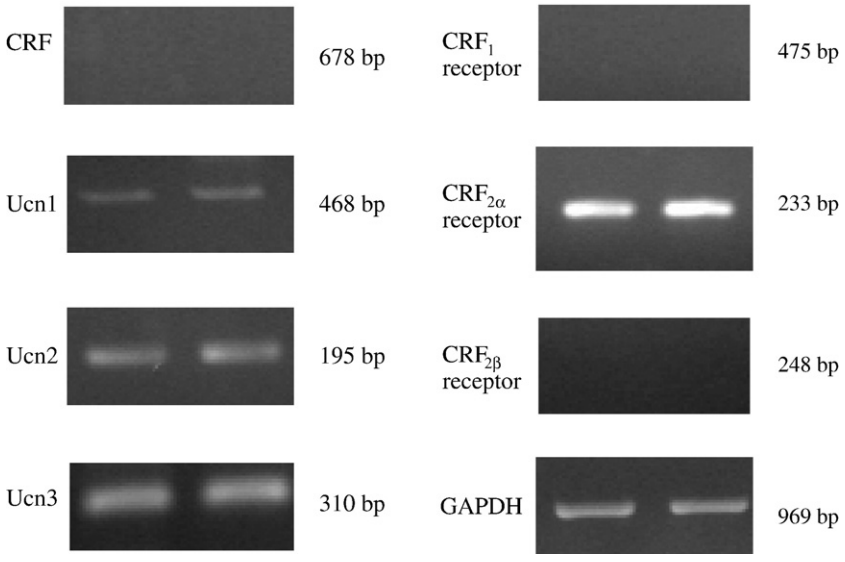

Fig. 1. Expression of Ucns and CRF receptors mRNA in HUVECs. A representative image of RT–PCR for Ucns and CRF receptors mRNA in HUVECs.