Abstract

Urocortin (UCN1) is a member of corticotrophin-releasing factor (CRF) family, which has been proven to participate in inflammation. Previous work showed that dihydrotestosterone (DHT) could promote the inflammatory process. Little is known about the effect of DHT on UCN1 expression. The aim of our study is to investigate the effects and underlying mechanisms of DHT on endothelial UCN1 expression in the absence and presence of induced inflammation. Therefore, we tested the alterations of endothelial UCN1 expression treated with DHT in the presence or absence of lipopolysaccharide (LPS). Our data showed that DHT alone decreased UCN1 levels, which were attenuated in the presence of the androgen receptor (AR) antagonist flutamide. Conversely, in the presence of LPS, DHT augmented the LPS-induced increase in UCN1 expression, which was, interestingly, not affected by flutamide. When cells were treated with DHT alone, AR was upregulated and translocated into the nuclei, which might repress UCN1 expression via a potential androgen-responsive element found in human CRF family promoter. In the presence of LPS, DHT did not influence AR expression and location while it increased toll-like receptor 4 expression and activation, which was not altered by flutamide. DHT enhanced LPS-induced p38MAPK, ERK1/2, and nuclear factor κB pathway activation, which may contribute to the elevated expression of UCN1. These data suggest that DHT differentially influences UCN1 levels under normal and inflammatory conditions in human umbilical vein endothelial cells, which involves AR-dependent and -independent mechanisms respectively.

Key Words
- androgen
- urocortin
- inflammation
- lipopolysaccharide

Introduction

Epidemiological and clinical data have indicated that the incidence of cardiovascular diseases is greater in men compared with women of similar age (Vitale et al. 2007). Among women, the incidence of cardiovascular disease is greater in postmenopausal women compared with premenopausal women (Orshal & Khalil 2004). Despite numerous reports that have suggested the beneficial effects of estrogen replacement therapy (Gerhard & Ganz 1995), the risk/benefit is still controversial (Enstrom et al. 2002, Grimes & Lobo 2002). Following the recent reevaluation of the estrogen protection orthodoxy, empirical research has flourished into the role of androgens in the progression of cardiovascular disease (Liu et al. 2003). In addition, clinical data have demonstrated that male hormones are independent factors contributing to the higher male susceptibility to cardiovascular diseases.
promoter region (Bao et al. 2006). AR on CRF expression, was identified in the CRF gene. Furthermore, an androgen-responsive element (ARE), which initiated a repressing effect of homology to UCN1. Furthermore, an androgen-responsive factor (CRF), which shares 45% amino acid sequence to UCN1. Moreover, an inflammatory process. UCN1 is proven to be involved in the vascular and intercellular adhesion molecule 1 in rat aortic lipopolysaccharide (LPS)-induced expression of COX-2 (Kageyama et al. 2009). In addition, UCN1 was reported to augment cyclooxygenase-2 in inflammatory diseases. Importantly, both AR and urocortin (UCN1) have been identified in vascular ECs and systemic inflammation. Importantly, both AR and androgens in inflammation owing to the significance of inflammation in the pathogenesis of cardiovascular diseases.

Dihydrotestosterone (DHT), a more potent androgen, is the form of testosterone that undergoes 5α-metabolite action (Mareck et al. 2008). DHT has threefold greater affinity than testosterone and 15- to 30-fold greater affinity than adrenal androgens for androgen receptor (AR; Ikeda et al. 2012). Reportedly, DHT enhanced the binding of monocytes to the endothelium via increased expression of vascular cell adhesion molecule-1 in male human endothelial cells (ECs; McCrohon et al. 1999, Death et al. 2004). Furthermore, both in vivo and vitro, DHT treatment was found to enhance nuclear factor kB (NF-kB) activity in cerebral arteries and augmented levels of cyclooxygenase-2 (COX-2) and inducible NO synthase, two well-known pro-inflammatory products of NF-kB (Gonzales et al. 2009).

All these findings suggest that the pro-inflammatory actions of DHT may contribute to sex-related differences in inflammatory diseases.

Situated at the interface between the circulating blood and vasculature, endothelium is not only a protective barrier but also a key player for cardiovascular homeostasis. Particularly, ECs, ingredients of endothelium, are paramount to vascular biology, such as modulating local and systemic inflammation. Importantly, both AR and urocortin (UCN1) have been identified in vascular ECs (Liu et al. 2003, Kageyama et al. 2012). Endothelial UCN1 is upregulated by inflammatory cytokines (Kohno et al. 2001). Our previous studies and others demonstrated that UCN1 could increase EC permeability in the presence of induced inflammation (Cureton et al. 2009, Wan et al. 2013). In addition, UCN1 was reported to augment lipopolysaccharide (LPS)-induced expression of COX-2 and intercellular adhesion molecule 1 in rat aortic ECs by our group (Zhang et al. 2009). Thus, endothelial UCN1 is proven to be involved in the vascular inflammatory process.

There is evidence that a subpopulation of neurons in the PVN expresses both AR and corticotrophin-releasing factor (CRF), which shares 45% amino acid sequence homology to UCN1. Furthermore, an androgen-responsive element (ARE), which initiated a repressing effect of AR on CRF expression, was identified in the CRF gene promoter region (Bao et al. 2006). Similarly, our in vivo data showed that UCN1 exacerbated the inflammatory process in a rat model of vasculitis induced by sodium laurate (Xu et al. 2009). Furthermore, the inflammatory state was more grave in male rats than in female rats. Interestingly, pronounced gender-specific differences in UCN1 production were also seen and the gender bias in UCN1 production accounts for the sex difference in vasculitis (original article submitted).

Based on the above results, we hypothesized that DHT may increase UCN1 level and that the role of DHT under normal and inflammatory conditions in induction of endothelial UCN1 expression may be different.

Materials and methods

Cell culture and reagents

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

DHT, AR antagonist flutamide, and LPS were obtained from Sigma. AR antibody was purchased from Signalway Antibody (Pearland, TX, USA). Specific antibody to toll-like receptor 4 (TLR4), NF-kB p65, and p-NF-kB p65 were provided by Cell Signaling Technologies (Danvers, MA, USA). UCN1 antibody and HRP-conjugated rabbit anti-chicken IgG were obtained from Abcam (Cambridge, UK). Antibodies to p38MAPK, p-p38MAPK, ERK1/2, p-ERK1/2, JNK, and p-JNK were from Bioworld Technology (Minnesota, MN, USA). Lipofectamine 2000 transfection reagent, TRIZol, and Moloney murine leukemia virus (MMLV) were from Invitrogen. Cytoplasmic and nuclear protein extraction kit was from Fermentas (Burlington, ON, Canada). Dual-Luciferase Reporter Assay System was obtained from Promega. SYBR green PCR mix was from Bio-Rad. The other reagents were derived from commercial sources.

RNA isolation and PCR

Total RNAs were extracted from HUVECs using TRIZol reagent according to the manufacturer’s protocol. For cDNA synthesis, MMLV was applied as the reverse transcriptase. Quantitative real-time PCR was performed using SYBR green with the ABI Prism 7300 (Applied Biosystems). Primers for UCN1 and β-actin are listed in Table 1. The expression levels were calculated based on the 2−ΔΔCT methods (Jin et al. 2012) and β-actin was set as the internal control. For semiquantitative RT-PCR, Tag DNA polymerase was used in the reaction system. Primer sequences for AR (He et al. 2006) and GAPDH...
were synthesized from published sequences as shown in Table 2.

### Western blot analysis

The protein of HUVECs was extracted and western blot was performed as described previously (Zhang et al. 2009).

Primary antibodies against UCN1 (1:1000), TLR4 (1:1000), p38MAPK (1:1000), p-p38MAPK (1:1000), ERK (1:1000), p-ERK (1:1000), JNK (1:1000), p-JNK (1:1000), p-p65 NF-κB (1:1000), NF-κB (1:10 000), histone (1:5000), GAPDH (1:5000), and β-actin (1:5000) were used. Immunoreactive proteins were visualized by LumiGLO chemiluminescent reagent and peroxide.

### Isolation of nuclear and cytoplasmic proteins

Nuclear and cytoplasmic proteins of HUVECs were isolated using the cytoplasmic and nuclear protein extraction kit according to the manufacturer’s protocol.

### Transfection and dual-luciferase reporter assay

The luciferase reporter construct pGL2-hUS2 containing 2215 nucleotides of hUcn promoter region and 70 nucleotides of the exon 1 (Zhao et al. 1998) was graciously provided by Dr Wylie W Vale (The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, San Diego, CA, USA). Cells were transfected using Lipofectamine 2000 transfection reagent. Firefly and Renilla luciferase activities were detected separately using the dual-luciferase kit according to the manufacturer’s instructions.

### Immunofluorescence staining

Immunofluorescence staining was carried out as described previously (Zhang et al. 2009), with specific antibodies to AR (1:200) and FITC-conjugated anti-rabbit secondary antibody. 4,6-Diamidino-2-phenylindole was used for staining of nuclei. The images were obtained at 20× magnification using LSM710 confocal microscope (Zeiss, Germany).

### Statistical analysis

Data were expressed as means ± S.E.M. and analyzed with GraphPad (San Diego, CA, USA) Prism 5 by one-way ANOVA followed by Tukey’s test. *P* values <0.05 were considered statistically significant.

### Results

#### DHT alone decreased UCN1 expression

To determine the effect of DHT treatment on UCN1 expression in the absence of induced inflammation, a time-course study was performed. HUVECs were treated with DHT (10⁻⁶ M) and harvested at indicated times. As depicted in Fig. 1A and B, DHT reduced UCN1 expression in a time-dependent manner, with the mRNA and protein levels reaching the bottom at 9 and 24 h respectively. Hence, the time points of 9 and 24 h were selected in the following studies. Furthermore, DHT decreased the UCN1 expression in a concentration-dependent manner and a marked effect was observed with 10⁻⁸ M DHT (Fig. 1C and D). Thus, 10⁻⁸ M DHT was selected as the best given concentration, which is used in the following experiments.

#### DHT increased the UCN1 expression in the presence of LPS

To determine the effect of DHT on UCN1 expression under the condition of induced inflammation, HUVECs were treated with LPS (10 μg/ml) in the presence of DHT. As shown in Fig. 2A and B, DHT alone decreased the expression of UCN1, which is consistent with the above-mentioned data. However, DHT augmented the LPS-increased UCN1 expression at both mRNA and protein levels. To explore whether AR was involved in the action of DHT, we pretreated the HUVECs with flutamide, the AR antagonist, for 0.5 h and then DHT and LPS were added in turn. Interestingly, flutamide could abolish the effect of DHT when cells were treated with DHT alone. But when stimulated with LPS, flutamide failed to inhibit the DHT action (Fig. 2C and D). All the data shown suggest that

### Table 1

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Product size (bp)</th>
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<tr>
<td>AR Sense: TCTCAAGAGATTGGATGCTCC</td>
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<td>Antisense: TCACTGGGTTGGGAAATAGATG</td>
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<td>Antisense: ATGAGTCCACACCCCTGGT</td>
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DHT decreases the UCN1 expression in an AR-dependent manner and increases the LPS-induced UCN1 expression in an AR-independent manner.

**AR repressed UCN1 promoter activity**

To determine whether DHT targeted UCN1 directly, we performed reporter assays. HUVECs were transiently cotransfected with pGL2-hUS2 or pGL2-Basic and treated with vehicle, DHT, LPS, and DHT + LPS. As shown in Fig. 2E, we found that DHT decreased the transcriptional activity significantly while increased the transcriptional activity together with LPS. The effect of DHT on UCN1 promoter activity was inhibited by flutamide. However, flutamide failed to reverse the enlargement action of DHT with LPS together on UCN1 transcriptional activity. Taken together, the above data suggest that DHT could directly regulate the transcriptional action in an AR-dependent manner and that in the presence of LPS, DHT plays as a pro-inflammatory part in an AR-independent manner.

**DHT activation altered the AR expression**

Previous data have shown that androgens could induce the upregulation of the levels of AR (Gonzalez-Cadavid et al. 1993). We investigated the AR protein levels after treatment with vehicle or DHT or DHT + LPS by western blot. DHT increased AR protein expression in HUVECs, but in the presence of LPS, AR expression had no alteration compared with vehicle (Fig. 3A).

**An ARE was found in UCN1 promoter**

It is well known that ligand-activated AR can regulate gene transcription by binding to specific DNA sequences named ARE in gene promoters (Lundqvist & Norlin 2012). In order to determine whether AR could act on the UCN1 promoter by an ARE, the human UCN1 promoter region was searched for consensus ARE motifs. The consensus sequence of ARE was determined by Roche et al. (1992). As shown in Fig. 3D, a sequence strongly resembling an ARE was found at the position −1690. The presence of ARE in UCN1 promoter suggested that AR may repress the UCN1 promoter by ARE.

**DHT augmented the TLR4 pathway**

Previous data demonstrated that DHT could increase UCN1 transcription in the presence of LPS. LPS is
demonstrated to increase UCN1 expression (Honjo et al. 2006). Thus, it is reasonable to hypothesize that DHT could augment the TLR4 pathway when stimulated by LPS. TLR4 is a member of the TLR superfamily that is required for LPS responsiveness and is involved in the host defense against gram-negative bacteria (O’Neill 2000). To explore the mechanisms of DHT in UCN1 transcriptional activity in the presence of LPS, we first measured the expression of TLR4 treated with DHT plus LPS. HUVECs were pretreated with DHT for 0.5 h and LPS was added. As shown in Fig. 4A, the expression of TLR4 was increased in the presence of DHT together with LPS compared with LPS treatment only.

TLR4-mediated MAPK and NF-κB signaling plays a key role in the onset of inflammation (Buchanan et al. 2010), which has been implicated in the regulation of UCN1 expression (Brar et al. 1999, Wang et al. 2012). In fact, the activation of MAPK signaling may contribute to the activation of AP-1, another important transcription factor besides NF-κB (Su et al. 2011). To investigate the role of these two pathways in the effect of DHT on LPS-induced UCN1 expression, the phosphorylation at both RNA and protein levels were analyzed by real-time PCR (C) and western blot (D). (E) Dual-luciferase reporter assay was performed to further explore the effect of DHT on UCN1 promoter. HUVECs were transfected with pGL2-hUS2 or pGL2-Basic and treated with vehicle, DHT, LPS, and DHT + LPS for 12 h. Luciferase activities were detected. *P < 0.05, **P < 0.01, and ***P < 0.001. All the data given are the means ± S.E.M. of values obtained from more than three independent cultures, and a representative experiment is shown.

Figure 2
DHT decreased UCN1 expression in an AR-dependent manner but increased UCN1 expression in an AR-independent manner when cells were co-treated with LPS. (A and B) HUVECs were treated with vehicle, DHT (10−8 M), LPS (10 μg/ml), and DHT + LPS for 9 h (RNA) or 24 h (protein), and the expression of UCN1 at both mRNA levels and protein levels was determined by real-time PCR (A) and western blot (B). (C and D) To determine the involvement of AR, flutamide (F), the AR antagonist, was added 0.5 h before the DHT or DHT + LPS treatment. The UCN1 expressions at both RNA and protein levels were analyzed by real-time PCR (C) and western blot (D). (E) Dual-luciferase reporter assay was performed to further explore the effect of DHT on UCN1 promoter. HUVECs were transfected with pGL2-hUS2 or pGL2-Basic and treated with vehicle, DHT, LPS, and DHT + LPS for 12 h. Luciferase activities were detected. *P < 0.05, **P < 0.01, and ***P < 0.001. All the data given are the means ± S.E.M. of values obtained from more than three independent cultures, and a representative experiment is shown.

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status of NF-κB, p38MAPK, ERK1/2, and JNK was analyzed by western blot.

HUVECs were pretreated with DHT for 0.5 h and LPS was added subsequently. As depicted in Fig. 4B, DHT induced a significant phosphorylation of p38MAPK and ERK1/2 with peak activation at 2 h in the presence of LPS. Consistent with the results observed in UCN1 expression, this DHT effect was independent of AR (Fig. 4C). However, DHT failed to alter the phosphorylation of JNK (Fig. 4B).

Next, we examined the effect of DHT on the NF-κB pathway. First, the protein levels of NF-κB were determined in nuclear fractions. The NF-κB expression was increased in nuclear fractions after 2 h of treatment with LPS in the presence of DHT (treatment for 0.5 h before LPS application; Fig. 4D). Next, the phosphorylation status of NF-κB was determined by western blot. As shown in Fig. 4E, LPS treatment increased the phosphorylation of NF-κB, and DHT application significantly enhanced it. Taken together, we came to the conclusion that DHT could increase LPS-induced phosphorylation of p38MAPK, ERK1/2, and NF-κB, which may contribute to the elevated level of UCN1.

Discussion and conclusions

The aim of this study was to explore the effects and mechanisms of androgens on UCN1 expression in ECs. We examined the effects of nonaromatizable androgen DHT on UCN1 expression in the absence or presence of LPS. In that, both AR and UCN1 express in HUVECs, and the cell line of HUVECs was chosen for the research. In this study, we focused on DHT actions on UCN1 expression in ECs. Hence, the obtained data can only display the action features of DHT in HUVECs. More experiments in adult ECs are needed for adult disease-related aspects. Our data indicated that DHT alone could significantly decrease UCN1 expression at both mRNA and protein levels in an AR-dependent manner. On the contrary, in the presence of LPS, DHT increased UCN1 expression in an AR-independent manner as the AR antagonist, flutamide, did not influence this effect of DHT. TLR4 expression was elevated and TLR4-mediated NF-κB and MAPK pathways were activated. Thus, we conclude that DHT differentially influences UCN1 levels under normal and inflammatory conditions in HUVECs, in which AR-dependent and -independent mechanisms were involved respectively (Fig. 5).
UCN1 is reported to be upregulated under inflammatory conditions. In our experimental animal model, a significantly increased expression of UCN1 mRNA was found in the asthma group (Wu et al. 2006). Other investigators have shown similar findings in inflammatory diseases such as arthritis (Kohno et al. 2001), hypertension (Florio et al. 2006), and atherosclerosis (Inada et al. 2009). UCN1 is demonstrated to promote the development of vasculitis in vivo by our group (Xu et al. 2009). Moreover, UCN1-positive cells were found to be correlated with the severity of inflammation in colonic mucosa with ulcerative colitis (Saruta et al. 2004). Our observations taken together with those of other’s suggest that UCN1 is an important factor in inflammation.

Testosterone, the major androgen in circulation, is responsible for androgenic effects, but its role in cardiovascular diseases is still controversial. A key factor that can account for the discrepancies of testosterone actions is whether testosterone exerts effects either directly on AR or converts to the potent androgen, DHT, or aromatization to estrogen (Gonzales et al. 2009). DHT is considered to be a clear marker of androgen action rather than testosterone. DHT failed to convert into estrogen by aromatase, which makes the results not subject to the confounding potential interpretations of ER- and AR-mediated effects (Death et al. 2004). Our in vivo data showed that, in a laurate-induced rat’s peripheral arterial vasculitis model, the inflammatory status was much more serious in male rats than in female

Figure 4
DHT enhanced the TLR4 pathway under an inflammatory condition. (A) HUVECs were treated with vehicle, DHT, LPS, or DHT + LPS for 24 h, and the expression of TLR4 was examined by western blot. (B) HUVECs were treated with DHT plus LPS and harvested at indicated times. The phosphorylation levels of MAPKs (p38MAPK, p-ERK1/2, and JNK) were determined by western blot. (C) To explore the involvement of AR, flutamide was added. After treatment with vehicle, LPS, DHT + LPS, or flutamide (F) + DHT + LPS for 2 h, the phosphorylation levels of p38MAPK and ERK1/2 were determined. (D) The phosphorylation levels of NF-κB treated at indicated times were determined by western blot. (E) HUVECs were treated with vehicle, LPS, or DHT + LPS for 2 h; the phosphorylation levels of NF-κB were determined by western blot. *P < 0.05 and **P < 0.01. All the data given are the means ± S.E.M. of values obtained from more than three independent cultures and a representative experiment is shown.
rats (unpublished data). Furthermore, in male rats, the serum UCN1 level is higher than that in female rats. All the data suggest that UCN1 may be a key factor in gender difference of vascular inflammation. Thus, to investigate the association between DHT and UCN1 is important.

Consistent with our in vivo data, DHT increased UCN1 expression in the presence of LPS. Interestingly, under normal conditions, DHT repressed UCN1 expression. It was of interest to find that there was a specific ARE present in the human UCN1 promoter region. Similarly, Bao et al. (2006) showed a potential ARE in the human CRF promoter and testosterone repressed CRF promoter activity through the ARE. Thus, it appears that, in the absence of an inflammatory stimulus, DHT acts via the classical AR to repress UCN1 expression, but in the presence of LPS, DHT performs via an AR-independent mechanism to increase UCN1 levels. Our results are in accordance with the report that DHT differentially influences COX-2 levels under physiological and pathophysiological conditions in human coronary artery smooth muscle cells (Osterlund et al. 2010).

Our results first demonstrated that DHT increased LPS-induced TLR4 expression and augmented the TLR4-mediated signaling activation in an AR-independent manner. MAPK kinases are a group of highly conserved serine/threonine protein kinases that are involved in LPS–TLR4-mediated cytokine expression, including UCN1 (Wang et al. 2012). In human placenta, the MAPK signal transduction pathway played a pivotal role in the regulation of CRF gene expression (Cheng & Handwerger 2005). At least three distinctly regulated groups of MAPK have been identified, ERK1/2, JNK, and p38MAPK (Krishna & Narang 2008). It is well known that p38MAPK ameliorated the stabilization of ARE-containing mRNAs in posttranscriptional regulation (Kaminska 2005). In addition, activated JNK and ERK1/2 are reported to increase AP-1 activity (Rahman & MacNee 1998, Uto et al. 2005), and the promoter sequence of CRH contains an AP-1 site (Behan et al. 1993). Consistent with others, in this study, we found that ERK1/2 and p38MAPK were activated by DHT+LPS, but JNK was not affected. These results highly suggest that DHT increased UCN1 expression by augmenting the LPS-induced MAPK activation. NF-κB is another important nuclear transcription factor that is mediated by TLR4 stimulation (Jiang et al. 2000). Recently, NF-κB was shown to be involved in the regulation of UCN2 mRNA, another member of CRF family (Ikeda et al. 2009). Importantly, CRF promoter contains multiple NF-κB binding sites (Kageyama et al. 2010). Our present work demonstrated that DHT could promote the activation of NF-κB stimulated by LPS. Taken together with the above reports, DHT increased UCN1 expression in the presence of LPS and the mechanism may involve NF-κB signaling.

In conclusion, we here demonstrate that DHT alone decreases endothelial UCN1 expression via AR while increases endothelial UCN1 expression in the presence of LPS. Under normal conditions, DHT may decrease the UCN1 promoter through ARE. Under the circumstance of induced inflammation, DHT increased endothelial UCN1 and augmented LPS–TLR4 signaling.

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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