Original Article
Dihydrotestosterone modulates endothelial progenitor cell function via RhoA/ROCK pathway

Hao Zhang[1*], Liang Shi[2*], Guo-Qing Ren[1], Wen-Wen Sun[1], Yi-Bin Wang[3], Yi-Kun Chen[1], Jiang-Ning Yin[1], Bing Wan[4]

[1]Emergency Medicine Center, Departments of [2]Nuclear Medicine, [4]ICU, Affiliated Hospital of Jiangsu University, Zhenjiang 212001, China; [3]Department of Cardiology, Chunan Branch of Zhejiang Provincial People's Hospital, 311700, Zhejiang, China. *Equal contributors.

Abstract: Background: Previous findings indicate that testosterone level is negatively correlated with the incidence and mortality of cardiovascular diseases in men. Endothelial progenitor cells (EPCs) play a critical role in endothelial healing and vascular integrity. This study aimed to examine the effects of dihydrotestosterone (DHT), an active metabolite of testosterone, on human EPC function and investigate the underlying mechanism. Methods: EPCs were isolated from peripheral blood of healthy adult males and incubated with a series of concentrations (1, 10, and 100 nmol/L in dimethyl sulfoxide) of DHT for 24 h or with 10 nmol/L DHT for different periods (6, 12, 24, 36, and 48 h). EPC proliferation, migration, and adhesion were determined by MTT assay, modified Boyden chamber assay, and cell counting, respectively. Furthermore, vascular endothelial growth factor (VEGF) production was examined by ELISA, RhoA activity was determined through pull-down assay. The protein level of RhoA was quantified by Western blot analysis. Results: DHT significantly increased the proliferative, migratory, and adhesive abilities of EPCs in a dose- and time-dependent manner and upregulated the levels of VEGF and activated RhoA. However, RhoA inhibitor C3 exoenzyme or ROCK inhibitor Y-27632 significantly inhibited DHT-induced proliferation, migration, and adhesion, as well as VEGF production. Moreover, C3 exoenzyme inhibited the activation of RhoA stimulated by DHT. Conclusions: DHT promotes EPC proliferation, migration, and adhesion activities via RhoA/ROCK pathway.

Keywords: Endothelial progenitor cells, dihydrotestosterone, cardiovascular disease, RhoA/ROCK pathway

Introduction

Reconstitution and maintenance of an intact endothelial layer play a fundamental role in the prevention of cardiovascular diseases (CVDs) [1-4]. Risk factors such as hypertension, hyperlipidemia, insulin resistance, obesity, metabolic syndrome, excessive drinking and smoking, and diabetes are thought to significantly contribute to endothelial dysfunction and atherosclerosis [5-11]. Recent studies have demonstrated that an injured endothelial monolayer can be restored not only by resident cells within the wounded vascular wall but also by circulating endothelial progenitor cells (EPCs) [12-15].

As a circulating pool of cells, EPCs originally reside in the bone marrow and other putative niches and migrate into peripheral circulation and home into the sites of endothelial injury in response to many stimuli of endothelial repair and neovascularization [16]. In addition, EPCs can differentiate into mature endothelial cells [17, 18]. Although the effects of androgens on EPCs are controversial, numerous studies have demonstrated that androgen level is associated with EPC function in men, and immunohistochemical analysis showed that EPCs express a testosterone receptor. Furthermore, a synthetic androgen called methyltrienolone (R1881) can augment the proliferation, migration, and colony formation of EPCs, which could be abolished by pretreatment with flutamide [19]. EPC proliferation and adhesion are regulated by several pathways such as androgen receptor-mediated pathway and phosphatidyl-inositol-3-kinase (PI3K) signaling [20]. However, the molecular mechanisms by which androgen modulates EPCs remain unclear. Therefore, in this study we aimed to determine the signaling pathway

underlying the effects of male hormones on EPCs.

Rho family is an important member of small G proteins that regulate a diverse array of cellular processes, including cytoskeletal dynamics, cell polarity, membrane transport, and gene expression [21]. RhoA and Rho-associated kinases (ROCKs) are widely studied as prototypical members of Rho family [22]. RhoA/ROCK signaling pathway plays an important role in angiogenesis and may be a promising target for antiangiogenic treatment [23]. RhoA/ROCK has been indicated as an upstream regulator of mitogen-activated protein kinase (MAPK) family members, including extracellular signal-regulated protein kinase, c-Jun-NH2 kinase, and p38 MAPK (p38) [24-26]. Recently, Sandra et al. demonstrated that EPCs proliferated through MEK-dependent p42 MAPK signaling pathway [27]. Thus we speculated that the activation of RhoA/ROCK pathway might play a significant role in the imodulation of EPC function by androgens. We investigated the effects of dihydrotestosterone (DHT), an active metabolite of testosterone, on human EPCs and explored the role of RhoA/ROCK signaling in this process.

Methods

EPC isolation and culture

This study conforms to the principles outlined in the Declaration of Helsinki for use of human tissue and was approved by the Institutional Review Board of the Affiliated Hospital of Jiangsu University. Detailed informed consent for the procedures was obtained from all subjects. EPC was isolated and cultured as previously described [5, 20]. Briefly, peripheral blood mononuclear cells (PB-MNCs) were fractionated from other blood components through density gradient centrifugation using Ficoll solution according to the manufacturer's instructions. PB-MNCs were plated on fibronectin-coated 24-well dish at a density of 1×10^6 cells/well and maintained in EGM endothelial cell growth medium (Clonetics, Lonza, MD, USA) at 37°C in a humidified 5% CO_2 incubator. EMG medium consisted of endothelial basal medium-2 (EBM-2), gentamicin, vascular endothelial growth factor (VEGF), 5% fetal bovine serum, fibroblast growth factor-2, insulin-like growth factor-1, epidermal growth factor, and ascorbic acid. Culture medium was first changed on day 4 and then

every 2 days. Starting from day 7, culture medium was supplemented with 1, 10, or 100 nmol/L DHT to examine the effects of on cultured EPCs. The adherent cells were selected for the experiment after 48 h.

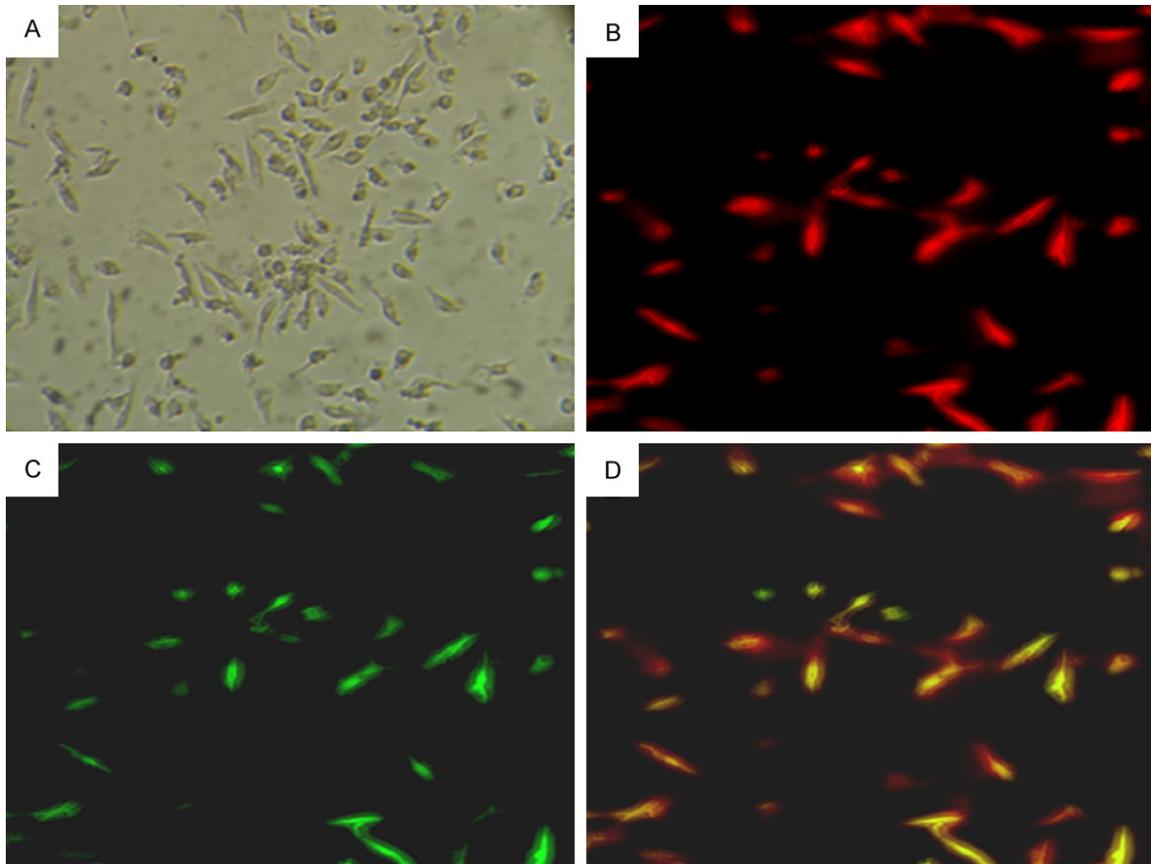### Characterization of cultured EPCs

Fluorescent detection of EPCs was performed on the attached MNCs after 7 days of culture. Those adherent cells were washed three times with PBS, incubated with 2.4 µg/mL 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated low-density lipoprotein (acLDL; Molecular Probe, Invitrogen, USA) at 37°C for 1 h and then fixed with 2% paraformaldehyde for 10 min. The cells were then incubated with 10 µg/mL fluorescein isothiocyanate (FITC)-conjugated *Ulex europaeus* agglutinin (UEA)-I (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. After staining, the samples were observed under a laser scanning confocal microscope (×200). Those attached cells with DiI-AcLDL and FITC-UEA-I double-positive were identified as early EPCs.

Proliferation assay

EPC proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [20, 22, 27]. Briefly, cells were digested with 0.25% trypsin (Keyi, Hangzhou, China) and then diluted to 2×10^5/mL. Cell suspension was seeded into 96-well plates (100 ml/well) and cultured in serum-free EGM-2 medium for 24 h. EPCs in each well were treated with DHT at different concentrations (0, 1, 10, and 100 nmol/L) and cultured for 48 h. Next 20 ml of MTT (5 g/L; Sigma-Aldrich) were added into each well and incubated for another 4 h. The supernatant was removed, and 150 ml dimethyl sulfoxide were added into each well and the plates were shaken for 10 min before the optical density (OD) was measured at 490 nm. RhoA inhibitor C3 exoenzyme (30 mg/mL; Alexis Biochemicals, Carlsbad, CA, USA) and ROCK inhibitor Y-27632 (10 mM; Sigma, St. Louis, MO, USA) were added 1 h before DHT treatment.

EPC migration assay

EPC migration was evaluated using a Transwell chamber (Greiner; Monroe, NC, USA) with 8 mm-pore filters as previously described [22].

Figure 1. Characterization of EPCs. (A) The attached cells exhibited an oval or spindle morphology after 7 days of culture. Adherent cells stained with Dil-AcLDL (B. Red; excitation wave length at 543 nm) and FITC-UEA-I (C. Green; excitation wave length at 477 nm) were detected under a fluorescent microscope. Double positive cells (D. Yellow) were identified as differentiating early EPCs. Magnification at ×200.

The cultured cells were digested with 0.25% trypsin and diluted to $2×10^5$/mL. EPCs were placed in the upper chamber of a Transwell chamber (100 μl/well). VEGF in serum-free EBM-2 medium was placed in the lower compartment of the chamber and EPCs were incubated at 37°C in 5% humidified CO_2 for 24 h. The lower face of the filter was washed with PBS, and cells remaining on the upper side were removed using a cotton wool swab. EPCs were then fixed with 2% paraformaldehyde for 10 min and stained with hematoxylin for 30 min. Migratory cells in the lower chamber were counted in five random microscopic fields (×200). RhoA inhibitor C3 exoenzyme or ROCK inhibitor Y-27632 was added 1 h before DHT treatment.

EPC adhesion assay

EPC adhesion was evaluated as previously described [20, 22]. Briefly, EPCs were washed with PBS and gently detached with 0.25% trypsin after treatment overnight with DHT at different concentrations (0, 1, 10, and 100 nmol/L) alone or in combination with RhoA inhibitor C3 exoenzyme or ROCK inhibitor Y-27632. An equal number of EPCs were seeded into fibronectin-coated 96-well plates and incubated for 30 min at 37°C in 5% humidified CO_2. The adherent cells were independently counted by blinded investigators in five randomly selected microscopic fields (×200).

ELISA

VEGF protein level in conditioned medium was detected by ELISA. EPCs were treated as described above. The conditioned medium was collected, and VEGF level in the conditioned medium was measured using a high-sensitivity ELISA kit (Bender, Vienna, Austria) according to the manufacturer's instructions.
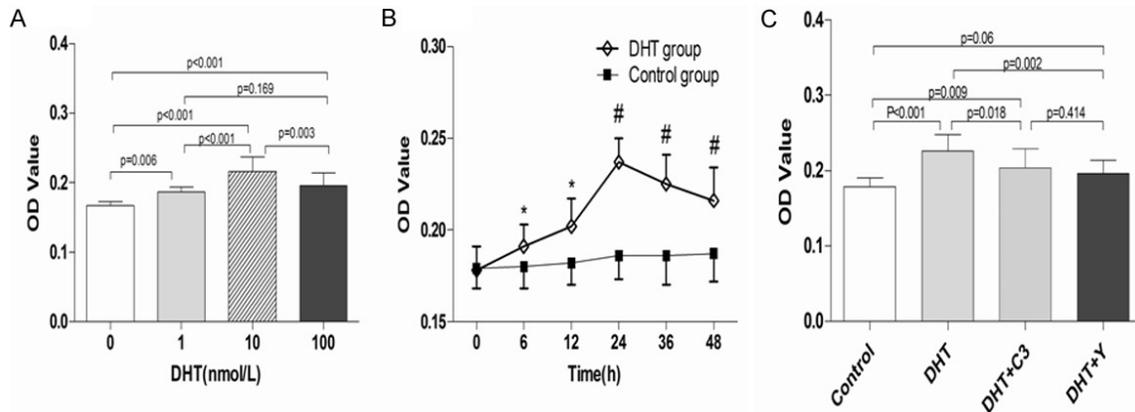
Figure 2. Effects of DHT, C3 exoenzyme, and Y-27632 on EPC proliferation. A. DHT dose-dependently promoted the proliferation of EPCs, with a maximal effect achieved at 10 nmol/L DHT. B. 10 nmol/L DHT increased EPC proliferation time-dependently. C. C3 exoenzyme and Y-27632 attenuated the stimulatory effects of DHT on EPC proliferation. The data are expressed as the mean ± SD; *n* = 10 separate experiments. *P < 0.05; #P < 0.01 versus control; *DHT:* dihydrotestosterone; *C3:* C3 exoenzyme; *Y:* Y-27632.

RhoA activation assay

RhoA activation assay was performed as previously described with some modifications [22, 28]. RhoA activity was determined by pull-down assay using a Rho activation assay kit (Cytoskeleton, USA) [29]. After culture in serum-free EBM-2 medium for 24 h, EPCs were treated with C3 exoenzyme for 48 h before treatment with 10 nmol/L DHT and then lyzed in lysis buffer. Cell lysates were incubated with agarose-conjugated Rhotekin-RBD at 4°C for 1 h, and then Agarose beads were boiled to release active Rho. Precipitation with Rhotekin GTP-Rho was performed thereafter. Finally, Rho protein was detected by specific anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). 20 µg of total cell lysate per sample was used to detect the total amount of RhoA.

Western blot analysis

EPCs were collected and lysed in lysis buffer. Total protein content was quantified using the BCA Protein Assay Kit (Boster, Wuhan, Hubei, China). Equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Bedford, MA, USA), which were blocked with 5% BSA for 1 h and then incubated with antibodies against RhoA (1:1000). The membranes were washed in TBST and subsequently incubated with HRP-conjugated goat anti-rabbit IgG antibody (1:2500; MultiSciences, Hangzhou, Zhejiang,

China). Antigen-antibody complex was quantified using an ECL Plus system (Amersham Bioscience, Piscataway, NJ, USA).

Statistical analysis

All data are presented as mean ± SD. Statistical significance was analyzed using unpaired Student's *t*-test or ANOVA for comparisons between two groups and multiple comparisons, respectively. SPSS version 17.0 was used for the analysis, and P < 0.05 was considered statistically significant.

Results

EPC characterization

MNCs were isolated from peripheral blood and cultured for 7 days, resulting in a spindle-shaped morphology and formed cell colonies as previously reported [17, 30, 31] (**Figure 1A**). EPCs were characterized as spindle-shaped adherent cells double positive for DiI-AcLDL (DiI-labeled AcLDL) uptake and FITC-UEA-I (FITC-conjugated Bandeiraea simplicifolia lectin I) binding using LSCM (87.2% ± 6.5%) (**Figure 1D**). Flow cytometry confirmed the expression of CD133 (82.2% ± 5.6%), VEGFR-2 (93.6% ± 5.7%) and CD34 (85.3% ± 6.2%) in EPCs (**Figure 1C**).

*DHT promotes EPC proliferation in vitro*

EPC proliferation was examined using MTT assay. We observed that DHT dose-dependent-
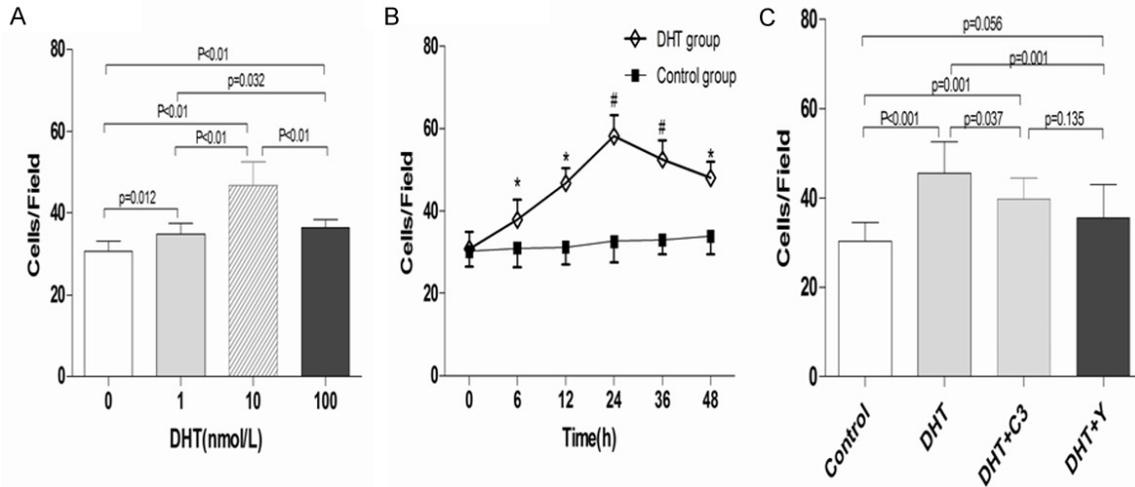
Figure 3. Effects of DHT, C3 exoenzyme, and Y-27632 on EPC adhesion. Cell counting assay was used to measure the adhesive activity of EPCs (×200). A. EPCs were incubated with DHT of different concentrations for 24 h. DHT concentration-dependently enhanced EPC adhesion, with a maximal effect achieved at 10 nmol/L. B. DHT time-dependently enhanced EPC adhesion. C. C3 exoenzyme and Y-27632 significantly decreased the adhesion of EPCs enhanced by DHT. *$P < 0.05$; #$P < 0.01$ versus control; data are presented as the mean ± SD; n =10 separate experiments. *DHT* dihydrotestosterone; *C3:* C3 exoenzyme; *Y:* Y-27632.
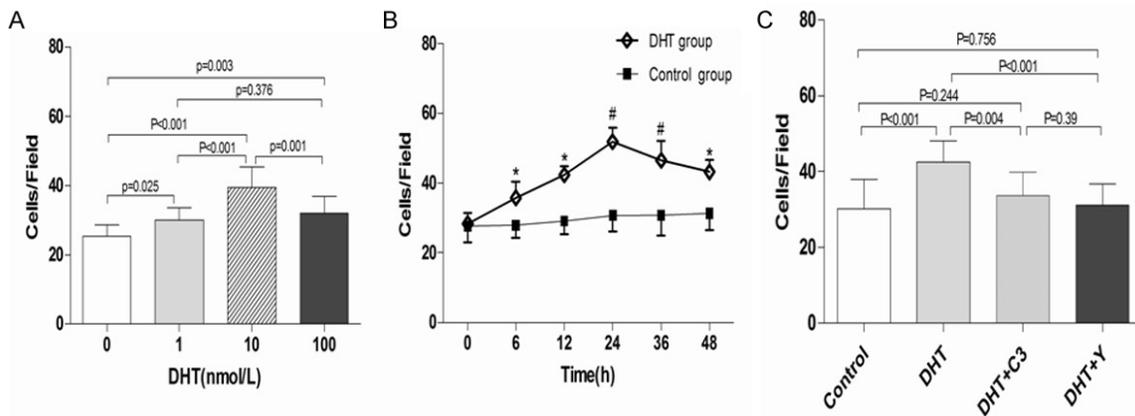
Figure 4. Effects of DHT, C3 exoenzyme, and Y-27632 on EPC migration. Transwell chamber assay was used to evaluate EPC migration (×200). A. EPCs were incubated with DHT at a series of concentrations for 24 h. DHT concentration-dependently promoted EPC migration. B. DHT time-dependently enhanced the migration capacity of EPCs. C. C3 exoenzyme and Y-27632 attenuated EPC migration enhanced by DHT. EPCs without treatment served as the control group. *$P < 0.05$; #$P < 0.01$ versus control; data are presented as the mean ± SD; n = 10 separate experiments. *DHT* dihydrotestosterone; *C3*: C3 exoenzyme; *Y:* Y-27632.

ly promoted the proliferation of EPCs, with maximal effect at 10 nmol/L DHT (10 nM DHT group vs. control group, 0.216 ± 0.204 vs. 0.167 ± 0.056, P < 0.001; **Figure 2A**). Time-course experiments showed that DHT improved EPC proliferation time-dependently, reaching a peak value at 24 h (P < 0.01; **Figure 2B**). However, after pretreatment with C3 exoenzyme or Y-27632, the proliferation activity of EPCs was significantly inhibited even in the presence of

DHT (C3 exoenzyme + DHT vs. DHT, 0.204 ± 0.026 vs. 0.226 ± 0.022, P = 0.018; Y-27632 + DHT vs. DHT, 0.196 ± 0.018 vs. 0.226 ± 0.022, P = 0.002) (n = 10, **Figure 2C**).

DHT promotes EPC adhesion in vitro

The adhesive capacity of EPCs was promoted by DHT in a dose-dependent manner, with maximal effect at 10 nmol/L DHT (10 nM DHT group
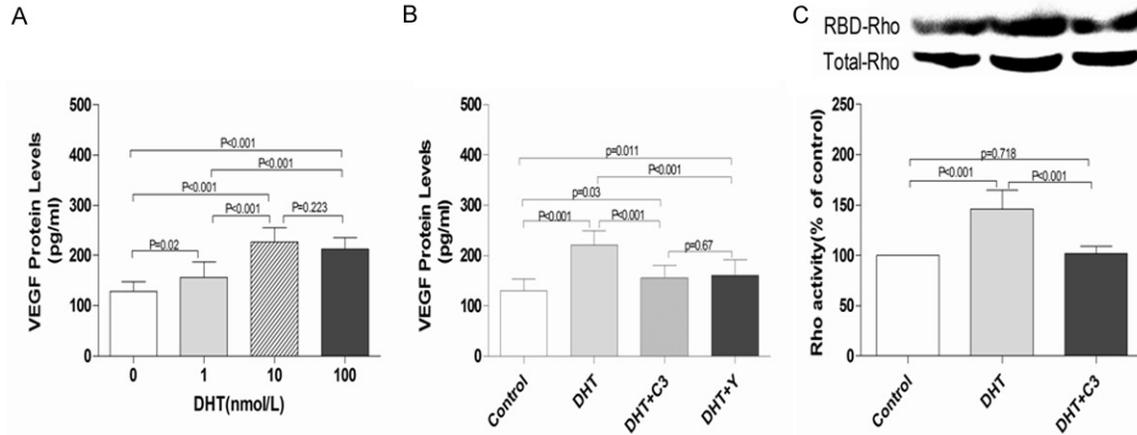
Figure 5. Effects of DHT, C3 exoenzyme, and Y-27632 on VEGF secretion and RhoA activation in EPCs. A. EPCs were incubated with DHT at different concentrations (0, 1, 10, and 100 nmol/L) for 24 h, VEGF protein levels in culture medium were determined by ELISA. DHT dose-dependently increased VEGF secretion with the maximal effect achieved at 10 nmol/L. B. C3 exoenzyme or Y-27632 markedly attenuated VEGF protein secretion induced by DHT. EPCs without treatment served as the control group. Data are presented as the mean ± SD; *n* = 10 separate experiments. *DHT* dihydrotestosterone; *C3*: C3 exoenzyme; *Y*: Y-27632. C. Serum-starved EPCs were pretreated with or without C3 exoenzyme (30 ng/mL) before incubation with 10 nmol/L DHT for 15 min. RBD-RhoA proteins and the total amount of RhoA were examined by Western blot analysis. The ratio of active RhoA vs. total RhoA was calculated to indicate the activation of RhoA. The results are representative of three independent experiments. EPCs without treatment served as the control group. Data are presented as the mean ± SD; *n* = 10 separate experiments. *DHT*: dihydrotestosterone; *C3*: C3 exoenzyme.

vs. control group, 46.8 ± 5.67 vs. 30.6 ± 2.63, P < 0.001; **Figure 3A**). Time-course experiments showed that DHT promoted the adhesive capacity of EPCs in a time-dependent manner, with maximum effect at 24 h and then gradually decreased (P < 0.01; **Figure 3B**). However, C3 exoenzyme or Y-27632 reversed the enhanced EPC adhesion ability induced by DHT (C3 exoenzyme + DHT vs. DHT, 39.7 ± 4.74 vs. 45.5 ± 7.06, P = 0.037; Y-27632 + DHT vs. DHT, 35.6 ± 7.31 vs. 45.5 ± 7.06, P = 0.001; **Figure 3C**).

DHT promotes EPC migration in vitro

Transwell chamber assay was performed to determine the effect of DHT on EPC migration. The results revealed that DHT dose-dependently increased the migratory activity of EPCs, with the maximum migration rate at 10 nmol/L DHT (10 nM DHT group vs. control group, 39.4 ± 5.97 vs. 25.4 ± 3.13, P < 0.001; **Figure 4A**). The migration capacity of EPCs reached a maximum at 24 h and then progressively declined in time-course experiments with 10 nmol/L DHT (**Figure 4B**). However, the enhanced effect of DHT on EPC migration was attenuated with the administration of C3 exoenzyme or Y-27632 (C3 exoenzyme + DHT vs. DHT, 33.6 ± 6.28 vs.

42.4 ± 5.76, P = 0.004; Y-27632 + DHT vs. DHT, 31.1 ± 5.7 vs. 42.4 ± 5.76, P < 0.001; **Figure 4C**).

DHT upregulates VEGF secretion by EPCs

The protein concentration of VEGF in conditioned medium of EPCs was examined by using an ELISA kit. The results showed that DTH increased the secretion of VEGF by EPCs in a concentration-dependent manner, with the maximal effect at 10 nmol/L (DHT vs. control, 227.25 ± 28.17 vs. 128.82 ± 19.51 pg/mL; P < 0.001; **Figure 5A**). However, RhoA/ROCK inhibitor C3 exoenzyme and Y-27632 attenuated DHT-induced EPC secretion of VEGF (C3 exoenzyme + DHT vs. DHT, 156.60 ± 23.73 vs. 221.04 ± 28.24, P < 0.001; Y-27632 + DHT vs. DHT, 161.63 ± 29.69 vs. 221.04 ± 28.24, P < 0.001) (n = 10, **Figure 5B**).

Effects of DHT and C3 exoenzyme on RhoA activation in EPC in vitro

RhoA activity was determined through pull-down assay. The results showed that the active form of RhoA (GTP-bound) was elevated to 146.00% ± 18.67% after DHT treatment compared with control (**Figure 5C**). Pretreatment

with C3 exoenzyme significantly inhibited RhoA activation induced by DHT (C3 exoenzyme + DHT vs. DHT, 101.90% ± 7.52% vs. 146.00% ± 18.67%, P < 0.01; **Figure 5C**).

Discussion

In this study, we demonstrated that DHT significantly enhanced the proliferation, adhesion, and migration of human EPCs and upregulated VEGF secretion in vitro in a dose-dependent manner within a specific concentration range. Furthermore, we revealed that the stimulatory effects of DHT on EPC activity are dependent on RhoA/ROCK pathway. These results support the potential role of DHT in upregulating EPC function and may provide novel strategy for the prevention and treatment of early atherosclerosis associated with EPCs and endothelial damage.

The role of androgens in the cardiovascular system is controversial. In clinical studies, androgen excess and deficiency have been reported to exert negative effects on cardiovascular parameters and endothelial function [32, 33]. Moreover, a low level of androgen is closely correlated to increased male CVD morbidity and mortality [34-36]. Integrated vascular endothelium is associated with the protection of cardiovascular system. Numerous environmental factors, especially cardiovascular risk factors, would damage the structure and function of the endothelium, resulting in the development of various diseases [37]. Endothelial dysfunction may induce a series of pathogenic events, including atherosclerosis, hypertension, diabetes, and thrombosis [38, 39]. Therefore, the repair of endothelial dysfunction is essential to the prevention of the initiation and development of atherosclerosis [40]. Accumulating evidence has suggested that EPCs are involved in multiple aspects of this repair process and play a significant role in the protection of cardiovascular function [41-43]. Accelerated re-endothelialization by EPCs may effectively abrogate smooth muscle cell proliferation, migration, and neointima formation, thereby preventing the initiation and development of atherosclerosis and restenosis following vascular injury [41, 44, 45]. Therefore, great efforts have been taken to improve EPCs function through different strategies ranging from available medication to the frontiers of cell therapy [18].

Recent studies have indicated that androgens or their derivatives have conspicuous protec-

tive effects on vascular function [46, 47]. Androgens can stimulate endothelial progenitor cells through an androgen receptor-mediated pathway [19]. Moreover, DHT can modulate EPC proliferation and adhesion via PI3K/Akt pathway [20]. These findings collectively suggest that androgens play important role in regulating EPCs in human. However, the molecular mechanisms remain unclear. In the present study, we explored the effects of DHT, an active metabolite of testosterone, on EPCs. Our results revealed that DHT could promote proliferation, migration, and adhesion of EPCs in a dose-dependent manner, with the peak value at 10 nmol/L DHT. These findings are partly consistent with the results of Liu et al. [20] but in contrast to other studies. Several reasons may explain the discrepancy, including the use of different androgens, androgen concentrations, various experimental conditions, as well as EPC culture methods.

We further demonstrated that DHT promoted the proliferative, migratory, and adhesive capacities of EPCs via RhoA/ROCK pathway. A growing body of experimental evidence revealed that RhoA/ROCK pathway is crucial to many cell physiological and pathological processes, including cytoskeletal dynamics, cell polarity, cell proliferation, survival and migration. A recent study showed that zoledronate attenuated the angiogenic effects of angiotensin II-stimulated endothelial progenitor cells through RhoA and MAPK signaling [22]. Bryan et al. revealed that RhoA/ROCK signaling is essential for multiple aspects of VEGF-mediated angiogenesis [23]. In this study, RhoA/ROCK signaling pathway was blocked by C3 exoenzyme or Y-27632, and the proliferative, migratory, and adhesive ability of EPCs were significantly attenuated. In addition, the effect of DHT on EPC secretion of VEGF was investigated in the present study. The results indicate that DHT enhanced the secretion of VEGF by EPCs, an effect that was effectively abrogated by C3 exoenzyme or Y-27632. These data suggest that the role of DHT in the promotion of EPC function may be due to the stimulation of paracrine effects of EPC through the secretion of VEGF.

Conclusion

This study demonstrates that DHT enhances the proliferative, migratory, and adhesive abilities of EPCs and promotes the secretion of VEGF by EPCs. Furthermore, these effects of

DHT are mostly likely mediated by RhoA/ROCK signaling. Our data suggest that DHT may play a protective role in vascular repair, especially for patients with low levels of androgens.

Acknowledgements

Disclosure of conflict of interest

None.

Authors' contribution

HZ and LS carried out the studies and were co-first author. GR and WS participated in the design of the study. YW, YC and JY helped to finish the studies. BW drafted the manuscript. All authors read and approved the final manuscript.

Address correspondence to: Dr. Bing Wan, Department of ICU, Affiliated Hospital of Jiangsu University, 438 Jiefang Road, 212001, Jiangsu, China. Tel: +86-511-8502-6995; Fax: +86-511-8502-6995; E-mail: 13511690653@163.com

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