Thyroid hormone induced angiogenesis through the integrin $\alpha v \beta 3$ /protein kinase D/histone deacetylase 5 signaling pathway

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Abstract

Thyroid hormone is reported to induce angiogenesis, which is mediated by the membrane receptor integrin $\alpha v \beta 3$, but the precise signaling pathway is still not very clear. Recently, studies have shown that protein kinase D (PKD) regulates the recycling of integrin $\alpha v \beta 3$, which is required for cell migration. Moreover, phosphorylated PKD stimulates histone deacetylase 5 (HDAC5) phosphorylation and nuclear export in endothelial cells. As a potent pro-angiogenic growth factor, basic fibroblast growth factor (*bFGF* (*FGF2*)) is a downstream target gene of HDAC5. Therefore, we examined the hypothesis that a novel signaling pathway through integrin $\alpha v \beta 3$ /PKD/HDAC5 might contribute to thyroxine (T_4)-induced angiogenesis. We selected human umbilical vein endothelial cells (HUVECs) for treatment. Angiogenesis was assessed using wound-healing and tubulogenesis assays. Signaling molecules, including phosphorylated PKD and HDAC5, were measured by western blotting. *bFGF* mRNA was analyzed by real-time PCR. Our results showed that T_4 (100 nmol/l) stimulated the migration and formation of tube-like structures of HUVECs, whereas tetraiodothyroacetic acid (Tetrac, 100 nmol/l) inhibited T_4 -induced cell migration. Importantly, T_4 promoted the phosphorylation of PKD and HDAC5. These effects were inhibited respectively by Tetrac, PKC inhibitor (2.5 μ mol/l) and PKD siRNA. Meanwhile, T_4 could promote the cytoplasmic accumulation of phosphorylated HDAC5 in HUVECs. In addition, *bFGF* mRNA expression in HUVECs significantly increased within 2 h of T_4 treatment, but was decreased by Tetrac. Our findings indicate that T_4 increases the expression of *bFGF* mRNA via the integrin $\alpha v \beta 3$ /PKD/HDAC5 signaling pathway, which plays an important role in angiogenesis.

Key Words

- thyroid hormone
- angiogenesis
- integrin $\alpha v \beta 3$
- protein kinase D
- histone deacetylase 5
- basic fibroblast growth factor
- human umbilical vein endothelial cells

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Introduction

Angiogenesis, the formation of new blood capillaries, is an important component of embryonic vascular development, wound healing and organ regeneration. It also contributes to pathological processes, including diabetic retinopathy, atherosclerosis and tumor growth

(Folkman 1995, Carmeliet *et al.* 2003, Ferrara *et al.* 2003). The process of angiogenesis includes coordinated endothelial cell proliferation, invasion, migration, and tube formation. It is orchestrated by different growth factors, such as vascular endothelial growth factor (VEGF),

angiopoietins, basic fibroblast growth factor (bFGF) and so on (di Blasio *et al.* 2010).

Recently, effects of thyroid hormones, including L-thyroxine (T₄), and 3,5,3'-triiodo-L-thyronine (T₃), on angiogenesis have been observed in studies of the chick chorioallantoic membrane model (Davis *et al.* 2004, Mousa *et al.* 2006a,b) and the human dermal microvascular endothelial cell microtubular model (Mousa *et al.* 2006a,b). Bergh *et al.* (2005) identified integrin $\alpha v \beta 3$ as a cell surface receptor for thyroid hormone (T₄) and as the initiation site for T₄-induced angiogenesis. The extracellular domain of integrin $\alpha v \beta 3$ includes an Arg-Gly-Asp (RGD) recognition site that is an important verification domain for the ligands of the protein (Plow *et al.* 2000). The thyroid hormone receptor on integrin $\alpha v \beta 3$ is located at or near the RGD recognition site. It is reported that T₄ is bound with greater affinity to integrin than T₃. By binding to integrin $\alpha v \beta 3$, the thyroid hormone signal is transduced by the MAPK (extracellular regulated kinase 1/2, ERK1/2) cascade (Davis *et al.* 2004, Bergh *et al.* 2005). Several complex downstream events occur as a result of the activation of ERK1/2, including regulation of the activity of the plasma membrane Na⁺/H⁺ antiporter (D'Arezzo *et al.* 2004), an increase in activity of cell membrane Na⁺, K⁺-ATPase (sodium pump) (Lei *et al.* 2008), secretion of some vascular growth factors including bFGF, consequent angiogenesis (Davis *et al.* 2004), etc. However, the precise signaling system of T₄-induced angiogenesis has not been discovered yet.

There is recycling of integrin $\alpha v \beta 3$ in cells, which is required for cell migration (Ramsay *et al.* 2007, Mai *et al.* 2011). di Blasio *et al.* (2010) found that protein kinase D1 (PKD1), a novel serine/threonine protein kinase, promotes integrin $\alpha v \beta 3$ recycling and inhibits its endocytosis. PKD1 is also reported to be phosphorylated in endothelial cells in response to VEGF and serves as a histone deacetylase (HDAC) kinase (Vega *et al.* 2004a,b). This signaling was confirmed by Ha *et al.* (2008a,b) to mediate VEGF-induced angiogenesis. They suggested that through a VEGF receptor 2-phospholipase C γ (PLC γ)–protein kinase C (PKC)–PKD1-dependent pathway, VEGF stimulates HDAC5 phosphorylation and nuclear export in endothelial cells, which induces gene expression and angiogenesis. Besides HDAC5, another class II a HDAC family member, HDAC7, which was also reported by Ha *et al.* (2008a,b), is activated through the VEGF–PKD1-dependent pathway and accumulates in the cytoplasm of endothelial cells.

HDAC5-dependent regulation of gene expression is well known to control angiogenesis. By binding to the promoter of the *bFGF* (*FGF2*) and *SLIT2* genes, HDAC5

represses the expression of these pro-angiogenic genes, whereas HDAC5 silencing promotes capillary sprouting of endothelial cells (Urbich *et al.* 2009). Accordingly, we propose that a new signaling pathway of integrin $\alpha v \beta 3$ /PKD/HDAC5 is involved in regulating *bFGF* expression, which further affects the angiogenesis induced by thyroid hormone.

Materials and methods

Materials

L-T₄ and tetraiodothyroacetic acid (Tetrac) were purchased from Sigma–Aldrich. MAB to GAPDH, β -actin and PKC inhibitor (bisindolymaleimide I, HCl, Bis) were purchased from Santa Cruz Biotechnology, Inc. The Nuclear and Cytoplasmic Protein Extraction Kit was obtained from KeyGEN BioTECH (Nanjing, Jiangsu, China). Anti-phosphorylated PKD, PKD, phosphorylated HDAC5 and HDAC5 were obtained from Cell Signaling Technology (Beverly, MA, USA). Matrigel was obtained from BD Biosciences (Franklin Lakes, NJ, USA). GenMute siRNA Transfection Reagent was purchased from SignaGen Laboratories (Ijamsville, MD, USA).

Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs, ATCC, Manassas, VA, USA) were cultured in DMEM/F12 (Invitrogen) supplemented with 1000 U/l penicillin, 1 mg/ml streptomycin and 10% (v/v) fetal bovine serum (FBS) in an atmosphere of 5% CO₂ at 37 °C. For experimental purposes, 80% confluent HUVECs were preincubated for 24 h in a serum-free medium, the cells were treated for 15, 30, and 45 min with vehicle or T₄ (100 nmol/l). Tetrac (100 nmol/l) and Bis (2.5 μ mol/l) were used to pretreat the cells for 30 min before adding T₄.

Western blotting

Whole-cell lysates were prepared in RIPA buffer (Sigma–Aldrich) containing complete protease inhibitors (Roche Diagnostics Ltd), and protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific UK Ltd, Leicestershire, UK). Thirty micrograms of proteins were separated on an 8% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane for immunodetection. The membrane was then blocked in Tris-buffered saline with 0.25% Tween 20 (TBST) containing 5% (w/v) dried skimmed milk powder

followed by overnight incubation at 4 °C with gentle shaking with primary antibody. The membrane was washed four times in TBST and incubated with the appropriate HRP-conjugated secondary antibody (Dako, Ely, UK) for 60 min at room temperature. The membrane was washed four times again in TBST. ECL plus reagents (GE Healthcare, Princeton, NJ, USA) were added to the membrane following the manufacturer's protocol. Any differences in protein phosphorylation due to variations in loading were routinely corrected by normalization to the levels of the appropriate total protein.

Nuclear and cytoplasmic protein extraction

HUVECs (5×10^6 – 1×10^7 /ml) were collected and washed with cold PBS. The pellet was added with 200 µl cytoplasmic lysis buffer (with 0.5 µl 10 mM DL-dithiothreitol, 1 µl 100 mM phenylmethylsulphonyl fluoride (PMSF), and 0.5 µl 1 mM protease inhibitors) and placed on ice for 15 min. Then the cells were centrifuged at 16 000 *g* for 5 min at 4 °C. The supernatant was carefully collected and cytoplasmic protein extract was obtained. One hundred microliters of nuclei lysis buffer (with 0.1 µl DL-dithiothreitol, 0.5 µl 100 mM PMSF, and 0.1 µl protease inhibitors) were added to resuspend centrifugal precipitate (nuclear), which was placed on ice for 40 min, then centrifuged at 16 000 *g* for 15 min at 4 °C. The supernatant was carefully removed and nuclear protein extract was obtained.

RNA interference

Cells were transfected with control siRNA (negative control, Shanghai GenePharma Co., Ltd, Shanghai, China) or siRNA specific to *PKD* (Shanghai GenePharma Co., Ltd) using GenMute transfection reagent (SignaGen

Laboratories) according to the manufacturer's protocols (Table 1). HUVECs were seeded at a concentration of 5×10^5 cells in six-well plates containing DMEM/F12 without FBS for 24 h. On the day of transfection, the siRNA–transfection reagent complex was prepared by diluting 25 nmol/l siRNA. This was followed by the addition of GenMute transfection reagent, gentle mixing and incubation for 20 min at room temperature. The siRNA–GenMute transfection reagent complexes were added dropwise to the cells. Cells were gently mixed and incubated for 6 h, following which *T₄* and DMEM/F12 without FBS were added. Cells were incubated for 48 h before RNA or protein extraction.

Real-time PCR

Cells were harvested in TRIzol (Invitrogen) and total RNA was extracted according to the manufacturer's protocol. Primer pairs for *bFGF* were designed using the Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>). Basic Local Alignment Search Tool (BLAST) was used to ensure the specificity of the primer pair. RNA (2 µg) was subjected to RT-PCR using the Access RT-PCR system (Promega). Total RNA was reverse transcribed into cDNA at 70 °C for 5 min, then at 42 °C for 60 min. Second-strand synthesis and PCR amplification were performed for 40 cycles with denaturation at 95 °C for 30 s, annealing at 58 °C for 60 s, and extension at 70 °C for 120 s, with final extension at 68 °C for 7 min after completion of all cycles. All of the quantitative real-time RT-PCR measurements were performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with SYBR Premix Ex Taq (Takara Bio, Inc., Shiga, Japan), according to the manufacturer's instructions. The primer pairs were as follows: *bFGF*, forward: 5'-TGG CTA TGA AGG AAG ATG GA-3' and reverse: 5'-ACT GCC CAG TTC GTT TCA GT-3'; and *UBC*, forward: 5'-GTG GAT CGC TGT GAT CGT CAC-3' and reverse: 5'-TGT CAC TGG GCT CAA CCT CG-3'. *UBC* was used as the control amplicon. The results were measured by the formula: $2^{-\Delta\Delta Ct}$. $-\Delta\Delta Ct = -((\text{target gene in sample Ct} - \text{UBC gene in sample Ct}) - (\text{target gene in control Ct} - \text{UBC gene in control Ct}))$.

Wound-healing assay

HUVECs were seeded in six-well plates with growth medium containing 10% FBS. The cells were allowed to grow to form a confluent monolayer. The wound-induced migration was triggered by scraping the cells with a plastic pipette tip, and the wound was imaged immediately.

Table 1 Oligonucleotide sequences of siRNA

	siRNA sequence
<i>PKD</i> (1)	Forward: 5'-CUGGUACGUCAAGGUCUUAATT-3' Reverse: 5'-UAAGACCUUGACGU ACCAGTT-3'
<i>PKD</i> (2)	Forward: 5'-CACCAUCAGAGUCGUUAUUT-3' Reverse: 5'-AUAAACGACUCUGAUGGUGTT-3'
<i>PKD</i> (3)	Forward: 5'-CGGCAAUGUAGUGUAUUATT-3' Reverse: 5'-UAAUACACUACAUUUGCCGTT-3'
Negative FAM control	Forward: 5'-UUCUCCGAACGUGUCACGUTT-3' Reverse: 5'-ACGUGACACGUUCGGAGAATT-3'
Negative control	Forward: 5'-UUCUCCGAACGUGUCACGUTT-3' Reverse: 5'-ACGUGACACGUUCGGAGAATT-3'
<i>GAPDH</i> positive control	Forward: 5'-GUAUGACAACAGCCUAAAGTT-3' Reverse: 5'-CUUGAGGCUGUUGUCAUACTT-3'

The cells were then treated with T₄ or Tetrac. The wound was imaged at different intervals as indicated using an inverted phase-contrast microscope. The wound gap was measured on the images and the distance of migration was calculated as wound gap at 0 h – wound gap at x h, where x is the time point of measurement, and the average wound-healing time was calculated based on at least nine determinations of the wound (Sharlow *et al.* 2008).

Tubulogenesis assay

The effect of thyroid hormone on tubulogenesis was tested by determining the formation of capillary-like tubes in 96-well plates loaded with Matrigel. Matrigel was thawed at 4 °C overnight before use. A total of 440 μ l of Matrigel were mixed with 2×10^4 HUVECs in a total volume of 220 μ l. A 50 μ l aliquot of the Matrigel cell mixture was then added to each well and incubated at 37 °C for 30 min to allow gel formation. This was followed by the addition of 250 μ l culture medium and incubation at 37 °C for 48 h. After washing, the cell medium was replaced with culture medium mixed with thyroid hormone (T₄: 100 nmol/l), which was replaced every 24 h for 2 days. The tube-like structures inside the wells were fixed with 4% formaldehyde and photographed (Zhang *et al.* 2010). The total length of the capillary-like network that was formed

within the Matrigel was measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The data were summarized as mean $\pm$ s.e.m. Unpaired Students' *t*-test was used for comparisons between two groups. For multiple comparisons, statistical analysis was performed by ANOVA. *P* values <0.05 were considered to be significant.

Results

T₄ promoted the migration and tubulogenesis of HUVECs

The effect of T₄ on cell migration was examined by wound-healing assay in HUVECs. Confluent HUVECs were wounded and then treated with T₄ or Tetrac. We found that the migration ability of HUVECs changed with time and was different in each group (Fig. 1). The T₄ (100 nmol/l) group showed a longer distance of migration than the control group, whereas the Tetrac (100 nmol/l) + T₄ group showed a shorter distance of migration than T₄ group at 8, 12, and 24 h (*P* < 0.05), which indicated that T₄ was able to induce HUVEC migration, and that Tetrac significantly

Figure 1

Effects of T₄ and Tetrac on migration of HUVECs in wound-healing assay. HUVECs were grown to 70% confluence in six-well plates. The monolayer was wounded and imaged immediately (0 h). Growth media containing a vehicle (PBS) or T₄, Tetrac, or and Tetrac + T₄ was added to every well. In the Tetrac + T₄ group, HUVECs were pretreated with Tetrac for 30 min, then treated with T₄. The concentration of T₄ and Tetrac were both 100 nmol/l. Wound closure was recorded at 8, 12, and 24 h. The width of

the wound was the average of nine determinations per time point. Distance of migration was calculated at each time point as described in Materials and methods. Phase-contrast images of the wounds were taken at $\times 100$ magnification. The experiment was repeated three times and results of a representative experiment are shown. **P* < 0.05 when compared with the control group, #*P* < 0.05 when compared with the T₄ group at the same time.

Figure 2

Effects of T_4 on tubulogenesis of HUVECs. HUVECs were mixed with Matrigel (total 50 μ l) for 30 min at 37 °C in an atmosphere of 5% CO_2 to allow formation of the gel. This was followed by the addition of 250 μ l HUVEC cell medium (5×10^3 cells per well) and incubation at 37 °C for 48 h. After washing with PBS, the cell medium was replaced with HUVECs medium mixed with thyroid hormone (T_4 : 100 nmol/l), which was replaced every 24 h for 48 h. The tube-like structures inside the wells were photographed. The total length and number of the capillary-like structures (indicated by arrows) that were formed within the Matrigel were measured. Compared with the control group, the number in the T_4 group was greater (*, $P < 0.05$ compared with the control group), and the length of tube-like structures ($P > 0.05$) was longer, but the difference was not statistically significant.

inhibited the effects of T_4 . The effect of Tetrac alone on cell migration did not result in a significant difference from the control group ($P > 0.05$). In addition, we investigated the effects of T_4 on the capability of HUVECs to form capillary-like structures in Matrigel and quantified it by measuring the number and length of capillary-like structures. Our results showed that after 48 h of T_4 treatment, although the length of tube-like structures in the T_4 group was not significantly different from that in control group ($P > 0.05$), the number of tube-like structures obviously increased in the T_4 group ($P < 0.05$) (Fig. 2). These results indicated that T_4 had a strong ability to induce capillary-like tube formation and migration of HUVECs.

The signaling pathway of angiogenesis induced by T_4

T_4 induced the phosphorylation of PKD To examine the potential role of PKD in the T_4 -stimulated signaling pathway, we first observed PKD phosphorylation in HUVECs in response to T_4 . HUVECs were stimulated with T_4 (100 nmol/l) at different times as indicated. As shown in Fig. 3A, T_4 rapidly induced PKD phosphorylation within 15 min ($P < 0.05$). This higher activation was

maintained until 45 min ($P < 0.05$). To confirm the role of integrin $\alpha v \beta 3$ in T_4 -induced angiogenic effects, we used Tetrac to inhibit the binding of T_4 to integrin $\alpha v \beta 3$ in HUVECs. Our results showed that, to certain extent, T_4 increased the phosphorylation of PKD, which was inhibited by Tetrac in a dose-dependent way (Fig. 3B). PKD, as a downstream target of the $PLC\gamma$ -PKC pathway, was reported to mediate the signal transduction and regulate many cellular functions. It is possible that PKC might be involved in the phosphorylation of PKD induced by T_4 . Our data showed that PKC inhibitor (Bis) also inhibited PKD phosphorylation induced by T_4 , and this inhibitory action diminished with time (Fig. 3C).

T_4 stimulated HDAC5 phosphorylation and cytoplasmic accumulation

Figure 3D shows that the expression of phosphorylated HDAC5 in the T_4 group was nearly 1.5-fold higher than that in the control group ($P < 0.05$). Compared with the T_4 group, different concentrations of Tetrac effectively inhibited T_4 -induced HDAC5 phosphorylation ($P < 0.05$). However, we barely observed any significant difference in HDAC5 phosphorylation with different concentrations of Tetrac + T_4 ($P > 0.05$, Fig. 3D). In addition, nuclear export of HDAC5 depended on its phosphorylation, which further affected the downstream gene expression. To confirm the subcellular location of phosphorylated HDAC5 in T_4 -activated signaling events, we examined the cytoplasmic localization of T_4 -induced HDAC5 phosphorylation in HUVECs. Our results showed that there was cytoplasmic accumulation of phosphorylated HDAC5 after administration of T_4 for 15 min (1.39-fold when compared with the control group, $P < 0.05$). While Bis and Tetrac inhibited T_4 -induced cytoplasmic accumulation of phosphorylated HDAC5 (Fig. 4). Collectively, these results suggested that T_4 induced the cytoplasmic accumulation of phosphorylated HDAC5 in HUVECs by activating the integrin $\alpha v \beta 3$ /PKC pathway.

T_4 -induced phosphorylation of HDAC5 mediated by PKD

To further define the role of PKD in T_4 -induced HDAC5 phosphorylation, we knocked down the endogenous PKD in HUVECs using siRNA specifically targeting human PKD. All three PKD siRNAs significantly inhibited the expression of PKD (PRKD1) mRNA and protein (Fig. 5A, B). Particularly, PKD siRNA significantly repressed T_4 -induced HDAC5 phosphorylation (Fig. 5C). Here, we confirmed an essential role of PKD in T_4 -induced HDAC5 phosphorylation in HUVECs.

Figure 3

PKD and HDAC5 phosphorylation in HUVECs. (A) T₄-stimulated PKD phosphorylation. HUVECs were starved without serum for 24 h and incubated with T₄ (100 nmol/l) for 15, 30, and 45 min. Cell lysates were prepared and subjected to western blotting using a specific antibody. GAPDH was used as a loading control. Quantification of bands was done by densitometric analysis. The results are representative of three independent experiments. **P* < 0.05 when compared with the control group. (B) Tetrac inhibited PKD phosphorylation induced by T₄. HUVECs were starved without serum for 24 h, then Tetrac (10⁻⁷, 10⁻⁶, and 10⁻⁵ mol/l) was added 30 min before T₄ (100 nmol/l, 30 min) addition. The results are representative of three independent experiments. **P* < 0.05 when compared with the control group. #*P* < 0.05 when compared with the T₄ group. (C) PKC inhibitor (bisindolymaleimide I,

Bis) inhibited PKD phosphorylation induced by T₄. HUVECs were starved without serum for 24 h, and then they were pretreated with Bis (2.5 μmol/l) for 30 min and finally with T₄ (100 nmol/l) for 15, 30, and 45 min. The results were representative of three independent experiments. **P* < 0.05 when compared with the control group. #*P* < 0.05 when compared with the T₄ group treated at the same time. (D) Effects of T₄ and Tetrac on phosphorylation of HDAC5. HUVECs were starved without serum for 24 h, and then they were pretreated with Tetrac (10⁻⁷, 10⁻⁶, and 10⁻⁵ mol/l) for 30 min and finally with T₄ (100 nmol/l) for 30 min. The results were representative of three independent experiments. **P* < 0.05 when compared with the control group, #*P* < 0.05 when compared with the T₄ group.

T₄ increased the expression of *bFGF* mRNA via the integrin α v β 3/PKD/HDAC5 signaling pathway

As a potent pro-angiogenic factor, *bFGF* is one of the numerous target genes regulated by HDAC5. To investigate whether the pro-angiogenic action of T₄ was related to

induction of *bFGF* expression via the integrin α v β 3/PKD/HDAC5 pathway, we examined the expression of *bFGF* with real-time PCR. HUVECs were treated with T₄ or Tetrac for 2–6 h. The results showed that an increase in *bFGF* expression was obvious after 2 h of T₄ treatment (2.637-fold when

Figure 4

Expression of phosphorylated HDAC5 in the cytoplasm. HUVECs were starved without serum for 24 h, and then they were pretreated with Tetrac (100 nmol/l) or Bis (2.5 μ mol/l) for 30 min and finally with T₄ (100 nmol/l) for 30 min. Cytoplasmic protein was extracted, and phosphorylation of HDAC5 was observed in the cytoplasm. GAPDH was used as a loading control. The results are representative of three independent experiments. * $P < 0.05$ when compared with the control group, # $P < 0.05$ when compared with the T₄ group.

compared with the control group, $P < 0.05$); whereas Tetrac effectively prevented T₄-induced expression of *bFGF* mRNA during the 2 h time period ($P < 0.05$) (Table 2). Expression of *bFGF* mRNA in HUVECs decreased gradually with time in all groups, and the difference between groups was not statistically significant at 6 h ($P > 0.05$).

Discussion

In recent years, many studies have focused on investigating T₄-induced angiogenesis. Schlenker *et al.* (2008) reported that hypothyroidism in rats induces a decrease in brain blood vessel density. Replacement with 3,5-diothyronic acid and T₄ effectively prevented the effects of hypothyroidism on cardiac function and increased blood vessel density. Thyroid hormones also exhibit direct pro-angiogenic effects on brain endothelial cells, i.e. increasing brain endothelial cell expansion and formation of tube-like structures (Zhang *et al.* 2010). One possible explanation for this is the ability of thyroid hormone to regulate the dynamics of cytoskeleton remodeling, a key structural component required for cells to migrate. This pro-angiogenic mechanism is confirmed by the findings of Farwell *et al.* (2006), which indicate that the T₄-dependent actin polymerization in developing neurons is necessary for migrating cells to recognize the laminin guidance

molecule. Both T₄ and 3,3',5-triiodothyronine (rT₃) exert direct, positive control of the quantity of polymerized actin in cultured astrocytes without affecting gene expression.

In our study, T₄ was also discovered to be associated with angiogenesis in HUVECs. We found that T₄ promoted HUVEC migration and formation of tube-like structures. Meanwhile, Tetrac, a pure inhibitor of the binding of T₄ and T₃ to integrin $\alpha v \beta 3$ (Bergh *et al.* 2005, Davis *et al.* 2009), prevented T₄-induced cell expansion. Our results indicated that T₄ showed a strong ability to regulate HUVEC angiogenesis, which was initiated at the membrane receptor integrin $\alpha v \beta 3$, and the use of Tetrac was appropriate for investigating the participation of the receptors in actions of thyroid hormone analogs. This result is consistent with previous research showing that integrin $\alpha v \beta 3$ is required in thyroid-hormone-induced angiogenesis. Studies report that the extracellular domain of integrin $\alpha v \beta 3$ includes an RGD recognition site, which is an important verification domain for the ligands of the protein. The thyroid hormone receptor domain is at or near the RGD recognition site on integrin $\alpha v \beta 3$ (Bergh *et al.* 2005, Cody *et al.* 2007). By binding to integrin $\alpha v \beta 3$, thyroid hormone activates several downstream signaling molecules to regulate signal transduction.

Integrin $\alpha v \beta 3$ contains two thyroid hormone-binding sites, denoted as S₁ and S₂, which translate the thyroid hormone signal differently (Davis *et al.* 2011). Several studies have shown that the signaling pathway of angiogenesis induced by T₄ is mediated by S₂, and ultimately activates the ERK1/2 pathway (Lin *et al.* 2009). Interestingly, there is recycling of integrin $\alpha v \beta 3$ in cells, i.e. from endosomal compartment to plasma membrane, which is required for cell migration (Pellinen & Ivaska 2006, Shattil *et al.* 2010). There are some signaling molecules involved in regulating this complicated cellular recycling. Among them, PKD1 can interact with integrin $\alpha v \beta 3$ in endothelial cells and regulate its trafficking in two different ways: on the one hand, it promotes integrin $\alpha v \beta 3$ recycling, and on the other hand, it inhibits integrin $\alpha v \beta 3$ endocytosis (di Blasio *et al.* 2010). Wong & Jin (2005) also confirmed that PKD, as a downstream target, is involved in VEGF-induced MAPK signaling and proliferation of endothelial cells. This evidence indicates a potential signaling role of PKD, which is activated by T₄ binding to integrin $\alpha v \beta 3$, involved in T₄-induced angiogenesis. Thus, we evaluated the phosphorylation of PKD in our model and found that T₄ increased PKD phosphorylation in HUVECs, whereas Tetrac inhibited T₄-induced PKD phosphorylation. However, the dose-dependent inhibitory effect of Tetrac

Figure 5

Effects of *PKD* siRNA on phosphorylation of HDAC5 induced by T₄. (A) Expression of *PKD* mRNA in HUVECs after transfection with *PKD* siRNA. HUVECs were starved without serum for 24 h. Transfection reagent with negative control siRNA (25 nmol/l) or one of the three *PKD* siRNAs (25 nmol/l) was added to cell culture medium for 6 h, then the transfection reagent was replaced with culture medium. HUVECs were cultured for another 24 h, and *PKD* and *UBC* cDNAs were isolated. The levels of *PKD* cDNA were corrected for variations in *UBC* cDNA. (B) Expression of *PKD* protein in HUVECs after transfection with *PKD* siRNA. HUVECs were transfected with a vehicle, negative siRNA (25 nmol/l) or *PKD* siRNA (25 nmol/l) for 6 h, and then the

transfection reagent was removed. After culturing for 48 h, HUVECs were treated with T₄ (100 nmol/l) for 15 min. β -actin was used as a loading control. * $P < 0.05$ when compared with the control group. # $P < 0.05$ when compared with the T₄ group. (C) Phosphorylation of HDAC5 induced by T₄ was inhibited by *PKD* siRNA. After HUVECs were transfected with a negative siRNA (25 nmol/l, control) or *PKD* siRNA (25 nmol/l), culture was continued for 48 h, and then cells were exposed to T₄ (100 nmol/l) for 15 and 30 min. Representative immunoblot data are shown ($n = 3$). * $P < 0.05$ when compared with the control group, # $P < 0.05$ when compared with the T₄ group.

was observed only for a certain range of concentrations, which was possibly attributable to the lower potency of the thyromimetic activity of Tetrac within the cell (Moreno *et al.* 2008). Our results confirmed that T₄ stimulated the phosphorylation of PKD by binding to integrin $\alpha v \beta 3$. By stimulating PKD phosphorylation, T₄ might promote the recycling of integrin $\alpha v \beta 3$, therefore generating a positive feedback, enhancing the effects of T₄. Many studies report that the phosphorylation of PKD depends on the activation of PKC (Vega *et al.* 2004a,b). To further clarify the role of PKC in T₄-induced PKD phosphorylation, we pretreated HUVECs with PKC inhibitor. Our results showed that T₄-stimulated PKD phosphorylation was reduced by PKC inhibitor. The data implied that the integrin $\alpha v \beta 3$ /PKC signaling pathway was involved in PKD activation induced by T₄.

In contrast to the previous studies, we first reported that through activation of the integrin $\alpha v \beta 3$ /PKC/PKD

pathway, T₄ induced the phosphorylation of HDAC5. We used both Tetrac and PKC inhibitor to block the signaling pathway and discovered that HDAC5 phosphorylation stimulated by T₄ was reduced. We also designed *PKD* siRNA to inhibit *PKD* expression and found that cellular HDAC5 phosphorylation significantly decreased. Class II a HDAC family members, especially HDAC5 and HDAC7,

Table 2 Expression of *bFGF* mRNA in HUVECs ($\bar{X} \pm S$)

	2 h	4 h	6 h
Control group	1.000	1.000	1.000
T ₄ group	2.637 $\pm$ 0.968*	1.760 $\pm$ 0.317*	1.396 $\pm$ 0.467
Tetrac group	1.409 $\pm$ 0.393*	1.471 $\pm$ 0.565*	1.238 $\pm$ 0.567
Tetrac + T ₄ group	2.260 $\pm$ 1.579* [†]	1.506 $\pm$ 0.431*	1.000

* $P < 0.05$ when compared with the control group at the same time, [†] $P < 0.05$ when compared with the T₄ group.

have been identified as important downstream regulators of PKD. They are reported to be involved in VEGF-induced gene expression and angiogenesis (Ha *et al.* 2008a,b). Ha *et al.* (2008a,b) also demonstrated that once HDAC5 is phosphorylated, it would be exported from the nucleus, resulting in the relaxation of nucleosomes, thereby beneficial for transcription of downstream target genes. To investigate the nuclear export of HDAC5 in response to T₄, we extracted cytoplasmic protein and found that phosphorylated HDAC5 was increased in the cytoplasm of HUVECs, while the effects of Tetrac and PKC inhibitor were in contrast to this, which indicated that there was nuclear–cytoplasmic shuttling of HDAC5 in response to T₄. Therefore, it is speculated that the pro-angiogenic action of T₄ is initiated at the cell surface integrin α v β 3 and ultimately activates the PKD/HDAC5 signaling pathway. HDAC5 phosphorylation and nuclear export promoted by T₄ regulate histone acetylation and chromatin remodeling and further affect pro-angiogenic factor transcription.

Acetylation of chromatin proteins and transcription factors is part of a complex signaling system that is mainly involved in the control of gene expression (McKinsey & Olson 2005, Backs & Olson 2006). Histone acetyltransferases and HDACs act in an opposing manner to control the acetylation state of nucleosomal histones. HDAC5 has been shown to act as a signal-responsive repressor of cardiac hypertrophy, skeletal muscle differentiation and bone development (McKinsey *et al.* 2000, Zhang *et al.* 2002, Vega *et al.* 2004a,b). There are numerous target genes regulated by HDAC5, among which *bFGF* was discovered and also the transcriptome of cells transfected with *HDAC5* siRNA was profiled by Urbich *et al.* (2009). Among the significantly regulated genes with altered RNA levels are the genes involved in angiogenesis, including angiogenic growth factors and receptors (e.g. *bFGF*, neuropilin 2, and VEGF receptor 2), guidance molecules (e.g. *Slit2*) and transcription factors. *bFGF*, *SLIT2*, and *EPHB4* are time-dependently and significantly upregulated in *HDAC5* siRNA-transfected HUVECs. Here, we selected the pro-angiogenic *bFGF* as a target gene of HDAC5 and assessed its mRNA expression. The results showed that T₄ increased the expression of *bFGF* mRNA within 2 h, and Tetrac decreased the expression of *bFGF* mRNA. Due to the shorter time of exposure, this effect is considered to be different from thyroid-hormone-induced genomic effects resulting from binding to thyroid hormone nuclear receptor. Our findings indicate that T₄-induced angiogenesis is regulated by the integrin α v β 3/PKD/HDAC5-dependent upregulation of *bFGF*.

Many studies report that T₄-induced angiogenesis is regulated by a complicated signaling system. Here, we

demonstrated a new signaling pathway through integrin α v β 3/PKD/HDAC5, in which T₄ stimulates HDAC5 nuclear export and increases *bFGF* mRNA expression that contributes to angiogenesis in HUVECs. The reduction of thyroid hormone in circulation or the inhibition of thyroid hormone actions at the integrin α v β 3 receptor would consequently produce a reduction in the proliferative and angiogenic effects of thyroid hormone. Al Hussein *et al.* (2013) also found that thyroidectomy mitigates hypoxia-induced pulmonary hypertension, and that the mechanism is associated with a reduced proliferation of endothelial cells and reduced angiogenesis. Therefore, targeting thyroid hormone actions could be an alternative adjuvant therapy against cancer proliferation and angiogenesis. Our study would facilitate exploration of the potential therapeutic targets and help us to control pathological angiogenesis.

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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