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Thyroid hormones stimulate L-arginine transport in human endothelial cells

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Abstract

Thyroid hormone activity is associated with L-arginine metabolism and nitric oxide (NO) production, which participate in the cardiovascular manifestations of thyroid disorders. L-arginine transporters play an important role in activating L-arginine uptake and NO production. However, the effects of thyroid hormones on L-arginine transporters in endothelial cells have not yet been evaluated. The following methods were used. We measured L-arginine uptake, mRNA expression of L-arginine transporters, endothelial nitric oxide synthase (eNOS) mRNA and NO generation after the administration of T₃, T₄ and the T₃ analog, 3,3',5-triiodothyroacetic acid TRIAC in human umbilical vein endothelial cells (HUVECs). We also analyzed the role of $\alpha\text{v}\beta 3$ integrin and of phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinases (MAPKs: ERK1/2, p38 and SAPK-JNK) and intracellular calcium signaling pathways as underlying mechanisms. To this end, $\alpha\text{v}\beta 3$ integrin was pharmacologically inhibited by tetraiodothyroacetic acid (TETRAC) or genetically blocked by silencing αv mRNA and PI3K, MAPKs and intracellular calcium by selective inhibitors. The following results were obtained. Thyroid hormones and the T₃ analog TRIAC increased L-arginine uptake in HUVECs, the sodium-independent y⁺/CAT isoforms, except CAT2b, sodium-dependent y⁺L system and sodium-independent system b⁰. L-arginine transporters, eNOS mRNA and NO production. These effects were suppressed by $\alpha\text{v}\beta 3$ integrin inhibition with TETRAC or αv integrin downregulation or by PI3K, MAPK or intracellular Ca²⁺ signaling inhibitors. In conclusion, we report for the first time that activation of L-arginine uptake by thyroid hormones is related to an upregulation of L-arginine transporters. This effect seems to be mediated by activation of $\alpha\text{v}\beta 3$ integrin receptor and subsequent PI3K, MAPK and intracellular Ca²⁺ signaling pathways.

Key Words

- thyroid hormones
- TRIAC
- TETRAC
- L-arginine transporters
- HUVECs
- $\alpha\text{v}\beta 3$ integrin
- PI3K
- MAPK

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Introduction

L-arginine transporters are expressed in endothelial and epithelial cells and play a major role in activating L-arginine transport and nitric oxide (NO) synthesis, which regulates vascular tone and, therefore,

arterial pressure (Pallone & Mattson 2002, Kakoki *et al.* 2004). The total intracellular concentration of L-arginine in endothelial cells is saturating for endothelial nitric oxide synthase (eNOS) and is consequently not limiting

for NO production (Block *et al.* 1995), and it has been reported that cellular L-arginine transport may be more important than intracellular L-arginine levels for NO production (McDonald *et al.* 1997). Thus, the supply of L-arginine may become limiting and reduce NO formation in physiological and pathological states. In this context, endothelial dysfunction can be improved by the administration of L-arginine in many diseases, including hypertension, atherosclerosis, hyperglycemia, restenosis/postcoronary angioplasty and reperfusion injury (Creager *et al.* 1990, Chen & Sanders 1991, Harrison 1991, Weyrich *et al.* 1992, Wascher *et al.* 1997). The cellular uptake of L-arginine is mediated by carrier-mediated transporters and passive diffusion. The saturable transport is produced by Na⁺-independent transporters (system y⁺, B^{0,+}) and a Na⁺-dependent active transporter system, y⁺L (Closs 2002, Mann *et al.* 2003). System y⁺, composed of the cationic amino acid transporter (CAT) family of proteins (CAT-1, CAT-2A, CAT-2B, CAT-3 and CAT-4), is the major cationic amino acid transport system and accounts for ~60% of the transport of the intracellular supply of L-arginine for NOS (Verrey *et al.* 2004).

Thyroid hormone excess is associated with a hyperdynamic circulation, which is characterized by increased cardiac output, heart rate and pulse pressure and decreased peripheral resistance (Klein 1990, Vargas *et al.* 2006). Thyroxine generates a concentration-related blood pressure (BP) elevation in rodents (Moreno *et al.* 2005, Vargas *et al.* 2006), increases NOS activity in tissues responsible for BP control (Quesada *et al.* 2002), raises plasma nitrite/nitrate levels (Rodríguez-Gómez *et al.* 2003), and preserves vascular endothelial function (Vargas *et al.* 1995). *In vitro*, T₃ augmented eNOS activity in endothelial cells (Hiroi *et al.* 2006). BP was increased in thyroxine-treated rats by oral treatment with the nonspecific NO inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME) and the iNOS inhibitor aminoguanidine, administered at concentrations without pressor effects in normal rats (Rodríguez-Gómez *et al.* 2003, 2005). Our group also reported that thyroid hormone levels modulate different L-arginine metabolic pathways and that changes in the abundance of eNOS and arginases I and II proteins in renal and cardiovascular tissues may be related to the hemodynamic and renal manifestations of thyroid disorders (Rodríguez-Gómez *et al.* 2016). Thus, chronic arginase inhibition with N[omega]-hydroxy-nor-L-arginine (nor-NOHA) prevented the increased systolic BP and heart rate and decreased proteinuria in hyperthyroid rats (Rodríguez-Gómez *et al.* 2015). All these observations

support an association of thyroid hormone activity with L-arginine metabolism and NO production.

L-arginine transport is regulated by NO donors (Ogonowski *et al.* 2000), NOS agonists (Bogle *et al.* 1991), inflammatory factors (Simmons *et al.* 1996), cellular membrane potential (Zharikov *et al.* 1997), free radicals (Patel *et al.* 1996) and pharmacological and vasoactive agents (Bogle *et al.* 1991, Grupper *et al.* 2013). It is affected in several diseases, including acute and chronic kidney diseases (Schwartz *et al.* 2002, Martens *et al.* 2014), intrauterine growth retardation (Casanello *et al.* 2009, Grupper *et al.* 2013), diabetes (Simmons *et al.* 1996) or obesity (Rajapakse *et al.* 2014). Hormones such as insulin (González *et al.* 2011) or collectrin (Malakauskas *et al.* 2007) also modulate L-arginine transport; however, the role of thyroid hormones has not yet been evaluated, despite their important participation in L-arginine metabolism and in NOS activity and function. Therefore, the objectives of this study were to evaluate the effects of thyroid hormones on L-arginine transporters and other variables in human umbilical vein endothelial cells (HUVECs) and to examine the contributions of αvβ3 integrin and of phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinase (MAPK) and intracellular calcium mobilization signaling pathways as underlying mechanisms.

Material and methods

Culture of HUVECs

Endothelial cells were isolated from human umbilical cord veins using a previously reported method with some modifications (Jiménez *et al.* 2010). Cells were cultured in medium 199 supplemented with 20% FBS, 2mM penicillin/streptomycin, 2mM amphotericin B, 2mM glutamine, 10mM HEPES, 30μg/mL endothelial cell growth supplement and 100mg/mL heparin in a humidified 5% CO₂ atmosphere at 37°C. All cells used in experiments from passages 2 to 5 were previously starved in serum-free medium for 2h.

HUVECs were incubated with a vehicle control (final concentration of 0.04N NaOH), the thyroid hormone 3,3',5-triiodo-L-thyronine (T₃, 10nM and 100nM), L-thyroxine (T₄, 10nM and 100nM) (diluted to its final concentration from 10⁻³M stock, using the vehicle as diluent) and thyroid hormone analogue 3,3',5-triiodothyroacetic acid (TRIAC, 10nM and 100nM), for 2 and 6h. In some experiments, cells were co-incubated with thyroid hormones and tetraiodothyroacetic acid

(TETRAC, 100 nM), a $\alpha\text{v}\beta 3$ blocker that inhibits binding of thyroid hormones to the plasmatic membrane (36), as well as with TETRAC alone. HUVECs were also co-incubated with T_3 (100 nM) for 6 h and PD98059 (10 μM , ERK1/2 inhibitor, Calbiochem, Darmstadt, Germany), SB203580 (10 μM , p38 MAPK inhibitor, Calbiochem), SP600125 (25 μM , SAPK-JNK inhibitor, Calbiochem), the PI3K inhibitor LY-294002 (1 μM) (all these inhibitors were diluted with DMSO, which was added to control cells) or 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid tetrakis (acetoxymethylester) (BAPTA/AM) a selective chelator of intracellular Ca^{2+} stores, added 1 h before T_3 administration. The effects of these inhibitors without T_3 were also analyzed. HUVECs were incubated in medium supplemented with 1% FBS in all experiments. Cells were then used to characterize the L-arginine transport system and gene expression.

Characterization of L-arginine transport system in HUVECs

Confluent cultures of HUVECs were prepared in 6-well plates. To determine the transporters for L-arginine supplied to the cells, HUVECs were incubated in an uptake buffer (in mM: 25 HEPES, 1.8 CaCl_2 , 5.4 KCl, 140 choline chloride, 0.8 MgSO_4 and 5 glucose) containing 20 nM L-[^3H] arginine for periods of 0 min, 2 and 6 h. In some experiments, 50 μM of unlabeled L-arginine was included in the solution to determine that the transporters measured were the expected. L-arginine uptake was stopped by addition of ice-cold uptake buffer, and cells were washed three times with 1 mL of this buffer. After the final wash, cells were lysed by addition of 125 μL 0.5% SDS in 0.1 N NaOH. Cellular lysates were added to 4 mL of Ecoscint-A scintillation fluid, and scintillation spectroscopy (Beckman Instruments) was used to determine the amount of L-[^3H]arginine, considered to represent the cellular transport of L-arginine as previously described (Ogonowski *et al.* 2000).

RT-PCR analysis

For RT-PCR analysis, total RNA was extracted from HUVEC by homogenization and converted to cDNA using standard methods. PCR was performed with a Techne Techgenethermocycler (Techne, Cambridge, UK). Quantitative real-time RT-PCR was applied to analyze mRNA expression, and the sequences of sense and antisense primers used for amplification are reported in Table 1. Preliminary experiments were carried out with

various amounts of cDNA to determine non-saturating conditions of PCR amplification for all genes studied. Under these conditions, the relative quantification of mRNA was assessed by the SYBR Green based-RT-PCR method. The efficiency of the PCR reaction was determined by using a dilution series of a standard tissue sample. Quantification was performed with the $\Delta\Delta\text{Ct}$ method. The housekeeping genes β -actin was used for internal normalization.

Transfection of $\alpha\text{v}\beta 3$ siRNAs

Confluent HUVECs were transfected with αv -specific siRNA (pooled, validated siRNA from Dharmacon, Lafayette, CO, USA) or a scrambled siRNA to serve as a negative control, using Lipofectamine RNAiMAX (Invitrogen Life Technologies) for 48 h, as previously described (Quintela *et al.* 2014).

Quantification of NO released by DAF-2

NO released by HUVECs was quantified with the NO-sensitive fluorescent probe diaminofluorescein-2 (DAF-2), as previously reported (Quintela *et al.* 2014). Briefly, cells were incubated as described earlier. After this period, TH-treated and un-treated cells were washed with PBS and pre-incubated with L-arginine (100 μM in PBS, 5 min, 37°C). Next, DAF-2 (0.1 μM) was incubated for 2 min, and the calcium ionophore calmycin (A23187, 1 μM) was then added for 30 min, followed by measurement of the fluorescence intensity (arbitrary units, AU) with a spectrofluorimeter (Fluorostart, BMG Lab technologies, Offenburg, Germany). The autofluorescence was subtracted from each value. In some experiments, L-NAME (100 μM) was added 15 min before the addition of L-arginine. The difference between the fluorescence signal without and with L-NAME was considered as the NO production.

Western blotting analysis

Cells underwent the siRNA procedure for 48 h followed by Western blotting, running HUVEC homogenates on a SDS-PAGE (40 μg of protein per lane) and then transferring the proteins to PVDF membranes. αv was detected after incubating the membranes with rabbit polyclonal anti-human integrin αv (Abcam) overnight at 4°C with the secondary peroxidase conjugated antibodies. Antibody binding was detected with an ECL system (Amersham Pharmacia Biotech, Amersham, UK), and densitometric

Table 1 Oligonucleotides for real-time RT-PCR.

mRNA targets	Descriptions	Sense	Antisense
CAT-1	Cationic amino acid transporter-1	ATCTGCTTCATCGCCTACTT	TCTCTGCCTCTGGTAAAAAC
CAT-2a	Cationic amino acid transporter-2a	TTCTCGTCCTTCTGTTTGTG	TTTGGGCTGGTCGTAAGATA
CAT-2b	Cationic amino acid transporter-2b	TTTTCCCAATGCCTCGTGTA	CATTTGGGCTGGTCGTAAGA
CAT-4	Cationic amino acid transporter-4	ATGGTGGGCTCGGGTCTCTA	TGCGGATGCTGTGGCTGAAC
y ⁺ LAT1	System y ⁺ L transport 1	CTTTGGTCTCTCTGGTCA	GCTTATGTTCTGGCACTCTG
y ⁺ LAT2	System y ⁺ L transport 2	CCTACCATCTTGTCCCTAAC	TTTCTGGGTTTTTGATTCT
B ^{0,+} system	System B ^{0,+}	GTCTTCTTGGCTTGGCTCATA	CAATCATACACCAGCCTAAAGCA
αv	Alpha-v	GCGGCGGAAAAGCCATCG	CGCCGCTTTTCGGTAGC
eNOS	Endothelial nitric oxide synthase	GTGGACGCACAGTAACATGG	ACCTCCCAGTCTTCACACG
Actb	Beta actin	CGGTGAAGGTGACAGCAG	TGTGTGGACTTGGGAGAGG

analysis was performed using Scion Image-Release Beta 4.02 software (<http://www.scioncorp.com>). αv/β-actin abundance ratios were calculated and expressed as a percentage of the values in control cells from the same gel.

Reagents

All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

Statistical analysis

Results were expressed as means ± s.e.m. Statistical analyses were performed using Graph Pad Prism 5 software. A two-factor ANOVA was used to test for drug or group interactions. When a significant interaction was detected, individual differences between groups were discerned by one-way ANOVA with Student–Newman–Keuls' *post hoc* test. Significance was accepted at $P < 0.05$.

Results

Thyroid hormones stimulated L-[³H] arginine uptake in HUVECs

Given that cellular L-arginine transport is very important for NO production, the effects of both thyroid hormones (T₃ and T₄ at 10 and 100 nM concentrations) on L-arginine uptake were analyzed after 0 min, 2 and 6 h after hormone administration to HUVECs (Fig. 1A and B). L-arginine transport was significantly stimulated by T₃ at 2 h with the 100 nM concentration and at 6 h with both 10 and 100 nM concentrations. T₄ increased L-arginine uptake at 2 h with both concentrations, but the slight increase observed at 6 h did not reach statistical significance.

Effects of thyroid hormones on the Na⁺-independent transporter (system y⁺) CAT-1, CAT-2a, CAT-2b and CAT-4

After observing that thyroid hormones increased L-arginine uptake in HUVECs, we examined the influence of thyroid hormones on the main family of L-arginine transporters in endothelial cells, the Na⁺-independent transporter system y⁺. The effects of 10 and 100 nM of T₃ and T₄ on the expression of system y⁺ were evaluated after 2 and 6 h in HUVECs. CAT-1 mRNA was augmented after 6 h of administration with either thyroid hormone at the higher concentration, while no significant effects were observed after incubation with either hormone (Fig. 2A).

Figure 1

Effects of thyroid hormones on L-arginine uptake. HUVEC monolayers were incubated with uptake buffer containing 20 nM L-[³H] arginine for 0, 2 or 6 h in the absence or presence of T₃ (A) or T₄ (B) at concentrations of 10 or 100 nM ($n = 6-8$ for each concentration or time). The amount of L-[³H] arginine taken up by cells over time was determined as described in materials and methods. Data are expressed as means ± s.e.m. * $P < 0.05$, ** $P < 0.01$ vs control (Ctrl) levels.

Figure 2

Effects of thyroid hormones on expression of mRNA related to system γ^+ in HUVECs. Cultures were incubated for the indicated times in the presence of T_3 and T_4 at concentrations of 10 and 100 nM with or without coinubation with TETRAC (100 nM), before RNA extraction and reverse transcription. Quantitative real-time RT-PCR was applied to analyze mRNA expression, using the housekeeping genes β -actin for internal normalization. mRNA levels of CAT-1 (A), CAT-2a (B), CAT-2b (C) and CAT-4 (D) after thyroid hormone exposure. The number of cases was 6–7 for each time and concentration. Data are expressed as means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ vs control (Ctrl) levels. # $P < 0.05$, ## $P < 0.01$ vs without TETRAC.

CAT-2a mRNA was augmented by the higher concentration of T_3 after 2 h and by both concentrations of T_3 after 6 h (Fig. 2B). CAT-2a mRNA was increased by both T_4 concentrations at 6 h, observing no significant effects at 2 h (Fig. 2B). CAT-2b mRNA was not increased by either concentrations of T_3 or T_4 after any time period (Fig. 2C). CAT-4 was only increased by the higher concentration of T_3 after 6 h, and no significant change in this transporter was observed with either hormone at other concentrations or times (Fig. 2D).

Effects of thyroid hormones on the Na^+ -dependent transporter (system γ^+) CATs γ^+ LAT1 and γ^+ LAT2

Because both thyroid hormones were found to stimulate system γ transporter, we also studied their effects on the

Na^+ -dependent transporter γ^+ L system, which works in additive cooperation with γ^+ system. Both concentrations of T_3 increased γ^+ LAT1 mRNA after 6 h, and the increase was statistically significant with the higher concentration (Fig. 3A). Similar increases were observed with T_4 after 2 h (Fig. 3A). Transporter γ^+ LAT2 mRNA was only upregulated by 10 nM T_3 after 6 h (Fig. 3B).

Effects of thyroid hormones on the Na^+ -independent transporter system $\text{B}^{0,+}$

L-arginine transport is also mediated by the CAT system $\text{B}^{0,+}$ in HUVECs, and we therefore analyzed the effects of thyroid hormones on this transporter. Both concentrations of T_3 significantly stimulated $\text{B}^{0,+}$ mRNA

Figure 3

Effect of thyroid hormones on expression of mRNA related to systems y⁺L and B⁰⁺ in HUVECs. Cultures were incubated for the indicated times in the presence of T₃ and T₄ at concentrations of 10 and 100 nM with or without coinubation with TETRAC (100 nM), before RNA extraction and reverse transcription. Quantitative real-time RT-PCR was applied to analyze mRNA expression, using the housekeeping genes β-actin for internal normalization. mRNA levels of y⁺LAT1 (A), y⁺LAT2 (B) and B⁰⁺ (C) after thyroid hormone exposure. The number of cases was 5–8 for each time and concentration. Data are expressed as means ± S.E.M. **P* < 0.05 vs control (Ctrl) levels. #*P* < 0.05, ##*P* < 0.01 vs without TETRAC.

generation after 6 h (Fig. 3C), and both concentrations of T₄ increased the expression of B⁰⁺ mRNA after 2 h, although the increase was only statistically significant at the 100 nM concentration (Fig. 3C).

Effects of TRIAC on L-arginine transporters

To further evaluate the impact of thyroid hormones on L-arginine transporters, we also explored the effects of TRIAC, a naturally occurring thyroid hormone metabolite that closely resembles the bioactive hormone T₃. We analyzed the effects of TRIAC at concentrations of 10 and 100 nM on L-arginine transporters after 2 and 6 h of exposure. CAT-1 mRNA was significantly increased with the higher concentration after 6 h (Fig. 4A), CAT-2a mRNA was upregulated by both concentrations after 2 and 6 h (Fig. 4A), while TRIAC had no effect on CAT-2b at either concentration (Fig. 4A) and CAT-4 was stimulated at 6 h by the high concentration (Fig. 4A). y⁺LAT1 mRNA, y⁺LAT2 mRNA (Fig. 4B) and B⁰⁺ system mRNA were only augmented with the higher concentration after 6 h (Fig. 4C). Thus, the effects of TRIAC on the different

L-arginine transport systems were similar to those of T₃ after 2 and 6 h.

Effects of T₃, T₄ and TRIAC on eNOS mRNA expression and NO production in HUVECs

Given the key role played by eNOS and NO in cardiovascular and renal functions and their contribution to the cardiac and renal manifestations of thyroid disorders, we studied the effects of T₃, T₄ and TRIAC on eNOS mRNA expression and NO production. Under our experimental conditions, exposure of HUVECs to T₃ produced a concentration-related increase in eNOS mRNA expression after 6 h but did not affect this expression after 2 h (Fig. 5A). However, T₃ incubation increased A23187-stimulated NO generation at both 2 and 6 h with the higher concentration tested (Fig. 5A). In contrast, T₄ produced a concentration-related increase in eNOS mRNA after 2 h but had no effect after 6 h, which correlated with the increased NO generation found at this time (Fig. 5B). As observed for T₃ and the T₃ thyroid hormone analogue, TRIAC increased eNOS mRNA expression with the higher concentration after 6 h,

Figure 4

Effects of TRIAC on L-arginine transporters. Cultures were incubated for the indicated times in the presence of TRIAC at concentrations of 10 and 100 nM with or without coinubation with TETRAC (100 nM) before RNA extraction and reverse transcription. The methods were the same as for the natural thyroid hormones. mRNA levels of CAT-1, CAT-2a, CAT-2b and CAT-4 (A), y+LAT1, and y+LAT2 (B) and B^{0,+} (C) after TRIAC exposure. The number of cases was 5–8 for each time and concentration. Data are expressed as means $\pm$ S.E.M. * P < 0.05, ** P < 0.01 vs control (Ctrl) levels. # P < 0.05, ## P < 0.01 vs without TETRAC.

but increased A23187-stimulated NO production at both times (Fig. 5C).

Pharmacological blockade of $\alpha\text{v}\beta 3$ integrin with TETRAC suppresses L-arginine transporters, eNOS expression and NO generation in HUVECs

T₄ and T₃ activate the cell-surface receptor for thyroid hormone on a plasma membrane structural protein, integrin $\alpha\text{v}\beta 3$, which is known to mediate local actions of the hormone on membrane ion pumps and the transport of chemotherapeutic agents and amino acids. We therefore investigated whether this membrane

receptor can be used to stimulate the mRNA expression of L-arginine transporters by thyroid hormones, using the integrin $\alpha\text{v}\beta 3$ thyroid hormone receptor blocker TETRAC. Coinubation of TETRAC with T₃, T₄ or TRIAC suppressed the stimulating effects of these agents on the different L-arginine transporters after 2 and 6 h of incubation (Figs 2, and 4). TETRAC also suppressed the stimulating effects of T₃, T₄ and TRIAC on eNOS mRNA expression (Fig. 5A, B and C). NO production stimulated by T₄, T₃ or its analog TRIAC was also suppressed by TETRAC (Fig. 5A, B and C). TETRAC administration did not have significant effect in control conditions (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

Figure 5

Effects of T_3 , T_4 and TRIAC on eNOS mRNA expression and NO production in HUVECs. Cultures were incubated for the indicated times in the presence T_3 , T_4 and TRIAC at concentrations of 10 and 100 nM with or without coincubation with TETRAC (100 nM) before RNA extraction and reverse transcription. Quantitative real-time RT-PCR was applied to analyze mRNA expression, using the housekeeping genes β -actin for internal normalization. Expression of eNOS mRNA was measured using the same experimental procedure as for the L-arginine transporters. eNOS mRNA after exposure to T_3 (A), T_4 (B) or TRIAC (C). A23187-stimulated NO production was measured with the NO-sensitive fluorescent probe diaminofluorescein-2 (DAF-2). After the exposure to T_3 (A), T_4 (B) or TRIAC (C), TH-treated and un-treated cells were washed with PBS before of A23187 addition. Data are expressed as means $\pm$ S.E.M. * P <0.05, ** P <0.01 vs control (Ctrl) levels. # P <0.05, ## P <0.01 vs without TETRAC (n =5–8).

Genetic blockade of $\alpha v \beta 3$ integrin suppresses L-arginine uptake, L-arginine transporters upregulation, eNOS mRNA and nitric oxide production induced by T_3 in HUVECs

The above observations were verified by conducting another set of experiments in which HUVECs were treated with control or αv -specific siRNA. At 48 h after transfection with αv -specific siRNA, HUVECs showed a $\approx 90\%$ decrease in αv mRNA and protein with respect to control siRNA-treated cells (Fig. 6A).

αv siRNA suppressed the stimulating effects of 100 nM T_3 on L-arginine uptake (Fig. 6B) and on the different L-arginine transporters (Fig. 6C) as well as on eNOS mRNA (Fig. 6D) and nitric oxide generation (Fig. 6E) after 6 h of incubation. These results are consistent with those obtained by the pharmacological blockade of $\alpha v \beta 3$ with TETRAC. The agonist (T_3), concentration (100 nM), and incubation time (6 h) were selected as providing the most effective stimulus in the previous experiments.

PI3K and MAPK inhibitors and chelation of intracellular Ca^{2+} suppress L-arginine transporters upregulation, eNOS mRNA and nitric oxide production stimulated by T_3 in HUVECs

Several thyroid hormone-stimulated membrane transport systems are activated through transduction pathways involving PI3K, MAPK and intracellular calcium. We therefore explored the possible involvement of these intracellular signaling pathways in the stimulatory effects of thyroid hormones on L-arginine transporters. For this purpose, we examined the effect of the PI3K inhibitor, LY-294002 on the stimulating effects of 100 nM T_3 on the different L-arginine transporters after 6 h of incubation. As shown in Fig. 7A, B and C, PI3K inhibition suppressed the mRNA expression of all L-arginine transporters studied. We also explored the role of the MAPK pathway by analyzing the impact of the ERK1/2 inhibitor PD98059, JNK inhibitor SP600125 and p38 MAPK inhibitor SB203580 on the stimulating effects of 100 nM T_3 . All three compounds inhibited the stimulating effect of T_3

Figure 6

Effects of genetic blockade of $\alpha v\beta 3$ integrin on L-arginine uptake, mRNA level of all L-arginine transporters, and NO production stimulated by T_3 (100 nM). Confluent HUVECs were transfected with control (Ctrl) or αv -specific siRNA (using Lipofectamine RNAiMAX). (A) mRNA and protein levels of αv after 48 h of siRNA transfection. Data are expressed as means $\pm$ s.e.m. ($n=5-8$). * $P<0.05$, ** $P<0.01$ vs siRNA-Ctrl. L-[3 H] arginine uptake (B), mRNA levels of CAT-1, CAT-2a, and CAT-4, γ -LAT1, system B $^{0,+}$ (C), and eNOS (D), and A23187-stimulated NO production (E) before and after 6 h of T_3 incubation (100 nM) in siRNA-Ctrl or siRNA- αv transfected HUVECs. Data are expressed as means $\pm$ s.e.m. * $P<0.05$, ** $P<0.01$ vs siRNA-Ctrl; # $P<0.05$, ## $P<0.01$ vs T_3 siRNA-Ctrl.

on the mRNA expression of all L-arginine transporters analyzed (Fig. 7A, B and C). Seemingly, PI3K and MAPK blockers also inhibited eNOS mRNA (Fig. 7D) and nitric oxide generation (Fig. 7E) stimulated by T_3 . However, the administration of these inhibitors did not have significant effect in control conditions (Supplementary Fig. 2). BAPTA/AM reduced the expression of all variables analyzed in the presence of T_3 , but did not have significant effects in control conditions (Fig. 8)

Discussion

The main findings of this study were that thyroid hormones stimulate L-arginine uptake and the mRNA expression of L-arginine transporters in HUVECs and that the thyroid hormone receptor on plasma membrane

integrin $\alpha v\beta 3$ plays an essential role in these effects. In HUVECs, L-arginine transport is mediated by the CAT isoforms γ -LAT1 (Sobrevia *et al.* 1995, Flores *et al.* 2003), γ -LAT2 (Casanello & Sobrevia 2002) and systems B $^{0,+}$ (Bussolati *et al.* 1993) and γ -LAT1 (Arancibia-Garavilla *et al.* 2003). All these transport systems except for CAT2b were upregulated by thyroid hormones. In fact, it has been reported that CAT-2b expression is only stimulated under cytokine or lipopolysaccharide treatment (Simmons *et al.* 1996). In the present study, T_3 and its analog TRIAC were the most active hormones in increasing mRNA of L-arginine Na $^{+}$ -independent and -dependent transporters after 6 h. It is likely that 6 h is the optimal period for the full development of intracellular signaling. The effects on the different L-arginine transport systems were similar between TRIAC and T_3 , in consonance with previous data showing that TRIAC and T_3 inhibit leptin secretion

Figure 7

Effects of PI3K, MAPK and inhibitors on L-arginine transporters and NO production stimulated by T₃ (100nM). Cultures were incubated for the indicated times in the presence of T₃ at concentrations of 100nM in the absence or presence of the following inhibitors: LY-294002 for PI3K, PD98059 for ERK1/2, SP600125 for JNK, SB203580 for p38 MAPK. The inhibitors were added 1 h before T₃ administration. mRNA levels of CAT-1, CAT-2a, CAT-4 (A), y⁺LAT1 (B), system B^{0,+}(C), and eNOS (D) and A23187-stimulated NO production (E). **P*<0.05, ***P*<0.01 vs control (Ctrl) levels; #*P*<0.05, ##*P*<0.01 vs without inhibitor.

with similar potency (Medina-Gómez *et al.* 2004). TRIAC exerts thyromimetic actions on the hypothalamus–pituitary–thyroid axis and on peripheral tissues including brain, heart, bone, liver, kidney, adipose tissue and skin, but its exact physiological role is unknown (Groeneweg *et al.* 2017). TRIAC binds efficiently to nuclear TRs (Oppenheimer *et al.* 1973, Smith *et al.* 1980), with a similar affinity as T₃ to TRα1 and a 3- to 6-fold higher affinity than T₃ to TRβ1 and TRβ2 (Schueler *et al.* 1990, Messier & Langlois 2000); and the present data indicate that TRIAC binds to the integrin αvβ3 plasma membrane receptor in endothelial cells, as reported D'Arezzo *et al.* (2004) in L-6 myoblasts.

Although the more active concentration of T₃ (100nM) is higher than physiological plasma levels (~6 fold), it should be taken into account that T₄ is supplied to cells at micromolar range and is transformed into T₃

by iodothyronine deiodinases. Thus, it has been reported that deiodinase 1 is present in endothelial cells (Sabatino *et al.* 2015). Deiodinase 1 transforms T₄ into T₃, releasing T₃ to the plasma, being responsible for the 80% of the plasma levels of T₃. This process of release might generate high levels of T₃ around the plasma membrane. Moreover, Bergh *et al.* (2005) reported that cultures incubated with 100nM total T₄ result in 1–0.1 nM free T₄, consistent with normal physiological levels. Hence, the data reported here can be considered of physiological relevance, playing a greater role in the hyperthyroid state.

Endothelial synthesis of NO requires the *de novo* import of extracellular L-arginine, and a correlation was found between NO production and L-arginine transport (Arancibia-Garavilla *et al.* 2003, Kakoki *et al.* 2004). The y⁺/CATs system is the principal cationic amino acid transport system for regulating the intracellular supply

Figure 8

Effects intracellular Ca^{2+} chelation on L-arginine transporters and NO production stimulated by T_3 (100 nM). Cultures were incubated for the indicated times in the presence T_3 at concentrations of 100 nM in the absence or presence of BAPTA/AM for intracellular Ca^{2+} chelation. BAPTA/AM was added 1 h before T_3 administration and was also studied in the absence of T_3 . mRNA levels of CAT-1, CAT-2a, CAT-4 (A), y+LAT1 (B), system B $^{0+}$ (C), and eNOS (D) and A23187 stimulated NO production (E).

* $P < 0.05$, ** $P < 0.01$ vs control (Ctrl) levels; # $P < 0.05$, ## $P < 0.01$ vs without inhibitor.

of L-arginine for NOS, and y+/CATs activity may be rate limiting for NO synthesis. In addition, human umbilical vein endothelium also requires the activity of the cationic and neutral amino acid transport system y+L for NO synthesis (Arancibia-Garavilla *et al.* 2003). After observing that L-arginine uptake and both y+/CAT and y+L systems were stimulated by thyroid hormone, we investigated the effects of T_3 , T_4 and TRIAC on eNOS mRNA expression and NO production, finding that both classic thyroid hormones and TRIAC stimulated eNOS mRNA expression and NO generation in HUVECs. These last results are in agreement with previous reports of the enhancement by T_3 of NO production in vascular myocytes and the expression of all three NOS isoforms (Carrillo-Sepulveda *et al.* 2010) and of the stimulation by T_3 of eNOS activity in endothelial cells (Hiroi *et al.* 2006). The present study reports for the first time that eNOS mRNA and NO production were also stimulated by TRIAC.

Recently, Flamant *et al.* (2017) have defined four types of thyroid hormone signaling. The classical genomic actions exerted by binding of T_3 to high-affinity nuclear thyroid hormone receptors (TRs) to regulate gene expression (Brent 1994) is now called type 1 of thyroid hormone action. By this type of signaling, thyroid hormones play important roles in metabolism, growth and cell differentiation (Lazar 1993). When thyroid hormone interacts with TRs and does not require direct interaction with DNA, it is called type 2 or 3 of thyroid hormone action. Thus, T_3 can also bind to cytoplasmic (Vicinanza *et al.* 2013) and membrane-localized (Kalyanaraman *et al.* 2014) TRs, which interact with intracellular signaling

pathways. Finally, the type 4 way of signaling is when TH acts independently of TRs, binding to other types of proteins. This type of thyroid hormone action can be included to the effects mediated by the binding of TTHH with the plasma membrane integrin $\alpha\text{v}\beta 3$ that contains a cell-surface receptor for thyroid hormones. T_4 and T_3 activate this receptor without entering the cell (Bergh *et al.* 2005). This integrin $\alpha\text{v}\beta 3$ receptor mediates a set of short- and long-term effects of thyroid hormone on membrane ion pumps and amino acids and an array of genes modulated by thyroid hormone (Davis *et al.* 2016). We studied whether this membrane receptor plays a role in increasing L-arginine uptake, the mRNA expression of L-arginine transporters, eNOS mRNA and the production of NO by thyroid hormones. Our results demonstrate that pharmacological blockade of integrin $\alpha\text{v}\beta 3$ by TETRAC suppressed the stimulatory effects of natural thyroid hormones and TRIAC on these variables. These observations were further verified by analyzing the effects of the genetic blockade of αv integrin, which confirmed the results obtained by pharmacological blockade of $\alpha\text{v}\beta 3$ with TETRAC. All these findings demonstrate the participation of integrin $\alpha\text{v}\beta 3$, mainly αv subunit, in these stimulatory effects of thyroid hormones.

We explored the participation of PI3K, MAPK and intracellular calcium in the signaling pathways of the stimulatory effects of thyroid hormones on L-arginine transporters and eNOS mRNA and NO. Results indicate that these intracellular pathways participate in these effects. These findings are consistent with the involvement of PI3K, MAPK and intracellular calcium

Figure 9

Proposed model for the sequence of events initiated by thyroid hormones in the plasma membrane that modify L-arginine transport in HUVECs. Scheme showing the proposed mechanisms underlying the stimulatory effects of thyroid hormones on sodium-dependent and -independent L-arginine transporters and eNOS in HUVECs. $\alpha v\beta 3$ integrin and PI3K, MAPK and intracellular Ca^{2+} signaling are critical steps in these effects of thyroid hormones. The binding of thyroid hormones to integrin $\alpha v\beta 3$ activates PI3K, MAPK and intracellular Ca^{2+} , which increased mRNA expression of L-arginine transporters, with subsequent L-arginine uptake and NO production. A full color version of this figure is available at <https://doi.org/10.1530/JOE-18-0229>.

in other effects of thyroid hormones on membrane transport systems in different cells. Thus, Na, K-ATPase and NHE1 ion transporter (D'Arezzo *et al.* 2004) and amino acid transport system A (Incerpi *et al.* 2002) were activated via a transduction pathway involving PKC, PI3K, the MAPKs and intracellular calcium (Incerpi *et al.* 2002, D'Arezzo *et al.* 2004). In addition, integrin $\alpha v\beta 3$ is linked to the activation of MAPKs (Bergh *et al.* 2005), and TETRAC blocks these hormonal actions, which are downstream from MAPKs (Yalcin *et al.* 2010). Accordingly, these factors may be sequential steps in the stimulatory effects of thyroid hormones on L-arginine transporters' upregulation. Finally, a schematic outline summarizing the sequence of events initiated by thyroid hormones in the plasma membrane is shown in Fig. 9. The cell-surface hormone signal is initiated at $\alpha v\beta 3$ integrin receptor and transduced through stimulation of PI3K, MAPKs (ERK1/2, JNK and p38) and intracellular calcium, culminating in the increased mRNA levels of the L-arginine transporter and of eNOS. Moreover, the present results suggest that

the continuous release of thyroid hormones, modulating L-arginine transporter activity, may contribute to the basal endothelial production of NO and vascular tone under physiological conditions; hence, alterations of this mechanism may participate in the cardiovascular abnormalities of thyroid disorders.

In conclusion, this paper shows for the first time that thyroid hormones stimulate L-arginine Na^{+} -dependent and -independent transporters and potentiate the NO production induced by calcium ionophore. These effects are blocked by pharmacological $\alpha v\beta 3$ integrin inhibition or genetic αv subunit downregulation or by PI3K, MAPK or intracellular calcium signaling inhibitors, indicating the key role of these mechanisms.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-18-0229>.

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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