L-Thyroxine promotes a proliferative airway smooth muscle phenotype in the presence of TGF-β1

Dekkers, Bart G J; Naeimi, Saeideh; Bos, I. Sophie T.; Menzen, Mark H; Halayko, Andrew John; Sadeghi Hashjin, Goudarz; Meurs, Herman

Published in:
American Journal of Physiology - Lung Cellular and Molecular Physiology

DOI:
10.1152/ajplung.00071.2014

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
l-Thyroxine promotes a proliferative airway smooth muscle phenotype in the presence of TGF-β1

Bart G. J. Dekkers,1,2 Saeideh Naeimi,1,3 I. Sophie T. Bos,1,2 Mark H. Menzen,1,2 Andrew J. Halayko,4 Goudarz Sadeghi Hashjin,5 and Herman Meurs1,2

1Department of Molecular Pharmacology, University of Groningen, Groningen, The Netherlands; 2Groningen Research Institute for Asthma and COPD, University of Groningen, Groningen, The Netherlands; 3Department of Pharmacology, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran; 4Department of Physiology, University of Manitoba, Winnipeg, Canada; and 5Department of Pharmacology, Faculty of Veterinary Medicine, University of Tehran, Teheran, Iran

Submitted 19 March 2014; accepted in final form 2 December 2014


Airway remodeling is a characteristic feature of chronic asthma which is considered to contribute importantly to airway hyperresponsiveness and lung function decline (6, 25). Airway remodeling is characterized by structural changes in the airway wall and includes epithelial shedding, goblet cell hyperplasia, increased vascularization, increased extracellular matrix (ECM) deposition, and increased airway smooth muscle (ASM) mass (22, 34). Of these changes, increased ASM mass is considered to be the major factor contributing to airway hyperresponsiveness and lung function decline (23, 31). Increased ASM mass may be explained by cellular hypertrophy as well as hyperplasia, the latter feature being consistent with the fact that mature ASM cells retain the ability to reenter the cell cycle (3, 14, 38). Thus exposure to mitogenic stimuli can induce a proliferative ASM phenotype that is associated with decreased expression of contractile marker proteins and decreased contractile function (11, 12, 17). Conversely, removal of mitogenic stimuli can increase contractile protein expression. Notably, hormones such as insulin, as well as the asthma-associated cytokine transforming growth factor-β1 (TGF-β1), can enhance expression of proteins associated with a contractile ASM phenotype (13, 30).

Asthma may coexist with a changed thyroid function. Indeed, hyperthyroidism is associated with increased asthma severity, whereas hypothyroidism is linked with amelioration of coexisting asthma (1, 7). Treatment of hyperthyroidism improves asthma symptoms, and vice versa, treatment of hypothyroidism increases asthma symptoms (2, 5, 19). Little is known about the mechanisms involved in the changes in asthma symptoms in patients with thyroid disease. However, some studies show that inflammatory responses are reduced in hypothyroid animals, potentially explaining, at least in part, the effects of thyroid hormones in asthma (24). In addition to inflammation, thyroid hormones could also affect remodeling processes, since serum thyroxine levels inversely correlate with reversibility in response to β2-adrenoceptor agonists (19).

The thyroid hormones triiodothyronine (T3) and thyroxine (T4) have diverse biological functions in normal development, growth, and metabolism. Classically, the thyroid hormones are thought to mediate their effects by affecting gene transcription via the nuclear thyroid receptors α (THRA) and β (THRβ). The receptors act as ligand-activated transcription factors upon binding of T3 but are not directly activated by the prohormone T4 (8). In the absence of T3, corepressors inhibit transcriptional activity. In the presence of T3, coactivators activate transcription (8). Alternatively, nongenomic pathways may also be involved, including cytoplasmic THRβ and integrin α1β3 that is localized in the plasma membrane. Activation of integrin α1β3 by T4, and to a lesser extent by T3, can induce proliferation of tumor cells via activation of ERK1/2 (8, 26).

The effects of thyroid hormones on ASM phenotype are currently unknown. Therefore, the aim of the present study was to explore the potential effects of thyroid hormones on ASM proliferation and contractile protein expression. Using proliferation assays and Western blot analysis, we demonstrate for the first time that T4, and to a lesser extent T3, increases ASM...
proliferation and decreases ASM contractile protein expression in the presence of the profibrotic growth factor TGF-β1. Notably, increased ASM proliferation induced by thyroxine in the presence of TGF-β1 required activation of integrin αvβ3, but not the thyroid receptors. These results indicate that thyroid hormones may enhance ASM remodeling in asthma, which could be of relevance for hyperthyroid patients with this disease.

MATERIALS AND METHODS

Materials. Dulbecco’s modified Eagle’s medium (DMEM), streptomyacin, penicillin, fetal bovine serum (FBS), and ITS (insulin, transferrin, and selenium) were obtained from Gibco BRL Life Technologies (Paisley, UK). Active, recombiant human TGF-β1 (Chinese hamster ovary cell line derived) was purchased from R&D systems (Abingdon, UK). 1–850 was obtained from Calbiochem (Nottingham, UK). Anti-α-smooth muscle actin (sm-α-actin), anticalponin, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibodies, T3, T4, and tetracycline were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). U0126 was obtained from Tocris (Bristol, UK). Anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alamar blue was obtained from BioSource (Camarillo, CA). [methyl-3H]thymidine (0.25 μCi/ml) was purchased from Amersham Biosciences (Amersham, UK). All other chemicals were of analytical grade.

ASM cell culture. Three nontransformed human bronchial smooth muscle cell lines, made quiescent by incubation in serum-free DMEM supplemented with streptomycin (50 U/ml), penicillin (50 μg/ml), and FBS (10% vol/vol). [3H]Thymidine incorporation. [3H]Thymidine incorporation in ASM cells was performed as described previously (11–13). ASM cells were plated on 24-well culture plates at a density of 20,000 cells per well and allowed to attach overnight in DMEM supplemented with streptomycin, penicillin, and FBS (10%). Subsequently, cells were washed with phosphate-buffered saline (PBS; composition: 140 mM NaCl, 2.6 mM KCl, 1.4 mM KH2PO4, and 8.1 mM Na2HPO4, pH 7.4) and made quiescent by incubation in serum-free DMEM supplemented with streptomycin, penicillin, and 1% ITS for 72 h. Subsequently, cells were washed and incubated in the absence or presence of T3 (1–100 nM), T4 (1–100 nM), TGF-β1 (2 ng/ml), the combination of TGF-β1 and T3, or the combination of TGF-β1 and T4 in serum-free DMEM supplemented with streptomycin and penicillin for 28 h, with the last 24 h in the presence of [methyl-3H]thymidine (0.25 μCi/ml). When used, cells were pretreated with 1–850 (3 μM), tetracycline (100 nM), or U0126 (3 μM) for 30 min before stimulation with T3 and/or TGF-β1. After incubation, the cells were washed with PBS at room temperature. Subsequently, the cells were treated with ice-cold 5% trichloroacetic acid on ice for 30 min, and the acid-insoluble fraction was dissolved in NaOH (1 M). Incorporated [3H]thymidine was quantified by liquid scintillation counting using a Beckman LS1701 beta-counter. Alamar blue conversion assay. Mitochondrial activity, as a measure of ASM cell number, was assessed by an Alamar blue conversion assay as described previously (11). Cells were plated as described above, allowed to attach overnight, and maintained in serum-free DMEM supplemented with streptomycin, penicillin, and 1% ITS for 3 days. Cells were then incubated with or without TGF-β1 (2 ng/ml), T3 (100 nM), T4 (100 nM), the combination of TGF-β1 and T3, or the combination of TGF-β1 and T4 in serum-free DMEM supplemented with antibiotics for 4 days. Thereafter, cells were incubated with Hanks’ balanced salt solution containing 5% Alamar blue solution for 30 min. Proliferation was assessed by conversion of Alamar blue, as indicated by the manufacturer.

Preparation of whole cell lysates. Cells were plated in six-well plates and grown until confluence. After serum deprivation for 24 h in serum-free DMEM supplemented with streptomycin and penicillin, the cells were stimulated with TGF-β1 (2 ng/ml), T3 (100 nM), T4 (100 nM), the combination of TGF-β1 and T3, or the combination of TGF-β1 and T4 in serum-free DMEM supplemented with antibiotics for 7 days. Culture medium was refreshed after 4 days of stimulation. To obtain whole cell lysates, stimulated cells were washed with ice-cold PBS, followed by lysis using cold SDS lysis buffer (composition: 62.5 mM Tris-HCl, 2% SDS, 1 mM NaF, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A, pH 8.0).

Western blot analysis. Equal amounts of protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose. To avoid nonspecific binding, membranes were blocked with blocking buffer (composition: 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dried milk powder) for 1 h at room temperature. Subsequently, the membranes were incubated with anti-sm-α-actin and anti-calcitonin antibodies diluted in blocking buffer for 1 h at room temperature. After membranes were washed 3 times with 0.1% Tris-buffered saline-Tween 20 (0.1% TBS-T; composition: 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) for 10 min, membranes were incubated with HRP-labeled secondary antibodies for 1 h at room temperature, followed by an additional 3 washes with 0.1% TBS-T. With the use of enhanced chemiluminescence reagents, bands were recorded in the G:BOX iChemi gel documentation system equipped with GeneSnap image acquisition software (Syngen, Cambridge, UK). Band intensities were quantified by densitometry using GeneTools analysis software (Syngen). All bands were normalized to GAPDH.

RNA isolation and real-time PCR. Total cellular RNA was isolated using the Nucleospin RNA II kit (Machery-Nagel, Bioke, Leiden, The Netherlands). RNA concentration was determined by Nanodrop ND1000 (ThermoScientific, Wilmington, MA). Total RNA was reverse transcribed using the Promega cDNA synthesis kit. Real-time quantitative PCR for THRA, THRB, integrin αv (ITGAV), and integrin β3 (ITGB3) was performed using an Illumina Eco Personal qPCR System (Westburg, Leusden, The Netherlands) and the specific primers listed in Table 1. Cycle parameters were as follows: denaturation at 94°C for 30 s, annealing at 58°C (THRA and THRB) or 60°C (ITGAV and ITGB3) for 30 s, and extension at 72°C for 30 s for 40 cycles. The abundance of the target gene was normalized to the endogenous reference 18S RNA (designated as ΔCq). Relative differences were determined by using the equation 2^{-ΔΔCq}.

Data analysis. Data are means ± SE. Statistical significance was determined by one-way ANOVA for paired observations, followed by a Newman-Keuls multiple comparisons test. Data were considered statistically significant when P < 0.05.

RESULTS

Effects of triiodothyronine and β-thyroxine on ASM cell proliferation. To investigate the potential effects of thyroid hormones on ASM proliferation, ASM cells were exposed to increasing concentrations of T3 (1–100 nM) or T4 (1–100 nM) for 28 h and proliferation was assessed by [3H]thymidine incorporation, a measure of DNA synthesis. Neither T3 nor T4 alone affected DNA synthesis compared with control (Fig. 1). When combined with the profibrotic growth factor TGF-β1 (2 ng/ml), a small but significant (P < 0.05) increase in DNA synthesis was observed for T3 at a concentration of 10 nM, whereas no effect was observed for the higher concentration of
100 nM. For T4, however, a strong synergistic effect was observed at the highest concentration used (100 nM; \( P < 0.001 \)). In line with previous findings from our laboratory (29), exposure to TGF-β1 alone for 28 h had no effect on DNA synthesis. Although less prominent, TGF-β1 and T3 also increased DNA synthesis in primary human tracheal smooth muscle cells, whereas likewise a smaller increase was observed for the combination of TGF-β1 and T3 (data not shown). In line with the findings on \([3H]\)thymidine-incorporation, we observed no effect for 1-850. Furthermore, there were no effects on basal proliferative responses or in the expression of contractile markers sm-α-actin and calponin by T3 alone for both antagonists. However, in striking contrast, T4 decreased expression by ~50% in TGF-β1-treated cultures (\( P < 0.001 \)). Although less pronounced, T3 also significantly decreased TGF-β1-induced contractile protein expression by 25% (\( P < 0.05 \)). Collectively, these results suggest that T4, and to a lesser extent T3, suppresses the acquisition of a contractile ASM phenotype in the presence of TGF-β1.

\[ \text{L-thyroxine increases airway smooth muscle proliferation via integrin } \alpha_\beta_3. \]

Actions of thyroid hormones may be initiated not only by the thyroid receptors but also via membrane-associated \( \alpha_\beta_3 \)-integrins (8, 26). To investigate the involvement of these receptors, we first determined their presence in ASM cells by PCR. In line with previous findings (15, 28), integrins \( \alpha_\beta_1 \) and \( \alpha_\beta_3 \) were both expressed by ASM cells in culture (Fig. 4A; \( \Delta \text{Cq} = 12.1 \pm 0.1 \) and 14.8 ± 0.3, respectively). In addition, ASM cells expressed transcripts for both the thyroid receptor \( \alpha \) and \( \beta \) (\( \Delta \text{Cq} = 15.0 \pm 0.1 \) and \( \Delta \text{Cq} = 16.7 \pm 1.6 \), respectively). To determine which of the receptors may be involved in functional synergism for proliferation between T4 and TGF-β1, ASM cells were incubated with the specific thyroid receptor antagonist 1-850 (3 \( \mu \)M) or tetrac (100 nM), an antagonist of the binding of T4 to the integrin \( \alpha_\beta_3 \). Treatment with tetrac fully inhibited T4 and TGF-β1 synergism to promote DNA synthesis (\( P < 0.001 \); Fig. 4B), whereas we observed no effect for 1-850. Furthermore, there were no effects on basal proliferative responses or in the presence of T3 or TGF-β1 alone for both antagonists.

Because activation of integrin \( \alpha_\beta_3 \) by T4 can activate ERK1/2 (MAPK) signaling (26), the contribution of \( \alpha_\beta_3 \) to

---

**Table 1. Primer sequences of thyroid receptor \( \alpha \), thyroid receptor \( \beta \), integrin \( \alpha_\alpha \), and integrin \( \beta_3 \) mRNA and 18S rRNA used for PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI Accession No.</th>
<th>Primer Sequence Product Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid receptor ( \alpha )</td>
<td>NM_199334</td>
<td>Forward 5'-TCG AGC ACT AGG TCA ACC AC-3' 120</td>
</tr>
<tr>
<td>Thyroid receptor ( \beta )</td>
<td>NM_000461</td>
<td>Reverse 5'-TCG ACT TCT AGG AGG AA-3' 78</td>
</tr>
<tr>
<td>Integrin ( \alpha_\alpha )</td>
<td>NM_002210</td>
<td>Forward 5'-AGG GCA TCG GTA GTT TCA ACC CT-3' 127</td>
</tr>
<tr>
<td>Integrin ( \beta_3 )</td>
<td>NM_000212</td>
<td>Reverse 5'-TGG ACA AAA TAG TCG AAA CCG-3' 105</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>M10098</td>
<td>Forward 5'-GCA TCC TTG GCA GAT GTC TTA A-3' 62</td>
</tr>
</tbody>
</table>

---

Fig. 1. Effects of triiodothyronine (T3; \( A \)) and L-thyroxine (T4; \( B \)) on ASM proliferation in the absence and presence of transforming growth factor-β1 (TGF-β1). Human airway smooth muscle (ASM) cells were treated with serum-free medium (control), T3 (1–100 nM), T4 (1–100 nM), TGF-β1 (2 ng/ml), the combination of TGF-β1 and T3, or the combination of TGF-β1 and T4 for 28 h, after which \([3H]\)thymidine incorporation was determined as a measure of DNA synthesis. Data represent means ± SE of 3–7 experiments, each performed in triplicate. *\( P < 0.05 \); **\( P < 0.001 \) compared with unstimulated controls.
TGF-β1- and T₄-induced proliferation was further explored using the MEK1/2 antagonist U0126 (3 μM). In line with the involvement of integrin α₃, we found that the proliferative effect of combined TGF-β1 and T₄ was fully inhibited by U0126 (P < 0.001; Fig. 5). In contrast, we did not observe any effect of U0126 on proliferative activity in basal conditions or in the presence of T₃ or TGF-β1 alone. Collectively, these results indicate that the synergistic effects of T₄ and TGF-β1 on ASM proliferation require the activation of integrin α₃β₃.

**DISCUSSION**

In the present study we have demonstrated for the first time that thyroid hormones synergize with TGF-β1 to induce a proliferative ASM phenotype. T₄ markedly increased ASM proliferation in the presence of TGF-β1, whereas T₃ only induced a minor increase in ASM proliferation. Furthermore, T₄, and to a lesser extent T₃, decreased the expression of the contractile marker proteins sm-α-actin and calponin in TGF-β1-exposed myocyte cultures. Remarkably, using pharmacological inhibitors, we revealed that the positive synergistic effects of T₄ and TGF-β1 on ASM proliferation do not require nuclear thyroid receptors, but instead are mediated via the α₃β₃-integrin. In line with these findings, inhibition of MEK1/2, a known downstream signaling effector of integrin α₃β₃ also inhibited ASM proliferation.

Airway hyperresponsiveness is a hallmark of asthma, which is defined by an exaggerated response to a variety of pharmacological, chemical, and physical stimuli (33). Acute, variable
Fig. 5. ASM proliferation induced by T4 and TGF-β1 is inhibited by the MEK inhibitor U0126. Human ASM cells were treated with serum-free medium (control), T4 (100 nM), TGF-β1 (2 ng/ml), or the combination of TGF-β1 and T4 for 28 h in the absence and presence of the MEK1/2 inhibitor U0126 (3 μM). ASM proliferation was determined using [3H]thymidine-incorporation. Data represent means ± SE of 3 experiments, each performed in triplicate. ***P < 0.001 compared with unstimulated controls. ###P < 0.001 compared with TGF-β1 + T4 in the absence of U0126.

Proliferation and contractile protein expression after 7 days of exposure) and cell number (4-day exposure). Moreover, in line with the switch from a contractile to a proliferative ASM phenotype, contractile protein exposure after 7 days of exposure to TGF-β1 and T4 was reduced as well. These effects on proliferation and contractile protein expression were more muted when T3 was combined with TGF-β1. These observations are of note because increased ASM mass contributes to airway hyperresponsiveness in asthmatic patients (23, 31). Collectively, these findings indicate thyroid hormones, in particular T4, may affect mechanisms that contribute to both acute and persistent airway hyperresponsiveness.

Recently, we and others have shown that integrins play an important role in airway remodeling in asthma (10, 27–29, 39). For example, we have identified a key role for integrin αvβ3 in mediating ASM proliferation in response to extracellular matrix proteins collagen I and fibronectin, growth factors, and serum (10, 27–29). In addition to integrin αvβ3, ~50% of the cultured ASM cells also express integrin αvβ1 (15, 28). Studies on the role of integrin αvβ3 in ASM function have found that it is required for cellular attachment to monomeric collagen type I (28), as well as enhanced IL-1β-induced cytokine release by ASM cells cultured on fibronectin (32). In the present study we show that this integrin may also promote ASM proliferation in vitro through a pathway involving both T4 and TGF-β1. The concentrations of T4 at which these synergistic effects occurred were comparable to concentrations previously shown to interact with the αvβ3-integrin and to activate the ERK1/2 signaling pathway (4). The precise mechanisms underlying cross talk between T4 and TGF-β1 are currently unknown, but our findings clearly position integrin αvβ3 in this process, perhaps through interaction with the TGF-β receptor. This scenario is supported by studies in lung fibroblasts, with αvβ3 subserving proliferation induced with TGF-β1 (36). Of note, signaling pathways downstream of TGF-β1 receptor and αvβ3, including ERK1/2, may also interact (8, 40). In line with this, we found that inhibition of MEK1/2, the upstream regulator of ERK1/2, prevents the synergism between TGF-β1 and T4 with respect to human ASM cell proliferation. These findings may be of relevance in vivo, because we have previously shown that the integrin blocking peptide Arg-Gly-Asp-Ser (RGDS), which can inhibit αvβ3, suppresses ASM remodeling in a guinea pig model of asthma (10). Collectively, these findings indicate that in addition to integrin αvβ3, integrin αvβ3 may play a role in ASM remodeling in asthma.

In conclusion, our findings indicate that T4 and TGF-β1 synergize to promote a proliferative ASM phenotype. Increased ASM proliferation induced by thyroxine in the presence of TGF-β1 requires activation of integrin αvβ3, but not thyroid receptors. These results indicate that thyroid hormones may enhance ASM remodeling in asthma, which could be of relevance for hyperthyroid patients with this disease.

ACKNOWLEDGMENTS

We thank Edwin Gerrits and Rene Westerhof for expert technical assistance.

Present address of B.G.J. Dekkers: Department of Clinical Pharmacy and Pharmacology, University Medical Centre Groningen, Groningen, The Netherlands.

GRANTS

A. J. Halayko is supported through the Canada Research Chair Program.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.