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

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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, Tehran, Iran

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Dekkers BG, Naeimi S, Bos IS, Menzen MH, Halayko AJ, Hashjin GS, Meurs H. l-Thyroxine promotes a proliferative airway smooth muscle phenotype in the presence of TGF-β1. Am J Physiol Lung Cell Mol Physiol 308: L301–L306, 2015. First published December 5, 2014; doi:10.1152/ajplung.00071.2014.—Hypothyroidism may reduce, whereas hyperthyroidism may aggravate, asthma symptoms. The mechanisms underlying this relationship are largely unknown. Since thyroid hormones have central roles in cell growth and differentiation, we hypothesized that airway remodeling, in particular increased airway smooth muscle (ASM) mass, may be involved. To address this hypothesis, we investigated the effects of triiodothyronine (T3) and l-thyroxine (T4) in the absence and presence of the profibrotic transforming growth factor (TGF)-β1 on human ASM cell phenotype switching. T3 (1–100 nM) and T4 (1–100 nM) did not affect basal ASM proliferation. However, when combined with TGF-β1 (2 ng/ml), T4 synergistically increased the proliferative response, whereas only a minor effect was observed for T3. In line with a switch from a contractile to a proliferative ASM phenotype, T4 reduced the TGF-β1-induced contractile protein expression by ~50%. Cotreatment with T3 reduced TGF-β1-induced contractile protein expression by ~25%. The synergistic increase in proliferation was almost fully inhibited by the integrin αvβ3 antagonist tetrac (100 nM), whereas no significant effects of the thyroid hormone receptor antagonist v1 (TGF-β1), l-thyroxine; triiodothyronine; transforming growth factor-β1

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 (THRB). 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 αvβ3 that is localized in the plasma membrane. Activation of integrin αvβ3 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 contractile protein expression. Using prolifera
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), streptomycin, penicillin, fetal bovine serum (FBS), and ITS (insulin, transferrin, and selenium) were obtained from Gibco BRL Life Technologies (Paisley, UK). Active, recombinant human TGF-β1 (Chinese hamster ovary cell line derived) was purchased from R&D systems (Abingdon, UK). 1-850 was obtained from Calbiochem (Zwijndrecht, The Netherlands). U0126 was obtained from To cors (Bristol, UK). Anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alamar blue was obtained from BioSource (Camarillo, CA). [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 senescence resistant by stable expression of human telomerase reverse transcriptase (hTERT), were used for all experiments (18). hTERT ASM cells were generated from primary human bronchial smooth muscle cells obtained from nonsmoking, asthma-free donors as described previously (18). All procedures were approved by the Human Research Ethics Board of the University of Manitoba. For all experiments, ASM cells were grown on uncoated plastic dishes in 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 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, or the combination of TGF-β1 and T3 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-calponin 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 (Syngene, Cambridge, UK). Band intensities were quantified by densitometry using GeneTools analysis software (Syngene). 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 α3 (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 L-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 T4 (data not shown). In addition, ASM cells expressed transcripts for both T3 (1–100 nM), T4 (1–100 nM), TGF-β1(2 nM), or 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 T4 or TGF-β1 alone 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.

**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 T4, 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 αvβ3, 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 T4 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 αvβ3.

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 αvβ3-integrin. In line with these findings, inhibition of MEK1/2, a known downstream signaling effector of integrin αvβ3 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...
Airway hyperresponsiveness is considered to reflect airway inflammation (9). Animal studies investigating thyroid hormones in airway inflammation in asthma reveal a reduction in allergen-induced inflammatory responses in thyroidectomized animals (24). Treatment of the animals with T4 for up to 16 days, but not for only 1 or 3 days, normalized the inflammatory response (24), indicating that thyroid hormones may promote airway inflammation. Persistent airway hyperresponsiveness is present in the majority of patients with chronic asthma and is considered to reflect airway remodeling (9). Studies on the effects of thyroid hormones on airway hyperresponsiveness in vivo have shown that mild thyreotoxicosis induced by 4 wk of treatment with T3 does not affect lung function, exercise capacity, or airway hyperresponsiveness in asthmatic patients (20). Similarly, T3-induced hyperthyroidism does not affect airway reactivity or lung function in healthy volunteers (21). Conversely, treatment of hypothyroid patients with T4, which leads to prolonged elevation of serum thyroxine levels compared with healthy controls, increases airway responsiveness and inflammatory cell counts (5). In addition, in asthmatics, T4 levels inversely correlate with reversibility in response to β2-adrenoceptor agonists (19), suggesting that T4 may promote airway remodeling. In our current studies, we found no effects of T3 and T4 on basal proliferative responses of ASM cells, a finding that contrasts with reports that T3 can increase proliferation of vascular smooth muscle cells (35, 37). In line with our previous studies, exposure to the asthma-associated profibrotic growth factor TGF-β1 for short time periods did not increase ASM proliferation (29). However, when we combined T4 with TGF-β1, we observed significant synergy to enhance ASM proliferation on the level of both DNA synthesis (28-h exposure) and cell number (4-day exposure). Moreover, in line with the switch from a contractile to a proliferative ASM phenotype, contractile protein expression 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 α3β1 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 α3β1, ~50% of the cultured ASM cells also express integrin αvβ3 (15, 28). Studies on the role of integrin α3β1 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 α3β1-integrin and to activate the ERK1/2 signaling pathway (4). The precise mechanisms underlying this cross talk between T4 and TGF-β1 are currently unknown, but our findings clearly position integrin α3β1 in this process, perhaps through interaction with the TGF-β receptor. This scenario is supported by studies in lung fibroblasts, with α3β3 subserving proliferation induced with TGF-β1 (36). Of note, signaling pathways downstream of TGF-β1 receptor and α3β3, 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 α3β1 and α3β3, suppresses ASM remodeling in a guinea pig model of asthma (10). Collectively, these findings indicate that in addition to integrin α3β1, integrin α3β3 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 α3β3, 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.

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**DISCLOSURES**

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


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