Nonclassical ligands for the thyrotropin receptor: functional studies on thyrostimulin and Graves' disease immunoglobulins
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CHAPTER 9

Complete inhibition of rhTSH and Graves’ disease IgG and M22 induced cAMP production in differentiated orbital fibroblasts by a low molecular weight TSHR antagonist

In preparation

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The TSH receptor (TSHR) on orbital fibroblasts (OF) is a likely target of the autoimmune attack in Graves ophthalmopathy (GO). We recently showed that both recombinant human TSH (rhTSH) and immunoglobulins isolated from serum of patients with Graves disease (GD-IgG) increase cAMP production in differentiated OF from patients with GO. However, only GD-IgG increased the production of hyaluronan (HA) by these cells. In the present study we used a novel low molecular weight (LMW) TSHR antagonist to further evaluate the role of the TSHR in the cAMP-induction by rhTSH and GD-IgG in differentiated OF from GO patients. In addition, we evaluated the effect of the LMW TSHR antagonist on cAMP and HA production in these cells by M22, a potent human monoclonal TSHR-stimulating antibody. cAMP production significantly increased after incubation of differentiated OF cultures with 10 mU/ml rhTSH (3-fold; \( P \leq 0.05 \)), 1 mg/ml GD-IgG (2-fold; \( P \leq 0.05 \)) and 500 ng/ml M22 (5-fold; \( P \leq 0.05 \)). Incubation with the LMW TSHR antagonist blocked the rhTSH-induced, the GD-IgG-induced as well as the M22-induced cAMP production in a dose-dependent way. In all three experimental conditions, the stimulated cAMP production was completely blocked by \( 10^{-6} \) M antagonist. Surprisingly, preliminary results show that M22 fails to increase HA synthesis by differentiated OF. Our results demonstrate that GD-IgG and M22-induced cAMP production in differentiated OF is completely mediated via the TSHR and that stimulation of cAMP signalling pathways via the TSHR might not be involved in HA synthesis.
INTRODUCTION

The close association between Graves’ hyperthyroidism and Graves’ ophthalmopathy (GO) suggests a shared underlying pathophysiological mechanism. The TSH receptor (TSHR) has been reported to be expressed in orbital adipose/connective tissue of patients with GO and has been proposed to be an important target of autoimmunity in GO. In line with this hypothesis, elevated TSHR expression has been demonstrated in orbital tissues from patients with active GO compared to patients with inactive disease. Moreover, the titre of circulating TSHR autoantibodies correlates with the clinical activity of GO and has some prognostic value for the severity and the outcome of GO. The heterogeneous population of TSHR-expressing orbital fibroblasts are currently considered target cells in the immunopathogenesis of GO. Orbital fibroblasts function as structural cells in the extracellular matrix, but a subpopulation that is overexpressed in GO shows exaggerated inflammatory responses. Various cytokines, such as interleukin-1β (IL-1β), leukoregulin and interferon-γ (IFN-γ), induce orbital fibroblasts to produce excessive amounts of the highly hydrophilic glycosaminoglycan (GAG) hyaluronan (HA), which is the major GAG in orbital tissues of GO patients. Another subpopulation of orbital fibroblasts, termed preadipocytes or lipofibroblasts, can differentiate into mature adipocytes. Inflammation promotes the differentiation of fibroblasts into adipocytes in various tissues. Interestingly, functional TSHR expression increases in orbital fibroblasts after adipocytic differentiation, suggesting that the differentiated orbital fibroblasts may play a distinct role in the immunopathogenesis of GO.

The precise role for the TSHR in the immunopathogenesis of GO has not been established beyond doubt. Binding of the TSHR by TSH or by immunoglobulins isolated from the serum of patients with Graves’ disease (GD-IgG) results in activation of the adenylyl cyclase/cyclic adenosine monophosphate (cAMP) signalling pathway via G-protein coupled pathways in several cell types, among which differentiated orbital fibroblasts. In addition, activation of the cAMP signalling cascade can induce HA synthesis in several cell types, among which orbital fibroblasts. We previously found that stimulation with GD-IgG, but not rhTSH, resulted in increased HA production in differentiated GO orbital fibroblasts. This intriguing finding prompted us to further investigate the putative role of the TSHR in the pathogenesis of GO. Recently, novel low molecular weight (LMW) antagonists for the human TSHR have been developed at MSD (NV Organon, Oss, The Netherlands). In the present study we used a nanomolar potent LMW TSHR antagonist to evaluate the role for the TSHR in the cAMP induction...
by rhTSH, GD-IgG and M22 – a potent human monoclonal TSHR-stimulating antibody 231 – in differentiated orbital fibroblasts from patients with GO.

MATERIALS & METHODS

Chemicals

The LMW human TSHR-antagonist was synthesized at MSD (NV Organon, Oss, The Netherlands) 230. Recombinant human TSH (rhTSH) was purchased from Genzyme Therapeutics (Thyrogen®; Cambridge, MA), IL-1β was obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). We used immunoglobulins isolated from sera of a GO patient (GD-IgG) and a healthy control subject (c-IgG) using protein G Sepharose 4 Fast Flow (ProtG; Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands), after obtaining informed consent 215. The GO patient had TSH-binding inhibitory immunoglobulin (TBII) titre of 256 U/l and had been diagnosed with moderately severe GO (CAS score 4/7) 196. The patient had not been treated with corticosteroids and GO was inactive at the time of blood sampling. The healthy control subject was TBII-seronegative (<1.0 U/l) 215.

We also studied the effect the LMW TSHR-antagonist on cAMP production and HA synthesis induced by a potent human monoclonal TSHR-stimulating antibody (M22 IgG; RSR Ltd, Cardiff, UK) 42, 231-233, commercially available control monoclonal human IgG [h-IgG; #A01006; GenScript, Piscataway, USA] was used as a control in these experiments.

Human TSHR-expressing Chinese hamster ovary cell line

Chinese hamster ovary cell line (CHO-K1; ATTC No. CCL-61, LGC Standards, Middlesex, UK) stably transfected with the human TSHR (CHO.hTSHR) was generated at MSD (NV Organon), Oss, The Netherlands and cultured in DMEM/F12 modified medium (Gibco, Breda, The Netherlands) containing 5% FSC, 100 U/ml penicillin G/ 0.1 mg/ml streptomycin, 0.4 mg/ml hygromycin B (Invitrogen, the Netherlands; Greiner, Alphen a/d Rijn, The Netherlands). The human TSHR complimentary DNA was provided by Prof. E. Milgrom, INSERM 234. For the cAMP assays, M22, control IgG or recombinant human TSH in 10 μl assay medium [Hank’s balanced salt solution, 5 mM Hepes pH7.4, 20 μM rolipram (Sigma) and 0.1% BSA] were added in triplicate to a 384 well plate. In addition, 10 μl of the LMW TSHR antagonist or 10 μl assay medium were added in triplicate to the wells. The cells were resuspended in assay medium (DMEM/F12 modified medium (Gibco, Breda, The Netherlands), 1 μg/ml bovine insulin (Sigma), 5 μg/ml human apo-transferrin (Sigma), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin and added to
the wells in a volume of 10 μl (7.5×10^5 cells/ml). Total incubation volume was 30 μl. The LMW human TSHR antagonist (final concentrations of 3.16E-10, 1.00E-9, 3.16E-9, 1.00E-8, 3.16E-8, 1.00E-7, 3.16E-7, and 1.00E-6 M) was incubated in the presence of 10 mU/ml rhTSH or 1500 ng/ml M22 for 120 min at 37°C, 5% CO₂.

**Orbital fibroblast cell cultures and adipocytic differentiation**

Orbital fat tissue explants were obtained from 8 patients with severe GO undergoing orbital decompression surgery. Two patients were treated with 1 g of corticosteroids on the day of surgery or the day before and suffered from dysthyroid optic neuropathy. Six patients underwent decompression surgery in the inactive stage of the disease and had not received corticosteroids or orbital irradiation in the previous six months. Collection of these tissues was approved by our Institutional Review Board.

Orbital fibroblast cell-lines were initiated as described previously and cultured in 6 wells plates in culture medium (199 (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 10 U/ml penicillin, streptomycin, fungizone, and 10% fetal calf serum (FCS; all from Cambrex, East Rutherford, NJ, USA) 179, 215, 235. We used the fibroblast cultures between the 3rd and 7th passage from initiation, implying that not all cultures were subjected to all experiments.

For each experiment, 5 x 10^4 fibroblasts per well were grown to 90% confluence in 6-well plates in ~48h. We carried out adipocytic differentiation as reported previously 93. The differentiation protocol was continued for two weeks, replacing the media every 3-4 days and adipocytic differentiation was evaluated using phase contrast microscopy. For the dose-response experiment of h-IgG and M22-IgG on cAMP production in differentiated orbital fibroblasts, we incubated the cells in 750 μl of the differentiation medium containing 0.5 mM IBMX for 30 min at 37°C, 5% CO₂ which was followed by adding 0, 1, 10, 100 or 1000 ng/ml IgG. After 6h, the supernatants were collected and stored at -80°C until use.

For the dose-response experiments of the antagonistic effect of the LMW TSHR antagonist on rhTSH-, GD-IgG- and M22-IgG-induced cAMP production in differentiated orbital fibroblasts, plates were preincubated in 750 μl of the differentiation medium containing 0.5 mM IBMX for 30 min at 37°C, 5% CO₂. Then, we added in triplicate increasing concentrations of LMW TSHR-antagonist. After 5 min cells were incubated with 10 mU/ml rhTSH, 1 mg/ml c-IgG or GD-IgG, or 500 ng/ml h-IgG or M22-IgG for 6h at 37°C, 5% CO₂. Supernatants were collected and stored at -80°C until analyzed for cAMP or HA content.
cAMP assay

CAMP measurements were performed in CHO.hTSHR cells and in the supernatants of differentiated GO orbital fibroblast cultures. We measured CAMP concentrations in triplicate using the Alphascreen cAMP Assay (PerkinElmer, Groningen, The Netherlands) and corrected for total amount of protein as described previously 215.

Hyaluronan assay

HA measurements were performed in culture supernatants of differentiated orbital fibroblasts of one GO patient (4 wells/group). Plates were preincubated in 750 μl differentiation medium with increasing concentrations TSHR-antagonist and after 5 min cells were incubated with 500 ng/ml h-IgG or M22 for 48h. As a positive control for HA synthesis, we stimulated the cells with 10 ng/ml IL-1β. All experimental conditions were carried out in quadruplicate. After 48h supernatants were collected and stored at -20°C until use. The HA content was quantified in duplicate with a specific ELISA-kit (Echelon Biosciences, Salt Lake City, UT) according to the manufacturer’s instructions and data were corrected for total amount of protein per well, as described previously 215.

Statistical analysis

Normal distribution of the data was tested using the Kolmogorov-Smirnov test. To test statistical significance (P<0.05) between groups we used Student t-test if data were normally distributed or Mann-Whitney-U test when data were not normally distributed. All tests were performed using SPSS (SPSS, Chicago, IL, USA).

RESULTS

Effect of LMW TSHR antagonist in TSHR-expressing CHO cell line

Stimulation of TSHR-expressing CHO.hTSHR cells with rhTSH in the absence of the antagonist resulted in a significant increase in cAMP production (maximal increase of 108 nM CAMP/7,500 cells). Incubation in the presence of the LMW TSHR antagonist resulted in inhibition of the cAMP response in a dose-dependent manner with an IC50 of 11 nM (pIC50 of 8.00 and 7.89, n=2 independent experiments). The concentration of 10^-6 M antagonist blocked the TSH-induced cAMP response completely (figure 1a).

Stimulation with M22 increased cAMP levels in TSHR-expressing CHO cells (maximal increase of 47 nM CAMP/7,500 cells). The TSHR antagonist blocked the M22-induced cAMP response dose-dependently with an IC50 of 4 nM (pIC50 of 8.28 and 8.40, n=2 independent experiments). Again, 10^-6 M antagonist completely reduced the cAMP induction by M22 (figure 1b). As control, M22 did not stimulate human FSHR- or LHR-
expressing CHO cells (results not shown) as demonstrated before. In addition, control human IgG had no effect on cAMP levels up to the highest tested concentration of 10$\mu$g/ml.

**Figure 1** Dose-response curves illustrating the inhibition of rhTSH-induced (a) and M22-induced (b) cAMP production in a human TSHR-expressing Chinese hamster ovary (CHO) cell line by the low molecular weight (LMW) TSHR-antagonist. We incubated CHO cells (n=2) with 0, 3.16 x 10$^{-10}$, 10$^{-9}$, 3.16 x 10$^{-9}$, 10$^{-8}$, 3.16 x 10$^{-8}$, 10$^{-7}$, 3.16 x 10$^{-7}$, and 10$^{-6}$ M LMW TSHR antagonist in the presence of 10 mU/ml rhTSH (a) or 1500 ng/ml M22. cAMP was measured after 120 min of incubation. Mean values ± SEM are shown.

Dose-response curves of rhTSH and M22 in differentiated GO orbital fibroblasts

We performed a dose-response experiment in the differentiated orbital fibroblast cultures from two GO patients. rhTSH was added in the concentrations 0, 0.1, 1, 10, 100 and 316 mU/ml. cAMP production was maximally increased – approximately 3-fold – in the wells treated with 100 mU/ml rhTSH. Stimulation with 316 mU/ml rhTSH resulted in an approximately 2.5-fold cAMP increase (figure 2a). The dose of 10 mU/ml rhTSH yielded a 2.5-fold increase and was used in the subsequent experiments.

In previous studies we found that stimulation of differentiated orbital fibroblasts with 1 mg/ml GD-IgG resulted in increased cAMP production and HA synthesis, we therefore used 1 mg/ml GD-IgG in subsequent experiments.

We stimulated differentiated orbital fibroblasts from one GO patient with 0, 1, 10, 100 and 1000 ng/ml M22 in order to establish the optimal dose M22 in our experimental setup. cAMP production was increased by stimulation with 100 ng/ml M22 (3-fold) and 1000 ng/ml M22 (10-fold; figure 2b). For subsequent experiments, we used 500 ng/ml M22 which resulted in an approximately 5-fold increase in cAMP synthesis.
LMW TSHR antagonist in the differentiated GO orbital fibroblasts

We stimulated 5 differentiated GO orbital fibroblast cultures with 10 mU/ml rhTSH in the absence or presence of increasing concentrations of hTSHR antagonist and measured cAMP content in the supernatants of the cells. Incubation for 6h with increasing doses of the TSHR antagonist (10^{-10} – 10^{-6} M) blocked the cAMP response to rhTSH dose-dependently (IC50 of 2.6 nM, n=5; figure 3a).

Differentiated orbital fibroblast cultures from 3 GO patients were stimulated with 1 mg/ml c-IgG or GD-IgG in the absence or presence of increasing concentrations of the LMW TSHR antagonist. We observed a small increase in cAMP synthesis by incubation of the cells with c-IgG compared to vehicle that was not altered by co-incubation with the antagonist (figure 3b). In the absence of the antagonist, GD-IgG treated differentiated orbital fibroblasts increased cAMP production approximately 2-fold compared to c-IgG treated cells. Incubation with the LMW TSHR antagonist inhibited the cAMP response in a dose-dependent manner (IC50 of 0.5 nM, n=3). The cAMP increase by GD-IgG was completely blocked when the cells were incubated with 10^{-6} M of the TSHR antagonist (figure 3b).

We incubated differentiated orbital fibroblasts from one GO patient with 500 ng/ml h-IgG or M22 in the absence or presence of increasing concentrations of the TSHR antagonist. We observed a small increase in cAMP concentration after incubation with h-IgG compared to vehicle that was not altered by incubation with the TSHR antagonist (figure 3c). M22 induced cAMP production 5-fold. Incubation with increasing...
**Figure 3** Dose-response curves illustrating the inhibition of (a) recombinant human TSH (rhTSH)-stimulated, (b) GD-IgG-stimulated and (c) M22-stimulated cAMP production in differentiated GO orbital fibroblasts by the low molecular weight (LMW) TSHR-antagonist (anta). Differentiated GO orbital fibroblasts (a: 5 patients; b: 3 patients; c: 1 patient) were preincubated with 0, 10^{-10}, 3.16 x 10^{-10}, 10^{-9}, 3.16 x 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M LMW TSHR-antagonist for 5 min. Then (a) 10 mU/ml rhTSH (□); (b) 1 mg/ml control IgG (c-IgG) or GD-IgG (gray rounds); or (c) 500 ng/ml control monoclonal human IgG (h-IgG) or M22 (○) was added and supernatant was collected after 6h. Data were corrected for mg protein. Left panel: data were pooled and normalized to the mean value of the control group, which was set at 1. Right panel: data are shown as a percentage of the cAMP response to (a) rhTSH, (b) GD-IgG or (c) M22 in the absence of the LMW TSHR antagonist, which was set at 100%. Mean values ± SEM are shown.
concentration of the LMW TSHR antagonist resulted in a dose-dependent inhibition of the cAMP response with an IC50 of 0.5 nM. M22-induced cAMP production was reduced to control levels when the cells were coincubated with 10^{-6} M antagonist (figure 3c).

**M22, LMW TSHR antagonist and HA synthesis**

Incubation of differentiated orbital fibroblasts from one GO patient with 500 ng/ml h-IgG or M22 did not affect HA concentrations in the supernatant of the cells and the addition of increasing concentrations of the TSHR antagonist had no influence on HA concentrations (figure 4). Stimulation with 10 ng/ml IL-1β resulted in 3-fold induction of HA production in this cell line (data not shown).

**DISCUSSION**

We recently showed that both rhTSH and GD-IgG increase cAMP production in differentiated orbital fibroblasts from patients with GO. The aim of the present study was to evaluate the role of the TSHR in rhTSH-, GD-IgG- and M22-induced cAMP production in differentiated orbital fibroblasts from patients with GO using a nanomolar potent LMW hTSHR antagonist developed at MSD (NV Organon)²¹.

The potency of the LMW hTSHR antagonist was demonstrated in a hTSHR expressing CHO cell line. The antagonist inhibited the cAMP production induced by rhTSH and M22 in a dose-dependent manner in these cells with an IC50 in the nM range for both agonists. cAMP production was completely blocked by 10^{-6} M of the antagonist.

In differentiated GO orbital fibroblasts, cAMP significantly increased after rhTSH, GD-IgG and M22. Stimulation with 500 ng/ml M22 increased cAMP production efficaciously in differentiated GO orbital fibroblasts (approximately 5-fold). Incubation in the presence of the antagonist completely blocked the rhTSH-induced cAMP production. In addition,
both GD-IgG-induced cAMP production was completely abolished by the LMW TSHR antagonist and M22-induced cAMP production was reduced to control levels. Our results show that GD-IgG and M22-induced cAMP production in differentiated orbital fibroblasts is completely mediated via the TSHR. We previously reported that GD-IgG – unlike rhTSH – increases HA production in differentiated orbital fibroblasts from patients with GO. In the present study, we showed that M22 – a potent monoclonal, TSHR-stimulating antibody – failed to increase HA synthesis in differentiated orbital fibroblasts from one patient, despite adequate HA induction in response to IL-1β by these cells. Although we will have to repeat this experiment in additional number of differentiated orbital fibroblast cultures, this result suggests that stimulation of cAMP signalling pathways via the TSHR is not responsible for HA synthesis in differentiated GO orbital fibroblasts. This is in line with our previous finding that stimulation of differentiated GO orbital fibroblasts by rhTSH does not result in increased HA. If confirmed in future experiments this might suggest that GD-IgG, which are capable of activating HA synthesis in differentiated orbital fibroblasts, act either via the TSHR and non-cAMP post-receptor signalling pathways or via other receptors.

The type 1 insulin-like growth factor receptor (IGF-1R) has been proposed to represent a second autoantigen relevant for the pathogenesis of Graves’ disease and GO. IGF-1R expression is higher in GO orbital fibroblasts compared to normal orbital fibroblasts and GD-IgG can activate the IGF-1R resulting in the synthesis of T cell chemoattractants. More recently, it has been proposed that a close association exists between the TSHR and the IGF-1R and that the IGF-1R may enhance certain aspects of TSHR signalling. Although this needs to be confirmed by other investigators, our findings that GD-IgG induces HA synthesis in differentiated – but not nondifferentiated – GO orbital fibroblasts would be in line with this hypothesis.

In conclusion, stimulation of cAMP production in differentiated orbital fibroblasts by rhTSH and M22 can be completely blocked by a LMW TSHR antagonist, indicating that GD-IgG- and M22-induced cAMP-responses are TSHR-mediated. Although our preliminary results suggest that stimulation of cAMP signalling pathways via the TSHR is not involved in HA synthesis by differentiated orbital fibroblast, further studies on HA synthesis will be needed to support this notion.