Complete Inhibition of rhTSH-, Graves’ Disease IgG-, and M22-Induced cAMP Production in Differentiated Orbital Fibroblasts by a Low-Molecular-Weight TSHR Antagonist

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The close association between Graves’ hyperthyroidism and Graves’ ophthalmopathy (GO) suggests a shared underlying pathophysiological mechanism. The TSH receptor (TSHR) is expressed on fibroblasts in orbital adipose/connective tissue and in extraocular muscles of patients with GO and has been proposed to be an important target of autoimmunity in GO (1). In line with this hypothesis, elevated TSHR expression has been demonstrated in orbital tissues from patients with active GO compared with patients with inactive disease (2). Moreover, the concentration of circulating TSHR autoantibodies correlates with the clinical activity and severity of GO and has some prognostic value for the severity and the outcome of GO (3).

Thy-1 (CD90) negative orbital fibroblasts (OF), termed preadipocytes or lipofibroblasts, can in vitro differentiate into mature adipocytes (4–6). Functional TSHR expression by OF increases after adipocytic differentiation (4–7). The findings suggest that differentiated OF play a distinct role in the immunopathogenesis of GO (8, 9).

Ligation 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/cAMP signaling pathway via G protein-coupled pathways in several cell types (10), among which differentiated OF (11). Recently a novel low-molecular-weight (LMW) antagonist for the human TSHR has been developed at MSD (Oss, The Netherlands), Org 274179-0. This compound...
changes neither \([^{125}\text{I}]\text{TSH}\) binding nor the dissociation of \([^{125}\text{I}]\text{TSH}\) from the TSHR. These results are fully compatible with an allosteric action of the compound at a binding site within the transmembrane region of the receptor, thereby blocking signaling (but not binding) of TSH (12). In the present study, we tested whether Org 274179-0 inhibits cAMP induction by recombinant human TSH (rhTSH), GD-IgG, and M22, a potent human monoclonal TSHR-stimulating antibody (13), in cultured differentiated OF derived from GO patients.

**Materials and Methods**

**Chemicals**

The LMW human TSHR-antagonist Org 274179-0 was synthesized at MSD (Oss, The Netherlands) (12). rhTSH was purchased from Genzyme Therapeutics (Thyrogen; Cambridge, MA). Forskolin (FSK), a direct activator of the cAMP pathway, was purchased from Sigma (Zwijndrecht, The Netherlands). We used immunoglobulins isolated from serum of a GO patient (GD-IgG) and from 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 (14). The GO patient had a TSH-binding inhibitory immunoglobulin titer of 256 U/liter (15). The patient had been treated with corticosteroids, and GO was inactive at the time of blood sampling. The healthy control subject was TSH-binding inhibitory immunoglobulin seronegative (<1.0 U/liter) (14).

The potent human monoclonal TSHR-stimulating antibody M22 was obtained from RSR Ltd. (Cardiff, UK) (13), and a commercially available control monoclonal human IgG [h-IgG; no. A01006; GenScript, Piscataway, NJ] was used as a control in these experiments.

**Human TSHR-expressing Chinese hamster ovary (CHO) cell line, orbital fibroblast cell cultures, and adipocytic differentiation**

Chinese hamster ovary cell line (CHO-K1; ATCC no. CCL-61; LGC Standards, Middlesex, UK) stably transfected with the human TSHR (CHO.hTSHR) was generated at MSD (NV Organon) and cultured in a DMEM/F12-modified medium (Life Technologies, Inc., Breda, The Netherlands) containing 5% bovine calf serum, 100 U/ml penicillin G, 0.1 mg/ml streptomycin (Invitrogen The Netherlands; Greiner, Alphen a/d Rijn, The Netherlands). The human TSHR cDNA was provided by Professor E. Milgrom (Institut National de la Sante´ et de la RecherchCe, Le Kremlin Bicetre, France) (16).

Orbital fat tissue explants were obtained from eight 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 6 months. Collection of these tissues was approved by our Institutional Review board.

Of cell lines were initiated as described previously (11). We used the fibroblast cultures between the third and seventh passage from initiation. For each experiment, 5 × 10⁴ fibroblasts per well were grown to 90% confluence in six-well plates in approximately 48 h. We carried out adipocytic differentiation as reported previously (11).

**cAMP production**

CHO.hTSH cells (7.5E5 cells/ml), pretreated with LMW TSHR antagonist Org 274179-0 were stimulated with 500 ng/ml M22, control IgG, or 10 μM rhTSH in assay medium [Hanks’ balanced salt solution; 5 mM HEPES, pH 7.4; 20 μM rolipram (Sigma); and 0.1% BSA] for 60 min at 37 C, 5% CO₂. Control experiments showed that a 5-min preincubation of the cells with Org 274179-0 before addition of agonist did not change the IC₅₀ of Org 274179-0 (results not shown).

For the dose-response experiment of h-IgG and M22-IgG on cAMP production in differentiated OF, we incubated the cells in differentiation medium containing 0.5 mM 3-isobutyl-1-methylxanthine for 30 min at 37C, 5% CO₂, which was followed by adding 0, 1, 10, 100, or 1000 ng/ml M22-IgG for 6 h.

For the dose-response experiments of the antagonistic effect of Org 274179-0 on rhTSH-, GD-IgG- and M22-IgG-induced cAMP production in differentiated OF, cells were preincubated in differentiation medium containing 0.5 mM 3-isobutyl-1-methylxanthine for 30 min at 37 C, 5% CO₂. Subsequently, increasing concentrations of Org 274179-0 were added. After 5 min cells were incubated with 10 μM rolipram, 1 mg/ml c-IgG or GD-IgG, or 500 ng/ml h-IgG or M22-IgG or 50 μM FSK for 6 h at 37 C, 5% CO₂. Supernatants were collected and stored at −80 C until analyzed for cAMP, which was measured using the AlphaScreen cAMP assay (PerkinElmer, Groningen, The Netherlands) and corrected for total amount of protein as described previously (14).

**Statistical analysis**

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

**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 Org-274179-0 resulted in a significant increase in the cAMP production (maximal increase of 80 nm cAMP per 7500 cells). Incubation in the presence of Org-274179-0 resulted in the inhibition of the cAMP response in a dose-dependent manner with an IC₅₀ of 11 nm (pIC₅₀ [the negative logarithm of the IC₅₀ value] of 8.00 and 7.89, \(n = 2\) independent experiments). The concentration of 10⁻⁶ M Org-274179-0 blocked the TSH-induced cAMP response completely (Fig. 1A).

Stimulation with M22 increased cAMP levels in TSHR-expressing CHO cells (maximal increase of 47 nm cAMP
per 7500 cells). Org-274179-0 blocked the M22-induced cAMP response dose dependently with an IC$_{50}$ of 4 nM (pIC$_{50}$ of 8.28 and 8.40, n = 2 independent experiments). Again, 10$^{-6}$ M Org-274179-0 completely reduced the cAMP induction by M22 (Fig. 1B). As a control, M22 did not stimulate human FSHR- or LHR-expressing CHO cells (results not shown) as demonstrated before (12). In addition, control human IgG had no effect on cAMP levels up to the highest tested concentration of 10 µg/ml.

**LMW TSHR antagonist Org-274179-0 in the differentiated GO orbital fibroblasts**

We stimulated five differentiated OF cultures with 10 mU/ml rhTSH in the absence or presence of increasing concentrations of Org-274179-0. cAMP concentrations were significantly increased in response to rhTSH, and incubation with increasing doses of Org-274179-0 (10$^{-10}$ to 10$^{-6}$ M) blocked the cAMP response to rhTSH dose dependently (IC$_{50}$ of 2.6 nM, pIC$_{50}$ 8.58 ± 0.32, n = 5; Fig. 2a). Incubation of the cells with Org-274179-0 did not affect cell growth and vitality as shown by the unchanged protein content of the cells.

Differentiated OF cultures from three GO patients were stimulated with 1 mg/ml c-IgG or GD-IgG in the absence or presence of increasing concentrations of Org-274179-0. The IgG concentration was based on earlier experiments showing that stimulation of differentiated OF with 1 mg/ml GD-IgG results in increased cAMP production (11). We observed a small increase in cAMP synthesis by incubation of the cells with c-IgG compared with vehicle that was not altered by coincubation with the antagonist (Fig. 2B). In the absence of the antagonist, GD-IgG-treated differentiated orbital fibroblasts increased cAMP production approximately 2-fold compared with c-IgG-treated cells. Incubation with Org-274179-0 inhibited the cAMP response in a dose-dependent manner (IC$_{50}$ of 0.9 nM, pIC$_{50}$ 9.04 ± 0.35, n = 3). The cAMP increase by GD-IgG was completely blocked with 10$^{-6}$ M Org-274179-0 (Fig. 2B).

Stimulation of differentiated OF with 100 or 1000 ng/ml M22 increased cAMP production approximately 3- or 10-fold, respectively (data not shown). M22 (500 ng/ml) was used in subsequent experiments, which resulted in a 5-fold cAMP increase. Differentiated OF from one GO
patient were incubated with 500 ng/ml h-IgG or M22 in the absence or presence of increasing concentrations of Org-274179-0. We observed a small increase in cAMP concentration after incubation with h-IgG compared with vehicle that was not altered by incubation with the TSHR antagonist (Fig. 2C). Incubation with increasing concentration of Org-274179-0 resulted in a dose-dependent inhibition of the cAMP response with an IC$_{50}$ of 0.5 nM. M22-induced cAMP production was reduced to (almost) control levels when the cells were coincubated with $10^{-6}$ M antagonist (Fig. 2C). Org-274179-0 did not inhibit TSHR-independent cAMP production induced by FSK.

**Discussion**

The aim of the present study was to test whether the newly developed compound Org-274179-0 inhibits cAMP production upon ligation of the TSHR. Org-274179-0 dose dependently inhibited cAMP production induced by rhTSH in a hTSHR-expressing CHO cell line. In differentiated OF obtained form GO patients, Org-274179-0 also inhibited at nanomolar concentrations cAMP production induced by rhTSH, GD-IgG or M22; FSK-induced cAMP production was not affected by Org-274179-0. We conclude that Org-274179-0 is able to inhibit cAMP production in differentiated human OF, induced by ligation of the TSHR.

In principle, our findings suggest that Org-274179-0 might be used as a therapeutic agent in the treatment of GO, in analogy to other recently developed LMW TSHR antagonists that are proposed as a possible therapy for Graves’ hyperthyroidism (17, 18). These antagonists as reported by Neumann et al., however, block TSH and TSI signaling only at high micromolar potencies (i.e. IC$_{50}$ values ranging from 1 to 30 $\mu$M as measured in cAMP assays), in contrast to IC$_{50}$ values in the nanomolar range of Org-274179-0 (17, 18). Further experiments are clearly indicated to see whether Org-274179-0 also inhibits non-cAMP signaling pathways upon activation of TSHR in OF (19) and to evaluate whether the inhibitory effects of Org-274179-0 are followed by a reduction of glycosaminoglycan production in OF.

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