A Drug-Like Antagonist Inhibits Thyrotropin Receptor–Mediated Stimulation of cAMP Production in Graves' Orbital Fibroblasts

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Background: Fibroblasts (FIBs) within the retro-orbital space of patients with Graves' disease (GOFs) express thyrotropin receptors (TSHRs) and are thought to be an orbital target of TSHR-stimulating autoantibodies in Graves' ophthalmopathy (GO). Recently, we developed a low molecular weight, drug-like TSHR antagonist (NCGC00229600) that inhibited TSHR activation in a model cell system overexpressing TSHRs and in normal human thyrocytes expressing endogenous TSHRs. Herein, we test the hypothesis that NCGC00229600 will inhibit activation of TSHRs endogenously expressed in GOFs.

Methods: Three strains of GOFs, previously obtained from patients with GO, were studied as undifferentiated FIBs and after differentiation into adipocytes (ADIPs), and another seven strains were studied only as FIBs. ADIP differentiation was monitored by morphology and measurement of adiponectin mRNA. FIBs and ADIPs were treated with the TSH- or TSHR-stimulating antibody M22 in the absence or presence of NCGC00229600 and TSHR activation was monitored by cAMP production.

Results: FIBs contained few if any lipid vesicles and undetectable levels of adiponectin mRNA, whereas ADIPs exhibited abundant lipid vesicles and levels of adiponectin mRNA more than 250,000 times greater than FIBs; TSHR mRNA levels were 10-fold higher in ADIPs than FIBs. FIBs exhibited higher absolute levels of basal and forskolin-stimulated cAMP production than ADIPs. Consistent with previous findings, TSH stimulated cAMP production in the majority of ADIP strains and less consistently in FIBs. Most importantly, NCGC00229600 reduced both TSH- and M22-stimulated cAMP production in GOFs.

Conclusions: These data confirm previous findings that TSHR activation may cause increased cAMP production in GOFs and show that NCGC00229600 can inhibit TSHR activation in GOFs. These findings suggest that drug-like TSHR antagonists may have a role in treatment of GO.

Introduction

ALTHOUGH THE PATHOGENESIS of Graves' ophthalmopathy (or orbitopathy) (GO) has not been fully delineated, a consensus has arisen that fibroblasts (FIBs) expressing thyrotropin receptors (TSHRs) within the retro-orbital space are a target of TSHR-stimulating autoantibodies (TSABs) and TSAB activation of TSHRs on these cells is involved in causing GO [reviewed in Refs. (1,2)]. A number of laboratories around the world use FIBs derived from the retro-orbital space of patients with GO in *in vitro* studies to gain insight into this process [reviewed in Ref. (3)]. The appeal of primary cultures of Graves' orbital FIBs (GOFs) as models is that they are hu-

man cells that may have been preconditioned by exposure to the Graves' environment *in vivo* and therefore reflect the target cell in patients. Moreover, despite much effort put forth by several groups, there has not been a good animal model for GO although recently a potential mouse model has been reported (4,5).

We have developed a low molecular weight, drug-like compound (NCGC00229600), referred to here as C-1, that acts *in vitro* as an antagonist of activation of TSHR by TSH and by TSABs in the sera of patients with Graves' disease (6), and of signaling by constitutively active mutant TSHRs found in patients with nonautoimmune hyperthyroidism (7). We have shown inhibition of TSHRs ectopically overexpressed in a

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model cell system and of TSHRs endogenously expressed in human thyrocytes in primary culture. Other drug-like TSHR antagonists have been reported (7–9). Although it is predicted that these antagonists would inhibit TSHRs expressed in other cell types, it is important to demonstrate this, especially in GOFs.

Herein, we show that C-1 inhibits both TSH and stimulating antibody activation of TSHRs endogenously expressed in GOFs.

Methods

Cell culture

Three GOF strains were previously obtained from GO orbital decompression surgical specimens and frozen (10). Seven GOF strains were freshly isolated FIBs that had not been frozen (indicated in Results section). The clinical data of the tissue donors are summarized in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/thy). Use of these samples was approved by the Mayo Clinic Institutional Review Board and studies were carried out according to the Institutional Review Board guidelines. Thawed cells were initially proliferated as undifferentiated FIBs in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/gentamicin (growth medium) in a humidified 5% CO₂ incubator at 37°C. To differentiate cells into adipocytes (ADIPs), confluent cultures of FIBs were incubated in the same medium supplemented with 0.1 mM indomethacin, 0.1 μ M dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and 10 μ g/mL insulin (Sigma) (differentiation medium) for 10–14 days as described (10).

mRNA measurement

Cells were lysed, total RNA was purified using RNeasy Micro kits (Qiagen), and cDNA was prepared using a High-Capacity cDNA Archive Kit (Applied Biosystems) (6). RT-PCR was performed in 25 μ L reactions using the Universal PCR Master Mix (Applied Biosystems). Primers and probes were Assay-on-Demand (Applied Biosystems). Quantitative RT-PCR results were normalized to GAPDH.

Lipid vesicles

The number and size of lipid vesicles were estimated using oil Red O staining.

cAMP production

To study cAMP production in FIBs or ADIPs, cells were incubated in a medium without IBMX for 48 hours before the time of the measurement. Cells were washed and preincubated in Hank's Balanced Salt Solution containing 10 mM HEPES buffer, pH 7.4 (HBSS/HEPES) for 30 minutes at 37°C. Thereafter, the cells were incubated for an additional 15 minutes without or with 10 or 30 μ M C-1 and then in HBSS/HEPES containing 0.5 mM IBMX without or with 10 or 30 μ M C-1 and without or with 100 mU/mL bovine TSH (Sigma), 100 ng/mL M22 monoclonal antibody (Kronus) or 20 μ M BW 245C, a prostaglandin D2 receptor agonist (Cayman Chemical) at 37°C. After 1 hour, cells were lysed using the lysis buffer of the cAMP-Screen Direct™ System (Applied Biosystems), and

cAMP was determined as described by the manufacturer. Experiments with M22 used the Parameter™ System (R&D Systems) for cAMP determinations at 10, 30, and 60 minutes. The chemiluminescent signal was measured in a VICTOR3™ V 1420 Multilabel Counter (Perkin Elmer). A data analysis was performed with GraphPad Prism 4 for Windows.

Statistical analysis

A statistical analysis was performed by the *t*-test or ANOVA. Significance between the treatments was shown with $p < 0.05$.

Results

The GOF strains underwent ADIP differentiation under the protocol used as evidenced by, on average, a >250,000-fold increase in mRNA levels of adiponectin, which is expressed exclusively in ADIPs (Fig. 1). However, the acquisition of lipid vesicles was variable in ADIPs of different strains, and the numbers and sizes of lipid vesicles in cells of the same strain varied in a single experiment and from one experiment to another (not shown). As expected (3), the levels of TSHR mRNA increased when FIBs were differentiated to ADIPs; in our experiments, the average TSHR mRNA level increased 10-fold (Fig. 1).

As cAMP is a primary second messenger of TSHR signaling, we monitored TSHR activation by measuring TSH stimulation of cAMP production. We compared TSHR-mediated production of cAMP to that produced by forskolin, which is a general pharmacologic activator of cAMP production and is used to estimate the cAMP production capacity of a cell system. Figure 2 illustrates a number of interesting observations regarding cAMP production and the effects of TSH and forskolin in three strains of FIBs and ADIPs. Overall, the three strains exhibited qualitatively similar profiles. Although the

FIG. 1. Effect of adipocyte (ADIP) differentiation on adiponectin (ADIPOQ) and thyrotropin receptor (TSHR) mRNA levels. mRNA levels were measured in undifferentiated fibroblasts (FIBs) and ADIPs in three sets of strains of Graves' orbital fibroblasts (GOFs). The cycle thresholds (C_ts) were normalized to a GAPDH C_t=16; one C_t lower equals a twofold increase in mRNA level. The bars represent the mean $\pm$ SD of six replicates in three sets. The dotted line is the limit of detectability of the assay. * $p < 0.01$ vs. FIBs; ** $p < 0.05$ vs. FIBs.

FIG. 2. Effects of TSH to stimulate cAMP production in three GOF strains. Basal, TSH (100 mU/mL)-stimulated, and forskolin (10 μ M)-stimulated cAMP production were measured in FIBs and ADIPs of three strains as described in Materials and Methods section. The bars represent the mean $\pm$ range of duplicate determinations in a representative of 4 experiments. * p < 0.05 of TSH-stimulated vs. basal cAMP when analyzed for all four experiments.

absolute levels in the three strains were different, FIBs exhibited higher levels of basal and forskolin-stimulated cAMP production than ADIPs. That is, the cAMP-generating system was more robust in FIBs than in ADIPs. Importantly, TSH did not consistently stimulate cAMP production in FIBs of any strain; some FIB strains would exhibit TSH stimulation more consistently than others would. However, TSH did stimulate cAMP production in two of the three strains of ADIPs shown in Figure 2 and in the majority of other ADIP strains studied. Some ADIP strains exhibited TSH-stimulated cAMP production in some, but not in other experiments. Because of the above-noted findings, we decided to use pools of GOFs to minimize the variability in TSH-stimulated cAMP production and allow us to pool the data from several experiments. Figure 3 illustrates the data from three experiments. The data show that in these experiments TSH stimulated cAMP production in ADIPs, but not in FIBs. TSH stimulation was normalized to cAMP production stimulated by forskolin. As a percent of maximal (forskolin-stimulated) cAMP production, basal cAMP production was higher in FIBs (11%) than in ADIPs (3%). As noted above, TSH had no effect in FIBs, but stimulated cAMP production 2.3-fold in ADIPs.

We next determined whether C-1 inhibited TSHR activation in ADIPs and compared its effect on stimulation of cAMP production by another G protein-coupled receptor using a drug-like agonist BW245C of the prostaglandin D2 receptor (Fig. 4). It was shown that activation of the prostaglandin D2 receptor by BW245C can increase cAMP in GOFs (11). BW245C stimulated cAMP production to a much higher level than TSH. Although we did not estimate the number of functional receptors in these experiments, it is likely that the more robust effect of BW245C compared to TSH was because there are many more prostaglandin D2 receptors than TSHRs in these cells. C-1 had no effect on basal cAMP production in this pool of three strains of GOFs. Importantly, C-1 had no effect on BW245C stimulation, but inhibited TSH-stimulated

FIG. 3. Effects of TSH on cAMP production in pooled GOF strains. Basal, TSH (100 mU/mL)-stimulated, and forskolin (10 μ M)-stimulated cAMP production were measured in FIBs and ADIPs from a pool of three GOF strains as described in Materials and Methods section. The results are shown as % of forskolin-stimulated cAMP. The bars represent the mean $\pm$ SD of seven replicate determinations in three experiments. * p < 0.05 for TSH-stimulated vs. basal cAMP production.

cAMP production to basal levels. Thus, C-1 was an effective, specific antagonist of TSHR activation by TSH in ADIPs.

As we previously showed that C-1 was an effective antagonist of TSHR activation by TSABs in model cell systems and in human thyrocytes (6), we used the monoclonal antibody M22 derived from a polyclonal population of human TSABs to determine whether C-1 would antagonize antibody-stimulated TSHR-mediated cAMP production in GOFs.

FIG. 4. Effects of C-1 on cAMP production stimulated by TSH or BW245C (BW) in ADIPs from pooled GOF strains. Basal, TSH (100 mU/mL)-stimulated and BW245C (20 μ M)-stimulated cAMP production were measured in ADIPs from a pool of three GOF strains in the absence or presence of 10 μ M C-1 as described in Materials and Methods section. The bars represent the mean $\pm$ SD of triplicate determinations in one of three experiments. * p < 0.05 vs. basal; ** p < 0.05 vs. TSH.

FIG. 5. Effects of C-1 on cAMP production stimulated by M22 FIBs from 7 strains of GOFs. Basal and M22 (100 ng/mL)-stimulated cAMP production were measured in FIBs from GOF strains ($n=7$) in the absence or presence of 30 μ M C-1 as described in Materials and Methods section. The bars represent the mean $\pm$ SD of triplicate determinations in seven experiments. * $p < 0.05$ vs. basal; ** $p < 0.05$ vs. M22.

Figure 5 illustrates the effects of C-1 on M22-stimulated cAMP production in FIBs from strains of GOFs ($n=7$) derived from patients with GO. These seven GOF strains were freshly obtained and had not been frozen at the time of the experiments. C-1 inhibited M22-stimulated cAMP production by 66% in 7/7 strains studied. C-1 showed also an inhibitory effect on M22-induced TSHR activation in ADIPs (data not shown).

Discussion

In the present study, we tested whether the small molecule drug-like TSHR antagonist C-1 can inhibit TSH- and M22-induced cAMP production in FIBs and ADIPs.

The levels of TSHR mRNA increased when FIBs were differentiated to ADIPs. These data are consistent with previous findings (10,12). TSHR functionality in FIBs and ADIPs has been demonstrated by showing TSH stimulation of cAMP production (13,14). We also observed TSH-mediated cAMP production. However, the responses to TSH were small in FIBs, but higher in ADIPs, and TSH did not consistently stimulate cAMP production in all FIB strains. This variation in TSH stimulating efficacy has been reported previously (3) and might be expected in these primary cultures given the heterogeneity of the cells derived from different GO patients. The variability in TSHR responsiveness might also be due to the fact that the three FIB strains used in the experiments with TSH stimulation were revived from the frozen state (Figs. 2 and 3), while responsiveness to ligands is usually better in freshly isolated cells. This might also explain that M22 showed a good increase in cAMP production in the 7 FIB strains that had not been frozen at the time of the experiment (Fig. 5). Our data show that the use of pools of GOFs is one way to at least partially overcome the variability found with individual strains.

C-1 is an effective antagonist of both TSH- and antibody-mediated activation of TSHRs in GOFs. Although we had shown previously that C-1 was an inverse agonist at the TSHR, inhibiting basal signaling by high levels of wild-type TSHRs and constitutive signaling by mutant TSHRs (7), it is likely that the basal-signaling activity of TSHRs in ADIPs did not sufficiently increase cAMP production to be detected in our experiments. Our demonstration of inhibition of TSHR activation by C-1 in GOFs is potentially important in a therapeutic context. As M22 has been shown to stimulate adipogenesis (15) and other TSABs to enhance hyaluronan production in GOFs (16), the antagonism of TSHR activation in these cells would be expected to diminish the increased orbital fat volume and hyaluronan accumulation characteristic of the orbit in GO patients (1).

Thus, we have shown that C-1 antagonizes TSHR activation in GOFs *in vitro*. These data serve as proof-of-principle that if activation of TSHRs on retro-orbital FIBs or ADIPs in patients with Graves' disease is an important component of the pathogenesis of GO, that C-1, or other TSHR antagonists (7,9,17), could serve as lead drugs for the prevention of or treatment of GO (18,19).

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Disclosure Statement

The authors declare that no competing financial interests exist.

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