Modulation of expression of somatostatin receptor subtypes in Graves’ ophthalmopathy orbits: relevance to novel analogs

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Cozma I, Zhang L, Uddin J, Lane C, Rees A, Ludgate M. Modulation of expression of somatostatin receptor subtypes in Graves’ ophthalmopathy orbits: relevance to novel analogs. Am J Physiol Endocrinol Metab 293: E1630–E1635, 2007. First published September 11, 2007; doi:10.1152/ajpendo.00177.2007.—Apart from evaluating orbital inflammation in Graves’ ophthalmopathy (GO), somatostatin (SST) analogs have been proposed as a therapy, but recent trials were disappointing. We aimed to measure somatostatin receptor (SSTR) expression in orbital tissues ex vivo and determine whether the new broad-affinity analog SOM230 might be of therapeutically useful. Orbital adipose/connective tissues from 29 GO patients and 10 normal individuals were analyzed. Transcripts were quantified using SYBR Green and a light cycler. In vitro models were used to investigate whether thyrotropin receptor activation (as occurs via thyroid stimulating antibodies) or adipogenesis affected SST expression in primary preadipocytes and to compare the biological activity of octreotide and SOM230 in their modulation. The expression of SSTR1 was significantly higher in GO patients than normal controls (P = 0.024). Although differences in the expression of SSTR2 were not significant, 39% of GO samples had levels above the 97th percentile of the controls. SSTR3, -4, and -5 were at or below the limit of detection (LOD). The lymphocyte contribution was minimal, since CD3α transcripts were at the LOD. TSH receptor activation did not modulate SSTR expression. An in vitro model of adipogenesis indicated upregulation of SSTR1 and SSTR2 during differentiation. SOM230 produced significantly greater inhibition of orbital preadipocyte proliferation than octreotide. Ex vivo analysis of orbital tissues reveals upregulation of SSTR1 and -2 in a group of GO patients. Adipogenesis, a process occurring in GO orbits, provides one possible explanation for some of the observed increase.

SOMATOSTATIN (SST) analogs such as octreotide (OCT) have a wide application in clinical imaging. SST receptors (SSTR) are expressed in many tissues, including activated lymphocytes. Consequently, OCT scintigraphy was proposed as a method to evaluate orbital inflammation in Graves’ ophthalmopathy (GO), with the level of uptake correlating well with the clinical activity score (CAS) (7, 17).

Subsequently, it was suggested that SST analogs could provide a treatment for GO, with several studies (9, 11) reporting an improvement in CAS. However, in one of two recent double-blind, placebo-controlled trials, no significant therapeutic effect of OCT long-acting repeatable was found in patients with moderate to severe GO (4). In contrast, a second study reported an improvement in CAS (although the clinical benefits were questioned), and eyelid fissure was reduced (21).

A further prospective randomized study (2) showed no difference in the CAS between patients with active GO and controls treated with slow-release lanreotide at 12 wk. Although the diplopia in downward gaze improved, this parameter has limited clinical relevance.

The biological effects of SST are mediated via five different G protein-coupled receptors, SSTR1–5, with OCT binding with high affinity to SSTR2 and -5 and moderate affinity to SSTR3. Two publications have addressed the expression of SST in GO orbits. Both have employed a semiquantitative PCR protocol and have examined tissues following periods of in vitro culture. Normal and GO orbital fibroblasts (14), in common with normal dermal fibroblasts (6), were shown to express SSTR2 and -3; only GO orbital fibroblasts express SSTR1 and -5. Furthermore, pharmacological doses of OCT inhibited GO orbital fibroblast growth and forskolin-induced cAMP production (14). Subsequently, the same group reported moderate to strong expression of all five SST in lymphocytes obtained from GO orbits, in contrast to the equivalent peripheral blood mononuclear cells, which express only SSTR2, -3, and -4 (15).

In recent years adipogenesis has been recognized (3, 18) as an important pathogenic mechanism in GO. A recent study (20) has demonstrated that SST production is induced and SSTR expression modulated in adipocytes exposed to inflammatory stimuli, such as IL-1β. However, information regarding SST and SSTR expression during adipogenesis is lacking. Furthermore, SOM230, a synthetic SST analog with high affinity across a wider range of SSTR subtypes (SSTR1, -2, -3, and -5), has recently been developed (12), making a systematic evaluation of SST expression in GO orbits timely.

MATERIALS AND METHODS

Patients studied. For the quantitative PCR (qPCR) studies, adipose/ connective tissue samples were obtained from a total of 23 patients with GO. The surgical procedures were orbital decompressions (n = 17) and blepharoplasty (n = 6). These comprised 21 women and two men with a mean age of 47.2 yr (range 18–77 yr). They were compared with a group of eight individuals free of thyroid or other inflammatory eye disease who underwent orbital decompression (n = 4) or blepharoplasty (n = 4). The controls were six women and two men with a mean age of 53.1 yr (range 28–86 yr). The majority of patients were Caucasian, except for two Asians in each group.

There were 12 nonsmokers and 11 smokers in the Graves’ group and four nonsmokers and four smokers in the control group.

All GO patients except for two had inactive GO (CAS <2), as documented by the examining ophthalmologists (24). All GO patients

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had received systemic steroid treatment at some stage during their disease.

All samples were obtained with informed consent. The study was performed with the approval of the South East Wales local research ethics committee and was conducted in accordance with the principles of The Declaration of Helsinki.

**RNA extraction, DNase treatment, and reverse transcription.** Orbital adipose/connective tissues were snap-frozen and stored at −80°C until use. They were pulverized under liquid nitrogen, and the RNA was extracted using Trizol according to the manufacturer’s instructions. Following quantification, either 1 μg of total RNA or 8 μl (when RNA concentrations were measurable but <125 ng/μl) was treated with DNase (1 U; Promega) in a total volume of 10 μl. Subsequently, depending on the concentration of the input RNA, 1 (>200 ng/μl) or 2 μl (<200 ng/μl) was reverse transcribed in a total volume of 20 μl, with Moloney murine leukemia virus reverse transcriptase (0.1 U; Promega) using standard protocols (19).

Depending on the amount of RNA extracted, three to six reverse transcription reactions were performed for each sample; care was taken so that individual qPCR experiments (please see below) always used cDNA generated in the same batch.

**Preparation of plasmid standards: optimization of standard curves and qPCR.** The five SSTR are intronless genes. Consequently, we were able to amplify fragments of each subtype from genomic DNA in a PCR reaction using standard protocols (19).

All primers (designed with Primer 3 software) used are shown in Table 1.

The PCR fragments were subcloned into pGEM-T according to the manufacturer’s instructions, and the plasmid identity was confirmed by sequencing. qPCR was performed with SYBR Green incorporation measured on a Stratagene MX3000. Each reaction comprised 1 μl of plasmid standard containing 10⁶ to 10² copies in a total volume of 25 μl. Initially, the optimal primer combination, to generate a homogeneous amplification peak in the absence of primer dimers, was determined for each subtype.

qPCR measurement of SSTR transcripts in the samples used 1 μl of cDNA in the reaction. Comparison with standard curves for each subtype (included in each experiment) permitted calculation of absolute values for each sample (transcripts/μg input RNA). In addition, transcripts for a housekeeping gene, adenosine phosphoribosyltransferase (APRT), were measured (as previously described) so that values could be expressed relative to this (transcripts/APRT). In a single qPCR experiment, all measurements were made in triplicate; each subtype in all samples was evaluated on at least two different batches of cDNA. The standard curve was also run in at least duplicate in each reaction.

To assess the contribution of lymphocytes to the SSTR transcript measurements, qPCR for the T cell receptor constant region (α-chain lymphocyte marker (CD3α)) was undertaken on all 31 samples analyzed ex vivo.

**Table 1. Primers for SSTR subtypes CD3α and housekeeper gene APRT**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>SSTR1</td>
<td>TGAGTCAGCTGTCGGTCATC</td>
<td>GCGGTGGCCTGTAATTGCA</td>
</tr>
<tr>
<td>SSTR2</td>
<td>GCCGAATTCTCTTTTTC</td>
<td>TGCGTGTGTGTGTGTGTA</td>
</tr>
<tr>
<td>SSTR3</td>
<td>CCTGGTAACTCTGTTGTCAT</td>
<td>ACAGCCAGATATCGGTGTA</td>
</tr>
<tr>
<td>SSTR4</td>
<td>ACCAGCGTCTGTCGGTCATC</td>
<td>AGACGCGACGGTGAGAG</td>
</tr>
<tr>
<td>SSTR5</td>
<td>TCATGCCTGTTGTCGTCACA</td>
<td>GAGAGGAGATACGCCAGAG</td>
</tr>
<tr>
<td>CD3α</td>
<td>GGCTCTCGCAACAACAGATTA</td>
<td>GATTTAACCCGCCACCTTTC</td>
</tr>
<tr>
<td>APRT</td>
<td>GCTGTGTTGTCACCGAAGAAG</td>
<td>CTGTAACCCGAGGAGGAGGTT</td>
</tr>
</tbody>
</table>

SSTR, somatostatin; CD3α, α-chain lymphocyte marker; APRT, adenosine phosphoribosyltransferase.

**Preadipocyte culture, adipogenesis protocol, and evaluation of proliferation.** Orbital preadipocytes (<5 passages) were obtained from adipose tissue explants from an additional six patients with GO: one male and five females with a mean age of 52.1 yr (range 40 – 61 yr) and two women (aged 52 and 70) free of inflammatory eye disease. They were cultured in 6-cm dishes in DMEM-F-12 and 10% FCS (complete medium). Adipogenesis was induced in confluent cells by replacing with medium that had reduced FCS and contained a range of hormones and a peroxisome proliferator-activated receptor-γ (PPARγ) agonist (differentiation medium) for 10 – 12 days, as previously described (22). To determine whether adipogenesis had an effect on SSTR expression, RNA was extracted at various time points during the differentiation protocol for measurement of SSTR transcripts as described above. Total protein lysates were extracted at the same times in Laemmli buffer containing 1 mM sodium orthovanadate and 1 mM PMSF. Samples were separated by SDS-PAGE and the gels then electroblotted onto polyvinylidene difluoride membranes. The blots were probed with goat polyclonal antibodies (Santa Cruz Biotechnology) anti-SSTR1 (1:500 overnight, 4°C) and anti-actin (1:1,000, overnight, 4°C), followed by an anti-goat IgG-HRP conjugate (1:5,000, 1 h at room temperature) (Amersham Biosciences), and visualized by enhanced chemiluminescence (ECL Plus; Amersham Biosciences). Films were analyzed using the Alpha Imager 1200 digital imaging system (Alpha Innotech Corp, San Leandro, CA). The blots were initially probed with the SSTR1 antibody; they were then stripped and reprobed with antibodies that recognize actin.

Preadipocytes were cultured in 12-well plates to investigate the effects of varying concentrations of OCT and SOM230 on proliferation and adipogenesis (spontaneous and induced). Proliferation was assessed by direct cell counting of preadipocytes, 2 and 5 days after plating in 12-well plates, in complete medium, using a Coulter particle counter. Results are expressed as a percentage of the mean of time points of fold increase [(count day 5 – count day 2)/count day 2] of the cells in the absence of any analog. At each time point an aliquot of cells was stained with an equal volume of 0.1% trypan blue and the percentage of blue and white cells counted using a hemocytometer.

Microscopic examination provided a means of determining whether morphological changes that accompany adipogenesis, e.g., rounding up of cells and/or acquisition of lipid-filled droplets, had occurred. The degree of adipogenesis was also evaluated by measuring the adipocyte differentiation markers CCAAT/enhancer-binding protein-β (C/EBPβ), PPARγ, and lipoprotein lipase (LPL) as previously described (25). Briefly, the various cell populations were plated in six-well plates in complete or differentiation medium, supplemented or not with analog. Fourteen days later, RNA was extracted and reverse transcribed, and transcript copy numbers were measured using SYBR Green and a Stratagene MX3000 light cycler. Standard curves (the PCR amplicon subcloned into pGEM-T at 10⁶ to 10² copies) were included for each gene, and results are expressed as an absolute value either per microgram of input mRNA or relative to the housekeeping gene APRT.

A possible effect of TSH receptor (TSHR) activation on SSTR expression was examined. Activating mutant TSHR, L629F and M453T, and the wild-type TSHR were introduced into the orbital preadipocytes (2 of the GO and 1 normal) using retroviral vectors previously produced in our laboratory (5). Geneticin selection resulted in mixed populations stably expressing the various TSHR, which exhibit increased basal levels of cAMP compared with the equivalent nonmodified cell population, all as previously described (25). RNA was extracted from the nonmodified and mutant TSHR expressing cells for measurement of SSTR transcripts.

**Statistics.** The Mann-Whitney rank sum test was used for assessing nonparametric data (qPCR measurements) and Student’s t-test for parametric data (differentiation effects, evaluation of adipogenesis). The effects of SST analogs on proliferation were compared using one-way ANOVA and Tukey’s post hoc test (SPSS version 10.0 for Windows; SPSS, Chicago, IL). All results are expressed as means ±
RESULTS

**SSTR1** is overexpressed in all GO orbits and **SSTR2** increased in a proportion of GO orbits. SSTR1 was detected and quantified in all samples: 144.6 transcripts/µg input RNA (29.4–976) in GO orbits (n = 23) and 79.9 transcripts/µg input RNA (11.9–130) in normal controls (n = 8) (median and range, respectively). The difference was statistically significant (P = 0.024; Fig. 1A).

SSTR2 was detected in all samples and was measurable in GO [n = 13, 2.3 transcripts/µg input RNA (0.1–520.8)] and also in normal controls [n = 4, 0.2 transcripts/µg input RNA (0.1–1.9)]. We assigned an arbitrary value of 0.1 transcripts/µg input RNA to samples at the limit of detection (LOD) to facilitate statistical comparisons. The difference was not statistically significant (P = 0.092); however, 39% of the GO samples had levels above the 97th percentile of normals. Results are shown in Fig. 1B.

Ploting the expression of SSTR1 against SSTR2 results showed that the expression of SSTR1 in GO samples and controls does not parallel the expression of SSTR2 (data not shown). There was no statistically significant difference in the expression of SSTR1 and -2 between smokers and nonsmokers.

**SSTR3, -4, and -5 are at the limit of detection.** Transcripts for SSTR3, -4, and -5 were undetectable in the majority of samples and, even when they were present, were at the LOD. SSTR3 was detected in 10 of 23 GO and six of eight normal orbital samples, SSTR4 in two of 23 GO and none of nine normal orbital samples, and SSTR5 in three of 23 GO and none of nine normal orbital samples.

Are **SSTR transcripts derived from infiltrating lymphocytes or resident orbital cells?** Since lymphocytes have been reported to express SSTR (15), and since lymphocytic infiltration is a feature of GO (13), we measured transcripts for the T cell receptor constant region CD3ε to assess the lymphocyte contribution to SSTR expression. The lymphocyte contribution was minimal, since transcripts for CD3ε were at the limit of detection (LOD) in all 31 orbital samples tested ex vivo. This contrasted with Jurkatt lymphocyte cDNA, used as a positive control, in which >10^4 transcripts/µg input RNA were measured (data not shown).

**Does TSHR activation influence SSTR expression?** Having established that the SSTR were expressed by orbital cells, we sought an explanation for the increased levels of SSTR1 and SSTR2 in some GO samples. The most severe GO correlates with Graves’ patients having the highest titers of thyroid-stimulating antibodies (1, 8). We (3) have previously reported that a small proportion of fibroblast-like cells in GO orbits express the TSHR. Thus it seemed reasonable to investigate the effects of TSHR activation. We have used a previously described in vitro model (25) in which TSHR activation is achieved by stable incorporation of gain of function mutant TSHR. Examination of preadipocytes from two GO and one normal patient sample in this protocol revealed no consistent or significant change in the expression of any of the five SSTR subtypes.

**Does adipogenesis provide an explanation for the increased expression of SSTR1 and -2?** We and others (10, 23) have reported an increase in adipogenesis in GO orbits. Thus we investigated whether this process affects SSTR expression. As shown in Fig. 2A, transcripts for SSTR1 and SSTR2 are increased in the process. The experiment was repeated, with or without DNAse treatment, with five different populations of GO preadipocytes and one normal control, all with similar results. We were also able to demonstrate that the transcript levels for SSTR1 reflect those of the protein in a parallel Western blot performed on samples taken at the same time points, as shown in Fig. 2B. Transcripts for SSTR3, -4, and -5 were all at or below the limit of detection and were unchanged by adipogenesis.
SOM230 produces significantly greater inhibition of orbital preadipocyte proliferation than OCT. We compared the effects of SOM230 and OCT on orbital preadipocyte proliferation in vitro. As shown in Fig. 3, neither SOM230 nor OCT at $10^{-8}$ M had any effect on proliferation. The effect of analog at $10^{-6}$ was significant ($P \leq 0.001$) for proliferation measured by Coulter counting on days 2 and 5 compared with control; SOM vs. control, $P \leq 0.001$, OCT vs. control, $P = 0.019$, and the two analogs differed significantly from one another; SOM vs. OCT, $P = 0.017$. Proliferation inhibition by analog at $10^{-7}$ concentration was significant ($P \leq 0.001$). The individual effects were SOM vs. control, $P \leq 0.001$, OCT vs. control, $P = 0.001$, and the two analogs differed significantly from one another; SOM vs. OCT, $P \leq 0.001$.

In neither case was the reduced proliferation due to increased cell death, since the percentage of trypan blue-stained cells was <3% in all culture conditions.

Do SST analogs have any effect on adipogenesis? In complete medium, even when the cells had been confluent for ≤14 days, there was no obvious difference in the morphology of cells (e.g., rounding up and accumulation of lipid-filled droplets, which would be consistent with adipogenesis) in the presence and absence of $10^{-6}$ or $10^{-7}$ SOM230 or OCT, indicating that neither analog induced spontaneous adipogenesis.

PPARγ agonist induced differentiation of the cells produced the expected change in morphology. Similar signs of adipogenesis were present in cells incubated in differentiation medium supplemented with SOM230 or OCT, although the process appeared to be delayed, with foci being smaller and containing fewer lipid droplets.

To investigate further, we measured transcripts of gene markers of early (C/EBPβ), mid (PPARγ), and late (LPL) adipogenesis. Since the RNA was collected on day 14, results for the late marker are presented (C/EBPβ and PPARγ transcript numbers were unaffected by analog). In complete medium, transcripts for LPL were undetectable and increased to $2.0 \times 10^5$ (1.18) copies/µg input RNA after 14 days in differentiation medium. In the presence of $10^{-6}$ SOM230 or OCT, the increase was slightly, but not significantly, reduced.

Fig. 2. Expression of SSTR1 SSTR2 during adipogenesis. A: transcript copy numbers; data are presented as means ± SE. Preadipocytes were plated in 6-cm dishes, and differentiation medium was added at confluence. RNA was extracted at the indicated time points. The experiment itself was repeated 5 times with similar results (preadipocytes from 4 GO and 1 normal control). B: Western blot analysis performed at the same time points; top: SSTR1 (M, ~60 kDa), bottom: actin (M, ~40 kDa). D0, day 0; D4, day 4; D8, day 8; D12, day 12.

Fig. 3. Effects of SOM230 and octreotide (OCT) on orbital preadipocyte proliferation. Cells were counted on days 2 and 5 after $5 \times 10^4$ cells/well were plated. Results are expressed as %mean of time fold increase [(count day 5 – count day 2)/count day 2] of the cells in the absence of any analog. The mean numbers are the results of triplicates (which agree within 2%) for 1 representative of 4 independent experiments.
reaching levels of $1.6 \times 10^5$ (0.99) and $1.6 \times 10^5$ (1.06) copies/µg input RNA, respectively. The analogs at a concentration of $10^{-7}$ M did not reduce LPL transcript copy numbers induced by adipogenesis. Results are expressed as the mean (SD) of five independent experiments performed on preadipocytes from one normal individual and four GO patients.

**DISCUSSION**

To our knowledge, this is the first report of SSTR expression in GO orbits analyzed ex vivo and compared with tissue from normal controls. We found an upregulation in SSTR1 in the GO group as a whole and in SSTR2 in a proportion of the patients. Our observations of SSTR1 overexpression in GO orbits are consistent with, and extend those of, Pasquali et al. (14), who found expression of SSTR1 in five of 10 GO primary orbital fibroblast cultures compared with none of 10 controls. Our findings of measurable SSTR2 expression in all samples examined are also similar. Pasquali et al. were also able to demonstrate expression of SSTR3 and -5 in a significant number of samples (50 and 80% of GO samples, respectively). This contrasts with our observations where transcripts for the SSTR3, -4, and -5 were at or below the LOD. However, since all of the receptors have a single exon, we were obliged to treat our samples with DNase to avoid quantifying copies amplified from genomic DNA. Thus we may have missed differences in the expression levels of SSTR3, -4, and -5. Since we did not detect transcripts for CD3α, which we have used as a marker of lymphocytic infiltration in the ex vivo samples, we assume that the SSTR are expressed on the orbital preadipocyte/fibroblast population. The absence of lymphocytes may be expected in orbital tissue taken from individuals with inactive disease (as in 21 of 23 in the present study) and is probably the result of previous steroid therapy for immunosuppression.

Our samples were derived from within the orbit and also from the eyelid, but no differences were found in the expression levels. Similarly, when analyzing the data, taking into account the smoking status of the donor, no differences were apparent. Thus smoking does not seem to modify the expression of SSTR.

Application of in vitro models to investigate the effects of TSHR activation and adipogenesis on SSTR expression levels produced opposing results. We did not find a consistent or significant change in the level of SSTR1 and -2 expression in nonmodified orbital preadipocytes when comparing with the same population expressing an activating mutant TSHR; thus we can eliminate a role for thyroid-stimulating antibodies (which also result in TSHR activation) in this aspect of orbital biology. In contrast, we observed upregulation of SSTR1 and -2 during differentiation to mature adipocytes, suggesting that adipogenesis provides one explanation for the increased expression measured in the GO samples ex vivo. Furthermore, we were able to demonstrate that the transcript and protein levels of SSTR1 correlate, and our subsequent proliferation studies using somatostatin analogs validate the functional relevance of these receptors.

We (23) have previously reported increased adipogenesis in GO orbits (compared with normal) in samples obtained from patients with inactive disease (as in the current study), and this has been confirmed by others (10). Our findings suggest that, even in apparently inactive disease, as defined by CAS, pathological mechanisms may still be in operation. The expression of SSTR1 did not correlate with that of SSTR2 in either GO patients or controls analyzed ex vivo. Since both receptor subtypes are upregulated during adipogenesis, this indicates that additional mechanisms must be contributing to the increased expression, including the possibility that a fundamental difference exists in SSTR expression between GO preadipocytes and controls.

We then determined whether established and novel SST analogs exerted an effect on the biological activity of the orbital preadipocyte/fibroblasts. As expected, the effects of OCT and SOM230 were very similar, with both inhibiting proliferation, although SOM230 had a significantly greater impact on proliferation than OCT.

Our attempts to define whether the reduced proliferation was caused by increased apoptosis or a block/proliferation of the cell cycle were not successful due to the combination of slow growth, large size of cells, and limited availability. OCT has previously been shown to induce apoptosis in GO fibroblasts, accompanied by reduced Bcl-2 expression (15). Preliminary reports suggest that these findings are also replicable in GO fibroblasts treated with SOM230 (16).

In conclusion, we have examined expression of SSTR subtypes in GO orbital tissues ex vivo and find evidence of upregulation of SSTR1 for which the novel SST analog SOM230 has high affinity. Initial experiments indicate that SOM230 may be beneficial for GO by reducing preadipocyte proliferation. These observations could thus form the basis for a reevaluation of the role of newer somatostatin analogs in the management of patients with GO.

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