Gene expression of estrogen receptor-alpha in orbital fibroblasts in Graves' ophthalmopathy

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SUMMARY

Graves' ophthalmopathy (GO) is one of the most severe clinical manifestations of Graves' disease (GD), and its treatment might involve high-dose glucocorticoid therapy. The higher incidence of GO among females, and the reported association between polymorphisms of estrogen receptor (ER) and GD susceptibility have led us to question the role of estrogen and its receptor in GO pathogenesis. We, thus, assessed estrogen receptor-alpha (*ERA*) gene expression in cultures of orbital fibroblasts from a patient with GO before (controls) and after treatment with 10 nM and 100 nM dexamethasone (DEX). Orbital fibroblasts showed *ERA* gene expression. In the cells treated with 10 nM and 100 nM DEX, *ERA* gene expression was, respectively, 85% higher and 74% lower, than in the control group. We concluded that *ERA* gene expression is found in the orbital fibroblasts of patient with GO, which may be affected by glucocorticoids in a dose-related manner. Arch Endocrinol Metab. 2015;59(3):273-6

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INTRODUCTION

Graves' ophthalmopathy (GO) is one dramatic manifestation of Graves' disease (GD) and about 20-30% of Graves' patients have clinically apparent ophthalmopathy (1).

GO evolves from an autoimmune process directed against the thyrotropin receptor (TSHR) expressed on orbital fibroblasts, that is recognized by TSHR autoantibodies (TRAb). This results in inflammation, hyaluronan deposition and adipogenesis (2). The inflammatory nature of GO has directed treatments against inflammation, most frequently using corticoid therapy (3).

Although the overall mechanisms of GO pathogenesis are established, many endocrine and molecular aspects of the disease remain to be explored. Some nuclear receptors, such as peroxisome proliferator-activated receptor gamma (PPARG) that has a regulatory role in adipocytic differentiation, have been more investigated (4). However, the higher prevalence of GO among females suggests that estrogen and its receptors are also involved in GO pathogenesis.

The two main subtypes of estrogen receptors (ER), alpha (ERA) and beta (ERB), are expressed in several tissues (5), as well as in inflammatory cells and fibroblasts (6). Experimental studies have suggested that estradiol interferes with PPARG action and, consequently, with adipogenesis, by inhibiting adipocytic differentiation (7). Moreover, the relation between ER and inflammation has been described (5,8), with evidence that the regulation of pro-inflammatory cytokines expression by corticosteroids might be mediated by ERA and not by glucocorticoid receptor (GR) (9). These reports reinforce the hypothesis that ER, and particularly ERA, influences GO pathogenesis. However, although *ERB* polymorphism rs4986938 has been recently associated with GD susceptibility (5), the role of ER in GO is still uninvestigated. And, while broadly expressed in a wide variety of cells (10), it has not been found any study analyzing ER expression in orbital fibroblasts, neither whether the treatment with glucocorticoids alters such expression.

In this study, we assessed *ERA* gene expression in cultures of orbital fibroblasts from patient with GO

treated with different doses of dexamethasone. We observed that such expression increases as glucocorticoid doses decrease, suggesting a close dose-dependent relation between this receptor and the main treatment for GO.

MATERIALS AND METHODS

Cell culture and treatment

This study was approved by the Ethics Committee of Botucatu Medical School (process number 4037-2011). Orbital fibroblasts were obtained from a female patient with inactive (Clinical Active Score – CAS = 2), moderately severe-to-severe GO, during orbital decompression surgery performed in the Botucatu Medical School – Hospital of São Paulo State University. Fibroblast specimens were collected in Falcon tubes filled with 199 medium (LCG®) and antibiotics. Cultures were seeded in 25-cm³ bottles containing 199 medium supplemented with 20% fetal bovine serum (FBS) (Gibco®) and 1% antibiotic/antimycotic (ATB) (Sigma®) in 5% CO2 at 37°C (Figure 1). After reaching approximately 80% confluence, cells were transferred to a 75-cm³ bottle containing 10% FBS plus 1% ATB, where they were kept until reaching 80% confluence again. Subsequently, the cells were transferred to 6-well plates for treatment. After reaching 80% confluence in the wells, fibroblasts were treated in biological triplicates with either 10 nM or 100 nM of dexamethasone (DEX), and then assessed for *ERA* gene expression. Untreated fibroblasts were used as controls (control group – C group).

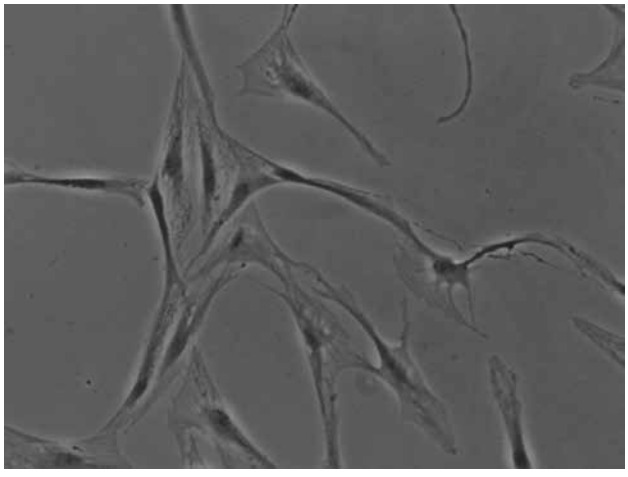

Figure 1. Culture of orbital fibroblasts from a patient with Graves' ophthalmopathy (light microscopy, X20).

RNA isolation and RT-PCR

After exposure to treatment, the culture medium was aspirated, and the cells were removed from the plates using 400 µl de Trizol® (Life Tech, USA). RNA was extracted also using Trizol®. RNA integrity was checked by visualization on 1% agarose gel for 30 min at 80 mV. RNA purity was determined by spectrophotometry; samples with 260/280 ratio < 1.6 were discarded for showing protein contamination.

Complementary deoxyribonucleic acid (cDNA) was synthesized from RNA using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). In Brief, RNA was added to 2 µl 10x buffer, 0.8 µl dNTP mix (100 mM), 2 µl randon primer, 1 µl RNase inhibitor, 1 µl Reverse Transcriptase, and 12.2 µl nuclease-free water. The samples were then incubated at 25°C for 10 min, at 37°C for 120 min, at 85°C for 5 seconds, and kept at 4°C.

cDNA analysis was performed by *TaqMan* arrays (Life Technologies, USA), using primers specific for the RNA targets (ESR1: estrogen receptor-alpha and cyclophilin: internal control). Each reaction contained 10 µL TaqMan® Universal PCR Master Mix (Life Technologies, USA), 3 µL reverse transcription reaction product, final volume adjusted to 20 µL with nuclease-free water. RT-qPCR experiments were performed in accordance with the *Minimum information for Publication of Quantitative Real-Time PCR Experiment* (MIQE) (11). Data were normalized against the corresponding cyclophilin internal control, and expression was quantified by the method $2^{-\Delta\Delta CT}$ (12). The C group was adjusted to 1 when groups were compared.

Statistical analysis

All data were expressed as means ± standard deviation. Two-way analysis of variance (ANOVA) was used to compare the effect of treatment within groups, followed by the test of Tukey for multiple comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

Orbital fibroblasts showed *ERA* gene expression. In the groups treated with 10 nM and 100 nM DEX, *ERA* gene expression was, respectively, 85% higher and 74% lower, than in the control group, with receptor gene expression being significantly lower in the group treated with 100 nM than in the group treated with 10 nM DEX (Figure 2).

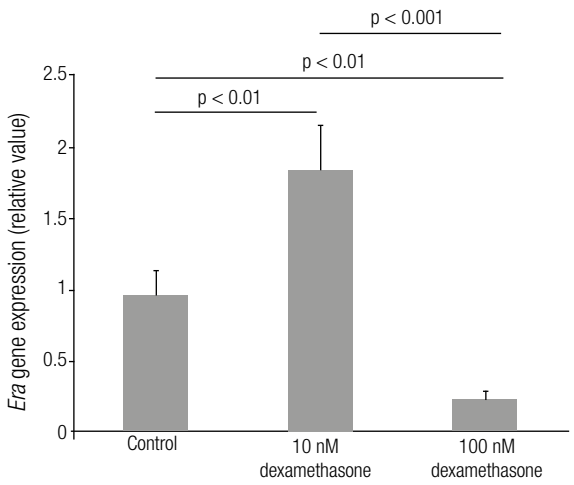

Figure 2. Effect of 10 nM and 100 nM dexamethasone on estrogen receptor alpha (*ER*-α) mRNA in orbital fibroblasts from a patient with Graves' ophthalmopathy. The experiment was performed in triplicate. Data were reported as mean and standard deviation. ANOVA was used in conjunction with Tukey's test ($p < 0.01$ or $p < 0.001$).

DISCUSSION

We observed *ERA* gene expression in cultures of orbital fibroblasts from patient with GO.

GO results from the autoimmune involvement of the orbit, and its progress involves, mainly, the processes of inflammation, hyaluronan deposition and increased adipogenesis (2). The orbital fibroblasts represent the target of the autoimmune process (2), through the expression of TSHR, which is recognized by TRAb (13). Some distinct orbital fibroblasts subtypes, when activated by TRAb, differentiate into adipocytes, while others, which express thymocyte antigen-1, are stimulated by cytokines to produce hyaluronan (13). The stimulus of the insulin growth factor (IGF) receptor present in such cells results in secretion of cytokines that increase the recruitment of activated T cells and other mononuclear immune cells into the orbit (13). The interaction of these cells with orbital fibroblasts results in synthesis of prostaglandins and hydrophilic hyaluronan that accumulates in the orbital tissue, expanding its volume (13). Moreover, prostaglandins bind to the nuclear receptor PPARG present in preadipocytes, thus stimulating its differentiation into mature adipocytes, further increasing orbital volume (13).

Sex hormones and its receptors are important for shaping the immune system differently in females versus males, with differing susceptibility to, and possibly differing pathogenetic mechanisms of, inflammatory autoimmune diseases (14). Although still controversial, the immunosuppressive role of estrogen in autoimmune diseases has been regarded as important (14). Thus, among the nuclear receptors possibly involved in GO, there are ERs, which have been associated with inflammatory processes, whether they are autoimmune or not (5,8). Although the mechanisms involved have not been elucidated yet, several factors seem to participate, such as, for instance, the IGF-1, which would exert an anti-inflammatory action mainly through ER, particularly ERA (15).

ERA expression is found in several tissues, such as breast and skin fibroblasts (16), for instance. However, the expression of *ERA* in orbital fibroblasts from a patient with GO, as seen in this study, has not been previously reported. Assuming estrogen involvement in GO development, it seems more likely that its immune modulatory effect is mediated through these cells, rather than directly through inflammatory cells, which express low levels of ER (14).

Furthermore, *ERA* and/or serum estrogen concentrations might be able to influence adipocyte differentiation. Bonofiglio and cols. report that, in breast cancer cell lines, ERA physically associates and functionally interferes with PPARG signaling (17). This suggests that ER may interfere with the adipocyte pathway, which is critical in GO development. In addition to adipocyte differentiation, peroxisome proliferator-activated receptors (PPARs) have also been associated with inflammatory processes (18). Thus, ERA would not only be involved in the onset of the inflammatory process, but also in the development of GO.

In this study, *ERA* gene expression was increased in orbital fibroblast cultures treated with 10 nM, and decreased in those treated with 100 nM DEX. This finding suggests a close dose-dependent relationship between ERA and glucorticoid, which had been described in other tissues and experimental models. Ulisse and Tata reported that DEX upregulates ER's mRNA (19), while Cuzzocrea and cols. demonstrated that a selective ERA antagonist reverses the anti-inflammatory activity exhibited by DEX, suggesting that the anti-inflammatory effect of DEX requires the participation of ER (20). Additionally, study performed with mice endometrial cells observed that the suppression of pro-inflammatory cytokines expression by glucocorticoids was mediated through ERA and not through GR, as it would be expected (9). In our study, *ERA* gene expression was modulated according to glucocorticoid

concentration. However, whether other glucocorticoid effects could be mediated by the ER, or whether estrogen status could influence the response to glucocorticoids, remains unclear.

The major limitation of this study is the fact that all orbital fibroblasts derived from a single patient with GO. Nonetheless, the findings reported herein, obtained using real-time PCR, confirmed the results observed in a prior pilot study using conventional PCR (unpublished data).

In conclusion, our results indicate that *ERA* gene expression is found in the orbital fibroblasts of patient with GO, and suggest that this gene expression may be affected by glucocorticoids in a dose-related manner. The clinical significance of these findings remains to be determined and further studies are necessary.

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Authors' contributions: Sarah Santiloni Cury participated in all stages of the study. Miriane Oliveira, Maria Teresa Síbio, Sueli Clara, Renata De Azevedo Melo Luvizotto and Sandro Conde standardized and implemented the techniques used in fibroblast culture and molecular analyses. Edson Nacib Jorge supplied the material and critically assessed the results. Célia Regina Nogueira and Gláucia Maria Ferreira da Silva Mazeto conceived the study, supervised the experiment, analyzed data, and discussed the results.

Disclosure: no potential conflict of interest relevant to this article was reported.

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