Fenofibrate increases the expression of high mobility group AT-hook 2 (HMGA2) gene and induces adipocyte differentiation of orbital fibroblasts from Graves’ ophthalmopathy

Daniela Pasquali1, Giovanna Maria Pierantoni2, Alfredo Fusco2, Stefania Staibano3, Vittorio Colantuoni4,5, Annamaria De Bellis1 Antonio Bellastella1 and Antonio Agostino Sinisi1

1Section of Endocrinology, Medico-Surgical Department of Clinical and Experimental Internal Medicine ‘F. Magrassi e A. Lanzara’, Research Center for Cardiovascular Disease, Second University of Naples, Departments of 2Cellular and Molecular Biology and Pathology ‘L. Califano’, 3Biomorphological and Functional Science and 4Biochemistry and Medical Biotechnologies, University of Naples ‘Federico II’, Naples, and 5Department of Biological and Environmental Sciences, University of Sannio, Benevento, Italy

Abstract

Expansion of adipose tissue in the orbits is a key feature of Graves’ ophthalmopathy. Recent evidence shows that orbital fibroblasts are committed to differentiate into adipocytes under appropriate stimuli. Rosiglitazone, an agonist of the nuclear hormone receptor, peroxisome proliferator-activated receptor γ (PPARγ) is able to induce both differentiation of orbital fibroblasts into mature adipocytes and expression of the TSH receptor (TSHr) gene. Several studies have suggested an important role of the high mobility group AT-hook 2 (HMGA2) gene in adipocytic cell growth and development. To investigate further the association between adipogenesis-related genes and orbital fibroblasts, we treated fibroblasts from Graves’ ophthalmopathy (FGOs) and from normal orbital tissues with fenofibrate, a specific agonist for PPARα. We then evaluated the expression of the PPARα, PPARγ2, HMGA2, leptin and TSHr genes before and after 24 h of fenofibrate treatment, using semiquantitative and real-time PCR. For up to 96 h after exposure to fenofibrate, FGOs differentiated into adipocytes. PPARα and PPARγ2 were expressed more in FGOs than in normal cultures, whereas TSHr mRNA was detected only in FGOs. Expression of HMGA2 mRNA and protein was significantly increased in FGOs from 6 to 24 h after fenofibrate, confirming its role in the early phase of adipocyte differentiation. Treatment with fenofibrate for 24 h significantly increased the expression of leptin and TSHr genes. Moreover, TSH treatment significantly increased the accumulation of cAMP, demonstrating that FGOs express functional TSHr. The high level of expression of PPARα other than PPARγ2 transcripts and the stimulating effect of fenofibrate on adipogenesis and on HMGA2, leptin and TSHr genes also indicate that the PPARα pathway plays an important part in the adipocyte differentiation of FGOs. These findings suggest that novel drugs to antagonize PPARα, other than the PPARγ signalling system, may also need to be considered in the treatment or prevention of Graves’ ophthalmopathy.

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Introduction

In Graves’ ophthalmopathy, mechanisms underlying orbital tissue proliferation remain unclear. On histology, the orbital tissue exhibits accumulation of glycosaminoglycan and overabundance of adipose tissue in the orbital muscles and connective tissues.
functional thyroid-stimulating hormone receptor (TSHr) (Feliciello et al. 1993, Bahn et al. 1998). This observation has led several groups to search for the link between adipocyte differentiation, the appearance of adipogenic markers and expression of the TSHr gene.

The process of adipogenesis involves the interplay of several transcription factors. The discovery of the peroxisome proliferator-activated receptors (PPARα, β and γ), members of the nuclear receptor superfamily, and the identification of fatty acids and their derivatives as ligands of PPARs have revealed a new regulatory model of lipid action as a direct modulator of gene expression (Desvergne & Wahli 1999). PPARγ is the major adipogenic transcription factor involved in terminal adipocyte differentiation (Tontonoz et al. 1994, Kubota et al. 1999, Rosen et al. 1999). PPARs act by binding to specific promoter response elements in association with the obligate heterodimerization partner, retinoid X receptor (RXR), which is activated selectively by 9-cis retinoic acid. Ligand-activated PPARs also regulate differentiation and clonal growth of several types of cancer cells (Kopelovich et al. 2002). Moreover, recent findings indicate a modulatory role for PPARs in controlling inflammation, which has potential therapeutic applications in chronic inflammatory diseases (Delerive et al. 2001).

Unsaturated fatty acids are specific natural ligands for PPARs. The thiazolidinedione hypoglycaemic agents such as rosiglitazone and pioglitazone are synthetic compounds that selectively bind PPARγ and can induce adipocyte transformation of orbital fibroblasts from patients with Graves’ ophthalmopathy and stimulate the expression of TSHr in vitro and in vivo (Valyasevi et al. 2002, Starkey et al. 2003). The possible role of PPARα agonists, such as the fibrates, in adipocyte differentiation of fibroblasts from Graves’ ophthalmopathy (FGOs) remains unexplored, but it has been shown that fibrates are able to induce adipogenesis in other fibroblast lines (Brandes et al. 1986). In addition, the pathways involved in adipocytic differentiation of orbital fibroblasts are putative.

Several studies have suggested an important role of the high mobility group AT-hook 2 (HMGA2) gene in adipocyte growth and development (Zhou et al. 1995, Battista et al. 1999, Anand & Chada 2000, Arlotta et al. 2000). The HMGA family is comprised of four proteins (HMGA1a, HMGA1b, HMGA1c, HMGA2) which are involved in the regulation of chromatin structure and function (Johnson et al. 1989, Manfioletti et al. 1991, Falvo et al. 1995). Overexpression of HMGA proteins is a necessary event in in vivo cell transformation (Chiappetta et al. 1995, Fedele et al. 1996, Bandiera et al. 1998). Moreover, HMGA2−/− mice express a pygmy phenotype, together with a drastic reduction (87%) in adipose tissue. Conversely, mice carrying a truncated HMGA2 gene showed an obese phenotype. Moreover, it has been found that HMGA2 gene was not expressed in wild-type adipose tissue, whereas it was expressed in fat deposits of both wild-type and genetically obese mice (Lep ob/Lep ob and Leprdb/Leprdb) after 1 week of a high fat diet, and that disruption of the HMGA2 gene prevents both diet- and gene-induced obesity (Zhou et al. 1995, Battista et al. 1999, Anand & Chada 2000, Arlotta et al. 2000).

In order to clarify the interplay between adipocyte differentiation of orbital fibroblasts and genes related to adipogenesis, we studied the expression of PPARα, PPARγ2, HMGA2, leptin and TSHr before and after exposure to fenofibrate, an agonist of PPARα, using fibroblasts derived from orbital tissues from patients with Graves’ ophthalmopathy and from individuals without thyroid-associated ophthalmopathy.

Material and methods

Origin of tissues and cell cultures

Orbital connective tissues were obtained from 12 patients with Graves’ ophthalmopathy during orbital decompression surgery. The diagnosis was based on endocrine and ophthalmological criteria, including laboratory determination of hormones and antibodies and imaging studies (ultrasonography, computed tomography or magnetic resonance scan) of orbits. The patients (nine women, three men; aged 31–68 years), currently euthyroid, had never been treated with steroids or had been off treatment for more than 6 months. Orbital tissue controls were obtained from seven individuals (five women, two men; aged 33–67 years) undergoing eye surgery for trauma, osteoma or strabismus. None of the control individuals had a history of autoimmune or thyroid diseases or was affected by orbital inflammatory disorders.
Tissue explants were minced and immediately placed on plastic culture dishes for incubation with the appropriate medium in a humidified incubator (37 °C, 5% CO₂). Fibroblasts were allowed to proliferate in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 1-glutamine, 10% (vol/vol) fetal bovine serum (FBS) and antibiotics (Gibco Brl, LifeTechnologies), as described previously (Pasquali et al. 2000). 

**Isolation of mRNA and semiquantitative RT-PCR**

Total RNA was isolated from the cell cultures at the first passage. Total RNA was recovered with TRIzol (Invitrogen). Residual DNA was removed by treatment with RNase-free DNase I (Promega). RNAs were reversely transcribed using 5 µg total RNA after annealing with 0·2 nM oligo(deoxythymidine) for priming of cDNA in the presence of reverse transcriptase (Superscript, 200 U; Invitrogen) at 37 °C for 1·5 h. The reaction was stopped by incubation at 95 °C for 5 min. To obtain a negative control for the amplification reactions, we carried out an RNA transcription without adding reverse transcriptase enzyme. Complementary DNA (400 ng cDNA) obtained by reverse transcription of RNAs was amplified in a total volume of 50 µl Tris HCl 10 mmol, 1·5 mmol MgCl₂ and 50 mmol KCl pH 8·3, 100 ng of 5’-3’ end primers as described previously (Pasquali et al. 1999, 2002).

To evaluate variability in the expression of PPARα, PPARγ2, HMGA2, leptin and TSHr, a semiquantitative RT-PCR was performed in which these genes were amplified with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described previously (Pasquali et al. 1999, 2002). Briefly, before semiquantitative PCR, the number of cycles was chosen from the middle of the exponential phase of the reaction, separately for each gene type. To establish the number of cycles, GAPDH was amplified at 15, 22, 32 and 40 PCR cycles; PPARα, PPARγ2, TSHr and leptin were subjected to 25, 35 and 40 amplification cycles in separate experiments (data not shown). The following PCR conditions were used: 35 cycles of amplification at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min for PPARα, PPARγ2, leptin and TSHr; 35 cycles at 94 °C for 1 min, 65 °C for 30 s, 72 °C for 30 s for HMGA2; 25 cycles for GAPDH. For the semiquantitative RT-PCR, 100 ng GAPDH primers were added to each PCR reaction after the first 10 cycles as internal control, and the 876 bp product of GAPDH was detected in each PCR reaction.

The levels of mRNAs, quantified by densitometry scanning of the amplification products electrophoresed on agarose gels, are expressed as the ratio between the density of each gene product and coamplified GAPDH as reported previously (Pasquali et al. 1999, 2002). The oligonucleotide sequences for PPARα, PPARγ2, HMGA2, leptin, TSHr and GAPDH are shown in Table 1. Using specific primers with PCR, we amplified the B–C region of the extracellular domain and the D–F region of the transmembrane domain of the TSHr gene, corresponding to nucleotides 158–537 and
1720–2306 respectively (Table 1). PCR products were then separated on a 1·2% agarose gel containing ethidium bromide using a 100 bp DNA ladder (Gibco Brl, Life Technologies) as size marker.

**Quantitative RT-PCR**

Real-time quantitative PCR was used to determine the amounts of PPARα, PPARγ2, HMGA2, leptin and TSHr mRNA. This PCR method monitors the progress of the PCR via detection of fluorescent signals released by Taq polymerase from a specific probe that contains both fluorescent dye and quencher. In these experiments, the amount of specific amplicon present was related to β2-microglobulin and subsequently to an internal control. RNA was extracted from primary cultures of orbital F from patients with Graves’ ophthalmopathy as described above. RNA was reverse transcribed to first cDNA as described previously.

Real-time PCR was repeated three times for each sample using the following primers: PPARα, 5’-ATGGCATCCAGAACAAGGAG-3’ (sense) and 5’-GGCGAATATGGCCTCATAAA-3’ (antisense); PPARγ2, 5’-TGACTGTAAGCCATTGAA-3’ (sense) and 5’-CTGCAGTAGCTGCAGTG TT-3’ (antisense); HMGA2, 5’-GGCCAGCTCAT AAATGGAA-3’ (sense) and 5’-TACTGTTCATA TTGGCCACAA-3’ (antisense); leptin, 5’-GGCTT TGGCCCTATCTTTTC-3’ (sense) and 5’-CCAA ACCGTTGACTTCTGT-3’ (antisense); TSHr, 5’-ACCCAGGGGACAAAGATACC-3’ (sense) and 5’-GAATGGATTGGCACAGGAGT-3’ (antisense). Real-time PCR was also repeated three times for a housekeeping gene, β2-microglobulin. The following primers were used: sense, 5’-CCACGGAG AGAATGGAAAAGTC-3’; antisense, 5’-CCATGCT GCTTACATGTCTCG-3’. The iQ SYBR Green Supermix kit (Bio-Rad Laboratories) was used in an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories). Data are expressed as the amount of specific PCR product of each gene in cells treated with fibrate (500 µM) divided by that in

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**Figure 1** Photomicrograph showing Oil Red O staining of human cultured orbital fibroblasts from a patient with Graves' ophthalmopathy and (inset) from a control patient treated for 96 h with 500 µM fenofibrate (original magnification ×100). The arrows indicate the presence of triacylglycerol inclusions in these cells.
untreated cells after normalization based on the housekeeping gene product, β2-microglobulin (which showed no significant difference in any treatment).

**Protein extraction, western blotting and antibodies**

Protein samples from orbital FGOs were extracted with Nonidet-P40 lysis buffer supplemented with 50 mM sodium fluoride, 0.5 mM sodium vanadate, 0.5 mM phenyl methylsulphonyl fluoride, 5 mg/ml aprotinin and 5 mg/ml leupeptin. Protein concentration was estimated by a modified Bradford assay (Bio-Rad Laboratories). The protein extracts were boiled in Laemmli sample buffer, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Hybond C, Amersham Inc.). Membranes were blocked with 5% non-fat milk proteins and incubated with the antibody at the appropriate dilution. The antibodies directed against the HMGA2 protein are described elsewhere (Santulli et al. 2000). Bound antibodies were detected by...
horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham Inc). As a control for equal loading of protein lysates, the blotted proteins were probed with antibodies against γ-tubulin.

**Oil Red O staining**

Orbital fibroblast cultures were plated in one-well culture chamber slides (Nunc plasticware, Life Technologies) in DMEM supplemented with 10% FBS, grown to confluence and, after 24 h of serum starvation, treated with or without 500 µM fenofibrate (Sigma) in DMEM supplemented with 1% FBS up to 96 h. Cells were washed with 1× PBS, fixed in 10% formalin overnight at room temperature, and rinsed in 60% isopropanol before staining with filtered 0.2% Oil Red O in isopropanol for 1 h. Washed cells were exposed to haematoxylin solution for 5 min, rinsed with tap water and visualized using a microscope (Leitz Laborlux, Leica Imaging System, Inc., Cambridge, England).

**Accumulation of cyclic adenosine monophosphate**

Confluent orbital FGOs and fibroblasts from control monolayers in six-well plates (Costar, Milan, Italy) were starved for 24 h in medium without FBS, then shifted to medium supplemented with 1% FBS and phosphodiesterase inhibitor. The cells were treated with forskolin 10 mM or bovine TSH 10 mU/ml and 3-methyl-1-isobutylxanthine (0.5 mM). After 30 min, cultures were interrupted by adding cold 70% ethanol. After overnight incubation in ethanol, the supernatant was collected, centrifuged and dried. The extracts were reconstituted with the appropriate buffer and processed for the cAMP assay using an RIA kit (Amersham Inc).

**Statistical analysis**

The data are expressed as the mean ± s.d. (n=3) from at least three separate experiments performed in triplicate. Data were subjected to analysis of variance, followed by Fisher’s LSD multiple comparison test using NCSS software (J. Hintze, Kaysville, UT, USA). Values of P<0.05 were considered statistically significant.

**Results**

**Fenofibrate treatment induces adipocyte differentiation in FGOs**

To test the hypothesis that fenofibrate could induce adipocyte differentiation in FGOs, we treated FGOs and control fibroblasts with or without 500 µM fenofibrate for up to 96 h. Few subpopulations (1–2%) of FGOs exposed to fenofibrate changed their typical fibroblastic appearance after 24 h of exposure to fenofibrate (data not shown).
Treatment with fenofibrate for up to 96 h resulted in strong adipocyte differentiation in 35% of FGOs, compared with 1–2% of control cell culture by Oil Red O staining (Fig. 1). Oil Red O staining was negative in untreated cells (data not shown). After 3–4 days of treatment, FGOs assumed a spherical shape with some intracytoplasmic vacuoles visible under phase-contrast microscope, indicating accumulation of triglycerides (Fig. 1).

PPAR\(\alpha\), PPAR\(\gamma\)2, leptin and TSHr mRNA are expressed in FGOs, and fenofibrate significantly increases leptin and TSHr mRNA levels

PPAR\(\alpha\) and PPAR\(\gamma\)2 mRNA were expressed at high level in FGOs, and were almost undetectable in control fibroblasts (Figs 2 and 3 respectively). Adipose cells from breast tissues obtained from patients undergoing mastectomy for gynaecomastia served as the positive controls (Figs 2 and 3). Fenofibrate treatment did not significantly modify the level of PPAR\(\alpha\) and PPAR\(\gamma\)2 transcripts (Figs 2, 3 and 4). Leptin (Fig. 5) transcript was detected in FGOs, and increased significantly after 24 h of fenofibrate treatment, while the level of expression in control fibroblasts was barely detectable (data not shown). The B–C region of the extracellular domain and the D–F region of the transmembrane domain of the TSHr gene were absent in control fibroblasts (Fig. 6). Both extracellular domain and transmembrane portions of TSHr were detected in untreated FGOs and were increased after 24 h of fenofibrate treatment (Fig. 6). The real-time PCR results were similar to those of semiquantitative analysis, confirming the above results. In particular, the levels of PPAR\(\alpha\) and PPAR\(\gamma\)2 transcripts were increased only slightly, and not significantly, by fenofibrate, whereas leptin and TSHr transcripts were increased significantly after 24 h of fenofibrate treatment (\(P<0.05\)) (Fig. 4).

HMGA2 mRNA and protein are expressed in FGOs and are modulated by fenofibrate

To investigate whether the expression of HMGA2 was related to adipocyte differentiation of FGOs, we treated FGOs with fenofibrate for different periods of time, to induce adipocytic differentiation. As shown in Fig. 7A, the early adipocyte differentiation of FGOs induced by fenofibrate was accompanied by a remarkable increase in HMGA2 mRNA levels. This increase was already detectable 6 h after the stimulation with fenofibrate; it persisted at 12 and 24 h, and was confirmed by real-time-PCR analysis (Fig. 4), which showed a significant increase in HMGA2 mRNA after 24 h of exposure to fenofibrate. This corresponded to the time at which adipocyte differentiation was first detected by Oil Red O staining. Findings from
western blot analysis of untreated and fenofibrate-treated FGOs paralleled those obtained with RT-PCR (Fig. 7B). We used a thyroid carcinoma cell line, FB-2, as positive control (Fig. 7) (Basolo et al. 2002). HMGA2 mRNA was undetectable in control fibroblasts (data not shown).

FGOs expresses functional TSHr

To demonstrate that the TSHr found by RT-PCR in FGOs was functional, we assayed the accumulation of cAMP under basal conditions and after 30 min of treatment with forskolin or bovine TSH. In FGOs, exposure to TSH or forskolin stimulated the accumulation of cAMP significantly \((P<0.05)\) (Fig. 8).

Discussion

The complex mechanism of the differentiation of preadipocytes into adipocytes is regulated by an interplay of several factors, including the PPAR isoforms \(\alpha\) and \(\gamma\). These function as important coregulators of lipid homeostasis: PPAR\(\alpha\) regulates fatty acid oxidation, primarily in liver and to a lesser extent in adipose tissue, whereas PPAR\(\gamma\) serves as a key regulator of adipocyte differentiation and lipid storage. Of the two PPAR\(\gamma\) isoforms, PPAR\(\gamma_1\) and PPAR\(\gamma_2\) – generated by alternative splicing of the same gene – the PPAR\(\gamma_1\) isoform is expressed in liver and other tissues, whereas PPAR\(\gamma_2\) is expressed exclusively in adipose tissue, where it regulates adipogenesis and lipogenesis. PPAR isotypes are often coexpressed in tissues and an emerging picture is that of a dual and complementary role of PPARs \(\alpha\) and \(\gamma\) in the regulation of lipid metabolism (Tontonoz et al. 1994, Desvergne & Wahli 1999).

Rosiglitazone, a synthetic agonist of PPAR\(\gamma\), is able to induce adipocyte differentiation in orbital FGOs (Valyasevi et al. 2002) and, recently, it has been reported that pioglitazone, a synthetic ligand
for PPARγ, induced reactivation of Graves’ ophthalmopathy in a diabetic patient (Starkey et al. 2003). Data on the PPARα pathway in FGOs are still lacking. Here, we have demonstrated that fenofibrate, a synthetic compound used as a hypolipidaemic agent that binds PPARα, was able to induce a marked appearance of adipocytes in FGOs in vitro. We found that FGOs coexpressed PPARα and PPARγ2 transcripts at a very high level. This was barely detectable in control fibroblasts. The reason why fenofibrate did not increase PPAR transcripts remains to be determined. One possible explanation could be that PPARs are already upregulated or that fenofibrate does not modulate their expression in this in vitro system but, through PPARα, induces transcription of genes, such as HMGA2, that are related to the early phase of adipocyte differentiation. In fact, treatment of FGOs with fenofibrate significantly increased the expression of HMGA2 evaluated at the mRNA and protein level. This increase had already appeared at 6 h after fenofibrate induction and there were further increases at 12 and 24 h.

Several studies have associated the expression of HMGA2 with adipocyte proliferation and differentiation in animal models, and here we have shown for the first time that HMGA2 is involved in the pathophysiology of Graves’ ophthalmology. This is a further demonstration of the ability of orbital fibroblasts to differentiate towards the adipocyte phenotype after stimulation by fenofibrate. Our data, obtained by both semiquantitative RT-PCR and real-time PCR, demonstrated that fenofibrate significantly increased the expression of leptin in

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Figure 7 Analysis of HMGA2 expression in FGO adipocyte differentiation. (A) Total RNA was extracted from FGOs that were untreated or treated with fenofibrate at different stimulation times (0, 6, 12 and 24 h) and analysed by RT-PCR. Aliquots (1 µl) of RNA were reverse transcribed, amplified as described in Materials and methods, transferred to nitrocellulose and hybridized with HMGA2 cDNA. GAPDH was used as internal control for uniform RNA loading. The thyroid carcinoma cell line, FB-2, was used as positive control. (B) Total proteins extracted from non-treated (−) and fenofibrate-treated FGOs were separated (50 µg/lane) on SDS-PAGE and transferred to nitrocellulose membranes. Western blots were first incubated with antibodies specific for HMGA2 protein, and then with horseradish peroxidase-conjugated secondary antibodies. As a control for equal loading, the blotted proteins were stained with Red-Ponceau. In addition, the same western blots were incubated with antibodies to the ubiquitous protein, γ-tubulin. Proteins were extracted from the cells at time 0, 6, 12 and 24 h of treatment as indicated.
FGOs, suggesting that actions mediated by PPARα are important for leptin signalling and maintenance of intracellular fatty acid homeostasis. Thus orbital FGOs may act as an endocrine compartment that could be linked to the systemic hormonal network; in fact, it expresses leptin, the product of the ob gene (Zhang et al. 1994), usually secreted by adipocytes as an indicator of the size of energy stores, and TSHr. One of the main targets of leptin are the cells of the hypothalamic nuclei that regulate food intake and energy expenditure (Woods et al. 1998). In recent years, several studies have suggested that TSHr may have a role in the pathogenesis of Graves' ophthalmopathy (Feliciello et al. 1993, Bahn et al. 1998, Valyasevi et al. 2002). Our results confirm the link between the adipocyte differentiation of orbital fibroblasts and the appearance of TSHr expression in FGOs (Feliciello et al. 1993, Soriski et al. 1996, Bahn et al. 1998, Valyasevi et al. 2002), showing that fenofibrate is also another inducer of adipogenesis via PPARα that is able to modulate expression of the TSHr gene. The TSHr protein appeared to be functional, because TSH treatment significantly stimulated the accumulation of cAMP. Pharmacological modulation of orbital fibroblast growth and adipocyte differentiation may be an important tool with which to control Graves' ophthalmopathy. Previously, we showed that retinoids may modulate the growth and differentiation of orbital FGOs, acting on the cell morphology and apoptosis (Pasquali et al. 2003). Regarding adipocyte differentiation, it is known that retinoic acid treatment induces the opposite effects; it is stimulatory during the early phases and inhibitory at a later stage of terminal differentiation (Shao & Lazar 1997, Barak et al. 1999). In the latter case, the inhibitory effect seems to be a result of inhibition of the expression of PPARs (Bost et al. 2002). This mechanism could account for the inhibition of cell growth and the apoptosis that are induced by retinoic acid in FGOs. In conclusion, fenofibrate induced adipogenesis and increased the expression of HMGIC, leptin and TSHr genes in orbital fibroblasts from patients with Graves' ophthalmopathy, showing that the PPARα pathway also has an important role in the adipocyte differentiation of FGOs. These findings suggest that novel drugs aimed at the antagonism of PPARα, other than the PPARγ signalling system, may also need to be considered in the treatment or prevention of Graves' ophthalmopathy.

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