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Expression of Peroxisome Proliferator-Activated Receptor alpha (PPAR α) in somatotropinomas: Relationship with Aryl hydrocarbon receptor Interacting Protein (AIP) and *in vitro* effects of fenofibrate in GH₃ cells



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ABSTRACT

Purpose: To search for a possible role of Peroxisome Proliferator-Activated Receptor α (PPAR α), a molecular partner of the Aryl hydrocarbon receptor Interacting Protein (AIP), in somatotropinomas.

Methods: Tumours from 51 acromegalic patients were characterized for PPAR α and AIP expression by immunohistochemistry (IHC) and/or Real Time RT-PCR. Data were analysed according to tumour characteristics and pre-operative treatment with somatostatin analogues (SSA). The effects of fenofibrate were studied in GH₃ cells *in vitro*.

Results: PPAR α was expressed in most somatotropinomas. A modest relationship was found between PPAR α and AIP expression, both being significantly higher in the presence of pre-operative SSA. However, only AIP expression was influenced by the response to treatment. Dual effects of fenofibrate were observed in GH₃ cells, consisting of cell growth inhibition and an increase in GH secretion inhibited by octreotide.

Conclusions: PPAR α is a new player in somatotropinomas. Potential interactions between PPAR α agonists and SSA may deserve further investigation.

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1. Introduction

Somatotropinomas represent a clinical challenge due to acromegaly or gigantism caused by GH/IGF1 hypersecretion and/or to pituitary mass effects (Melmed, 2006). About two thirds are macroadenomas and invasiveness towards the surrounding structures may reduce the chance for surgical cure (Melmed, 2006;

Katznelson et al., 2014). In such patients, somatostatin analogues (SSA) are used to reduce GH/IGF1 hypersecretion and to a lesser extent tumour volume (Katznelson et al., 2014). Because resistance to SSA may occur, peripheral inhibition of GH effects by pegvisomant and/or radiotherapy may be required (Katznelson et al., 2014). Improvement in the management of acromegaly relies on an earlier diagnosis and increasing knowledge of pathways involved in the control of somatotroph cell proliferation and hormone secretion.

In 2006, the Aryl hydrocarbon receptor Interacting Protein (AIP) gene was identified as a predisposing gene for GH and/or PRL-secreting pituitary adenomas (PA) (Vierima et al., 2006). Then,

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80% of PA associated with germline *AIP* mutations (*AIP*^{mut}) turned out to be GH-secreting (Beckers et al., 2013). *AIP*^{mut} somatotropinomas are more aggressive than unselected cases and typically present in a familial setting (Familial Isolated Pituitary adenomas, FIPA) or with an early sporadic onset (Beckers et al., 2013). *AIP* is involved in a variety of protein–protein interactions, mainly through its TPR domains and C-terminal α -helix. *AIP* partners include nuclear receptors such as the Aryl Hydrocarbon Receptor (AHR) (Bell and Poland, 2000), which expression correlates with *AIP* in PA (Jaffrain-Rea et al., 2009), and the peroxisome proliferator-activated receptor α (PPAR α) (Sumanasekera et al., 2003). Despite evidence for increased cAMP signalling in *AIP*^{mut} somatotropinomas (Formosa et al., 2013; Tuominen et al., 2015) and frequent *AIP* down-regulation in invasive sporadic cases (Jaffrain-Rea et al., 2009; Kasuki Jomori de Pinho et al., 2011), the tumour suppressing functions of *AIP* are not fully elucidated. Inactivating *AIP*^{mut} and/or *AIP* downregulation may result in defective interactions with its partner proteins (Bell and Poland, 2000; Jaffrain-Rea et al., 2009; Leontiou et al., 2008). Because *AIP* represses PPAR α transcriptional activity in rodent hepatic cells (Sumanasekera et al., 2003) and PPAR α is able to stimulate PRL transcription in pituitary GH₄C₁ cells, probably through an interaction with Pit1 (Tolon et al., 1998), PPAR α may also play a role in PA and in *AIP*-related pituitary tumorigenesis.

Peroxisome proliferator-activated receptors (PPAR)s are involved in a number of metabolic and differentiation processes and in several pathological conditions including cardiovascular and metabolic diseases, inflammation and cancer (Kota et al., 2005). The best characterized isotypes are PPAR γ and PPAR α . Human PA have been shown to express PPAR γ and rosiglitazone, a PPAR γ ligand, exerts anti-proliferative and anti-secretory effects on pituitary cell lines, including GH₃ cells (Bogazzi et al., 2004; Heaney et al., 2003; Winczyk and Pawlikowski, 2005). Although a role for PPAR α in the transcription of pituitary hormones has been suggested (Konig et al., 2009), little is known about its pituitary expression. In addition, despite a carcinogenic role in the rodent liver, PPAR α ligands generally exert anti-tumorigenic effects and PPAR α is expressed by a variety of solid neoplasia (Pozzi and Capdevila, 2008; Pyper et al., 2010), indicating PPAR α as a potential therapeutic target.

The aim of this study was to determine the expression of PPAR α in human somatotropinomas and its potential variations according to patients and tumour characteristics, including *AIP* status and pre-operative treatment with SSA. In addition, we aimed to evaluate the effects of fenofibrate, a PPAR α agonist drug used for the treatment of dyslipidemia (Adkins and Fauns, 1997), in the rodent somatotroph cell line GH₃.

2. Material and methods

2.1. Patients and samples

Tumours from 51 acromegalic patients were studied, most of which were collected during the 2007–2014 period at the Neuromed Institute (Pozzilli, Italy). Archive material from a subset of cases operated on in Liège (Belgium) and from familial somatotropinomas were also included. The study was performed according to the guidelines of the Declaration of Helsinki and approved by the Ethical committee at the Neuromed Institute (Pozzilli, Italy). Written informed consent was obtained from the patients, except for a minority of archive paraffin-embedded material from patients lost to follow-up. There were 27 females, 24 males, with a mean age of 42.0 \pm 14.9 years (range 8–78 yrs). Most were sporadic cases but a FIPA context was present in 6 patients. According to pre-operative MRI and intra-operative findings, a large majority of tumours were

macroadenomas (42/51, 82.3%) including 24 with a suprasellar extension (SSE) (47%). Half were invasive (26/51), in particular invasion of the cavernous sinus was recorded in 21 cases (41.1%). Thirty patients (58.8%) received pre-operative treatment with somatostatin analogues (SSA) for a median duration of 6 months (range 3–60). Noteworthy, the macroscopic characteristics of treated and untreated tumours were similar (24/30 vs 17/20 macroadenomas in treated vs untreated cases, *P* ns; 14/30 vs 11/20 invasive adenomas in treated vs untreated cases, *P* = ns). The pre-operative pharmacological response could be assessed in terms of plasma GH and IGF1 reduction in 25 patients. Individual hormone responses to SSA were classified in controlled, partially controlled and uncontrolled as reported previously (Jaffrain-Rea et al., 2013). Controlled disease was defined by pre-operative IGF1 levels within the normal range for age (*n* = 12), and partially controlled disease by pre-operative IGF1 levels above the normal range for age with a Δ IGF1 > 30% as compared to pre-treatment values (*n* = 7). Tumours from controlled and partially controlled patients were then grouped as “responsive” (*n* = 19). Uncontrolled disease was defined by pre-operative IGF1 levels above the upper limit for age with a Δ IGF1 < 30% or an increased IGF1 concentration as compared to pre-treatment values. All these patients also had pre-operative GH concentrations >2 ng/ml and a Δ GH < 50% as compared to pre-treatment values. The corresponding tumours were designed as “unresponsive” (*n* = 6). According to diagnostic immunohistochemistry for pituitary hormones, 15 tumours were mixed GH/PRL-secreting. Somatotropinomas were studied for PPAR α and *AIP* expression by semi-quantitative immunohistochemistry (IHC) (*n* = 39) and/or Real Time RT-PCR analysis (*n* = 26). Thirty-nine acromegalic patients, including all FIPA patients and those affected by early onset, aggressive and/or pharmacologically resistant tumours, gave written informed consent for leukocyte genomic *AIP* sequencing (gDNA), which was performed as previously described (Daly et al., 2007). *AIP* mutations (*AIP*^{mut}) were present in 6 patients (3 familial *AIP*^{R304X}, 2 familial *AIP*^{Q285fs}, 1 sporadic *AIP*^{A277P}). Four *AIP*^{mut} somatotropinomas were available for IHC.

2.2. GH₃ cells culture and treatment

GH₃ cells were freshly obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA, distributed by LGC Standards S.r.l., Milan, Italy) and cultured in Ham's F10 with 10% of fetal bovine serum, glutamine 1%, penicillin (100UI/ml) and streptomycin (100 mg/ml) in a humidified atmosphere at 37 °C with 5% CO₂. Cells were plated at a density of 1–2 \times 10⁶/well in 100 mm Petri dishes 18 h before treatment with fenofibrate (sc-204751, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 12.5–50 μ M final concentrations (Adkins and Faulds, 1997). Octreotide (Biorbyt Ltd, Cambridge, UK) was used at a 10⁻⁸ M final concentration and added 24 h after fenofibrate in combined experiments to obtain a significant effect of both drugs. Cells were counted with a Burkert chamber and viability was assessed using Trypan blu 0.5% exclusion (Euroclone, Pero, Italy).

2.3. Immunohistochemistry (IHC) and immunofluorescence (IF) in tissues and cells

IHC was performed on paraffin-embedded sections of pituitary adenomas as previously described (Jaffrain-Rea et al., 2009), using a mouse monoclonal anti-*AIP* at a 1:500 dilution for 3 h (clone 35–2, Novus Biologicals LLC, Littleton, CO, USA), a polyclonal rabbit anti-PPAR α antibody at a 1:250 dilution O/N (PA1-822A, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) and a multilink biotinylated antibody/avidin–biotin peroxidase system according

to the manufacturer's instructions (LSAB⁺ kit, DAKO Cytomation, Milan, Italy). Antigen retrieval was obtained by microwave boiling at 850 W in citrate buffer 0.1 M, pH 6.0 (5', 5' and 3') and cytoplasmic PPAR α background was reduced by introducing a further blocking step (5% milk in PBS for 60'). Normal pituitary fragments observed on a couple of microadenoma sections and normal hepatic fragments from surgical liver samples were used as positive and intensity controls for the study of AIP and PPAR α , respectively. Negative controls were obtained omitting the primary antibody. The specificity of PPAR α immunostaining was confirmed by pre-incubation with PPAR α synthetic peptide (PEP-025, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) on positive control sections. Immunostaining for AIP was determined semi-quantitatively according to intensity and expression pattern (range 0–6) as previously reported (Jaffrain-Rea et al., 2013; Kasuki Jomori de Pinho et al., 2011; Leontiou et al., 2008). PPAR α immunostaining was also quoted semi-quantitatively in the cytoplasm – PPAR α (c) – (0: negative, 1: weak, 2: moderate, 3: strong), and in the nucleus – PPAR α (n) – according to the percentage of positive nuclei (0: 0–10%, 1: 10–30%, 2: 31–60%, 3: >60%). The total PPAR α score – PPAR α (t) – was calculated by adding PPAR α (c) and PPAR α (n) scores (range 0–6). In both cases, positive immunostaining was defined by a score ≥ 2 and a high expression was defined for scores ≥ 3 .

IF was performed on paraffin-embedded sections of a normal post-mortem pituitary, using the same anti-PPAR α antibody (PA1-822A, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) co-incubated with a mouse monoclonal anti-GH or anti-PRL antibody (sc-51602 and sc-46698, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:250, 1:500 and 1:250, respectively. To this aim, sections were dewaxed in xylene and rehydrated through a descending ethanol series, antigen retrieval was performed by microwave boiling as reported hitherto and a 0.1% sodium borohydride solution was applied twice to reduce tissue auto-fluorescence (Baschong et al., 2001). Sections were then incubated for 60' in a blocking PBS solution (5% goat serum, 0.5% BSA) and subsequently with primary antibodies O/N at 4 °C. Next, sections were incubated 30' with appropriate secondary antibodies diluted 1:50 – anti-mouse Pierce® Rhodamine-conjugated and anti-rabbit ImmunoPure® Fluorescein-conjugated (31663 and 31635, respectively, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) –, washed with a DAPI (4',6-diamidino-2-phenylindol) containing PBS solution to identify cell nuclei, and mounted with a fluorescent solution (Fluoromont, NB900-66726, Novus Biologicals LLC, Littleton, CO, USA).

IF was performed on GH₃ cells grown on 6-well plates and fixed in formalin 10%. After washing in PBS, cells were permeabilized with Triton X-100 0.05% in PBS 1% for 10' at 4 °C, incubated in a 3% BSA blocking solution and O/N at 4 °C with the anti-PPAR α antibody diluted 1:250 (PA1-822A, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA). After further washing in PBS and blocking with 1% BSA, cells were incubated for 30' with a secondary anti-rabbit ImmunoPure® Fluorescein-conjugated antibody diluted 1:50 (31635, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA), washed and mounted on slides with ProLong Gold antifade reagent with DAPI (Invitrogen, Life Technologies, Monza, Italy). Negative controls were obtained omitting the primary antibody.

Data were analysed with a Zeiss Axioplan 2 microscope (Carl Zeiss Microimaging Inc, USA) and pictures were taken with a Leica DFC digital camera (Leica GmbH, Germany).

2.4. Gene expression analysis in tissues and cells

Total RNA was extracted from surgical fragments of somatotropinomas and GH₃ cells by EuroGOLD TriFast™ (Euroclone, Pero,

Italy). Surgical biopsies were collected in RNA later solution (Ambion®, distributed by Life Technologies, Monza, Italy) and frozen at –80 °C until use. After DNase treatment (Qiagen), 1 μ g RNA was reverse-transcribed with Euroscript MMLV (Euroclone, Pero, Italy) according to the manufacturer's instructions. Preliminary RT-PCR experiments using beta-actin or GADPH as housekeeping genes were performed for cDNA quality control, including PCR on DNase-treated samples to verify the absence of genomic DNA contamination (primers and conditions are available on request). In addition, potential contamination of somatotropinomas by normal pituitary tissue was checked for by RT-PCR for *Tpit* expression as previously described (Fratticci et al., 2007). Twenty-two macroadenomas and 4 microadenomas were finally retained for gene expression analysis, and four post-mortem normal human pituitaries were included. Intron-bridging primers for human PPAR α were first designed on the corresponding Genbank sequence AY206718.1 (forward: TTATCTGAA-GAGTTCCTGCAAGA, reverse: CCAGCTTGAGTCCAATCGTTC) to verify PPAR α gene expression by RT-PCR (amplification conditions are available on request) in a preliminary series of normal and tumour samples. AIP and PPAR α gene expression were then quantified in all samples by Real Time RT-PCR and corrected for β actin expression, using a Taqman methodology on an Applied Biosystems 7500 Fast Real-Time PCR (Life Technologies, Monza, Italy). Reactions were performed in duplicate on the same batch of cDNA. Ready-to-use gene expression assays were purchased from Applied Biosystems (Life Technologies, Monza, Italy), with the following identification numbers: Hs00610222_m1 (AIP), Hs00947536_m1 (PPAR α) and Hs_99999903 (β actin). Similarly, in cell culture experiments, PRL and GH gene expression were determined on the same batch of cDNA for each experimental condition and corrected for Cyclophilin B expression, which was unaltered by fenofibrate treatment. Ready-to-use gene expression assays were used, with the following identification numbers: Rn01495894_g1 (GH), Rn00561791_m1 (PRL) and Rn03302274_m1 (Cyclophilin B) (Applied Biosystems, Life Technologies, Monza, Italy).

2.5. Cell cycle and apoptosis analysis in GH₃ cells

Synchronized GH₃ cells were obtained by serum deprivation for 48 h and dose-dependent experiments with fenofibrate (12.5–50 μ M) were reproduced in 6-well plates. Cells were collected after 48 h of treatment for the analysis of cell cycle and apoptosis using the Tali® Image-Based Cytometer (Life Technologies, Monza, Italy), according to the manufacturer's instructions. Briefly, apoptosis was first studied on freshly collected cells using the Tali® Apoptosis Kit–Annexin V Alexa Fluor®488, whereas cell cycle analysis was performed on cells fixed in 70% ethanol at –20 °C O/N, washed and re-suspended in a Tali® Cell Cycle solution containing propidium iodide.

2.6. ELISA assays in GH₃ cells

Cell culture media were collected at each experimental time point and stored at –80 °C until hormone measurement. GH and PRL were determined by rat-specific enzyme-linked immunoassays (GH: A05104, PRL: A05101, SPI-BIO, Bertin Pharma, Montigny-le-Bretonneux, France) following the manufacturer's instructions and corrected for cell number. Experiments were performed in duplicate and repeated at least twice. Detection limits for GH and PRL assays were 0.5 ng/ml and 0.2 ng/ml, respectively. Cross-reactivity between PRL and GH was <1% in both assays.

2.7. Western blot analysis in GH₃ cells

Proteins were extracted from GH₃ cells using a RIPA buffer (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA). Sixty micrograms of each extract were resolved on 12% SDS-PAGE and electroblotted to PVDF membranes (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA). Membranes were blocked with milk 10% for 1 h and incubated O/N at 4 °C with the following primary antibodies: rabbit polyclonal anti-PPAR α , anti-GH (PA1-822A, PA1-85518, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA, respectively, both 1:400) and anti-cleaved Caspase 3 (9664, Cell Signaling, 1:400), mouse monoclonal anti-PPAR α (NB300-537, Novus Biologicals LLC, Littleton, CO, USA, 1:200), anti-PRL (sc-271773, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200) and anti-p21 (MA5-13293, Pierce Biotechnology, Rockford, IL, USA, 1:500), a rabbit polyclonal anti-Cyclophilin B antibody (PA1-027A, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA; 1:2000) being used as a loading control. PDVF membranes were then washed twice with Tris Buffered Saline with 0.1% Tween20 (TBS-T) at room temperature, blocked with 10% milk for 30' and incubated for 1 h with secondary anti-mouse or anti-rabbit HRP-conjugated antibodies (sc-2031 and sc-2030, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:2000 and 1:4000, respectively). After washing, a Pierce ECL WB substrate (Thermo Scientific, USA) was used for signal detection and imaging analysis was performed using a ChemiDoc™ XRS⁺ System (Bio-Rad Laboratories, Inc, Hercules, CA, USA).

2.8. Statistical analysis

Statistical analyses were performed using the JMP software for PC (SAS Institute, Cary, NC, USA). Continuous data obtained in patients and tumour samples are expressed in median (range) and analysed by non-parametric tests, using the Mann-Whitney U test for 2 groups-analysis and robust analysis for correlation studies, whereas distributions of nominal values were compared by the Pearson Chi-2 test. Data obtained from cell culture experiments are expressed as mean (\pm SD) analysed by ANOVA. $P < 0.05$ was considered significant.

3. Results

3.1. Expression of PPAR α in normal pituitaries and in somatotropinomas

3.1.1. Expression of PPAR α in the normal pituitary

PPAR α immunostaining was observed in normal post-mortem pituitary tissues as well as in normal pituitary fragments adjacent to PA, which was in agreement with the detection of PPAR α mRNA by RT-PCR in normal pituitary (NP) extracts (data not shown). Some endothelial cells also displayed nuclear immunostaining. Cytoplasmic and/or nuclear PPAR α staining could be observed in endocrine cells. According to IF, most were somatotrophs and lactotrophs (Fig. 1)

3.1.2. Expression of PPAR α in somatotropinomas and relationship with AIP

As shown in Fig. 2 (Panel A), PPAR α gene expression tended to be lower in somatotropinomas than in NP ($P = 0.07$). AIP was expressed at higher levels than PPAR α but showed a broader tumour distribution, ranging from under- to overexpression, with no significant difference as compared with NP. No correlation was found between PPAR α and AIP transcripts (data not shown).

PPAR α and AIP immunostaining are summarized in Table 1 and representative cases are shown in Fig. 2 (Panel B). PPAR α

immunopositivity was observed in most somatotropinomas (32/39, 82.0%). Both cytoplasmic and nuclear staining were observed in most cases, although exclusive cytoplasmic ($n = 6$) or nuclear ($n = 1$) localization could be found. AIP immunopositivity was observed in a similar proportion of somatotropinomas (32/39; 82.0%), a majority of tumours expressing both AIP and PPAR α (27/39, 69.2%). Overall, only a modest association was found between AIP and PPAR α immunostaining. Indeed, though the presence of a high PPAR α expression was twice more frequent in tumours showing a high AIP expression (76.0% vs 35.7% in the presence of a low AIP score, $\chi^2 = 6.15$, $P = 0.013$), no significant correlation was found between AIP and PPAR α scores. Of note, the PPAR α score was low in 3 out of 4 AIP^{mut} somatotropinomas (Table 2).

3.1.3. Factors influencing PPAR α expression in somatotropinomas

Overall, no significant difference in PPAR α mRNA or immunostaining was found according to patients' age, gender, tumour volume (macro- vs micro-adenomas, presence vs absence of suprasellar extension) or invasiveness (including presence vs absence of cavernous sinus invasion), or between GH- and GH/PRL-secreting phenotypes (data not shown).

In contrast, PPAR α immunopositivity was significantly more frequent in tumours treated with SSA before surgery (22/24) than in untreated cases (10/15) (91.7% in SSA⁺ vs 66.7% in SSA⁻, $\chi^2 = 3.9$, $P = 0.047$), so that a majority of treated somatotropinomas had a high PPAR α score (75% vs 40% in SSA⁻, $\chi^2 = 4.8$, $P = 0.029$), a high AIP score (75% vs 46.7% in SSA⁻, $\chi^2 = 3.2$, $P = 0.073$) or both (62.5% vs 26.7% in SSA⁻, $\chi^2 = 4.5$, $P = 0.029$). Excluding AIP^{mut} somatotropinomas from the analysis, the proportion of treated tumours showing a high AIP score was also significantly higher than in untreated cases ($P = 0.022$ for high AIP, $P = 0.020$ for high PPAR α , $P = 0.013$ for both). As shown in Fig. 3, this translated into trends towards higher PPAR α scores in treated tumours (Panel A), reaching significance after exclusion of AIP^{mut} cases ($P = 0.048$ and 0.043 for PPAR α (t) and PPAR α (c), respectively). Because neither the nuclear expression of PPAR α or PPAR α mRNA levels were affected by SSA (data not shown), this may in part reflect an enhanced cytoplasmic stability of the protein due to the higher AIP expression. However, in contrast to AIP, which expression was significantly higher in responsive than in unresponsive tumours ($P = 0.013$), neither PPAR α expression or intracellular localization appeared to be influenced by the outcome of treatment (Fig. 3, Panel B), and similar results were obtained excluding AIP^{mut} somatotropinomas from the analysis (data not shown).

3.2. Effects of fenofibrate on GH₃ cells in vitro

3.2.1. Cell growth and apoptosis

The expression of PPAR α and the effects of fenofibrate on GH₃ cells growth are illustrated in Fig. 4. In addition to the full length 52 kDa protein, a cleaved form of PPAR α was observed (33 kDa). Fenofibrate was found to significantly reduce GH₃ cell growth in a dose-dependent manner ($P < 0.0001$ vs control for each concentration, $P = 0.0019$ at 25 μ M vs 12.5 μ M, $P = 0.0003$ at 50 μ M vs 25 μ M). The maximal effect was obtained after 48 h of treatment and remained significant at 72 h ($P < 0.0001$ at 48 h and 72 h vs control cells at 25 μ M and 50 μ M). This was associated with a progressive increase of cells in the pre-G₁ (apoptotic) and G₂ fractions, with a reduction in the G₁ fraction, reaching statistical significance at 50 μ M. Accordingly, a dose-dependent increase in the percentage of annexin V-positive cells and in the expression of activated caspase 3 and p21 was observed.

3.2.2. Endocrine effects of fenofibrate

We subsequently analysed the effects of fenofibrate on GH and

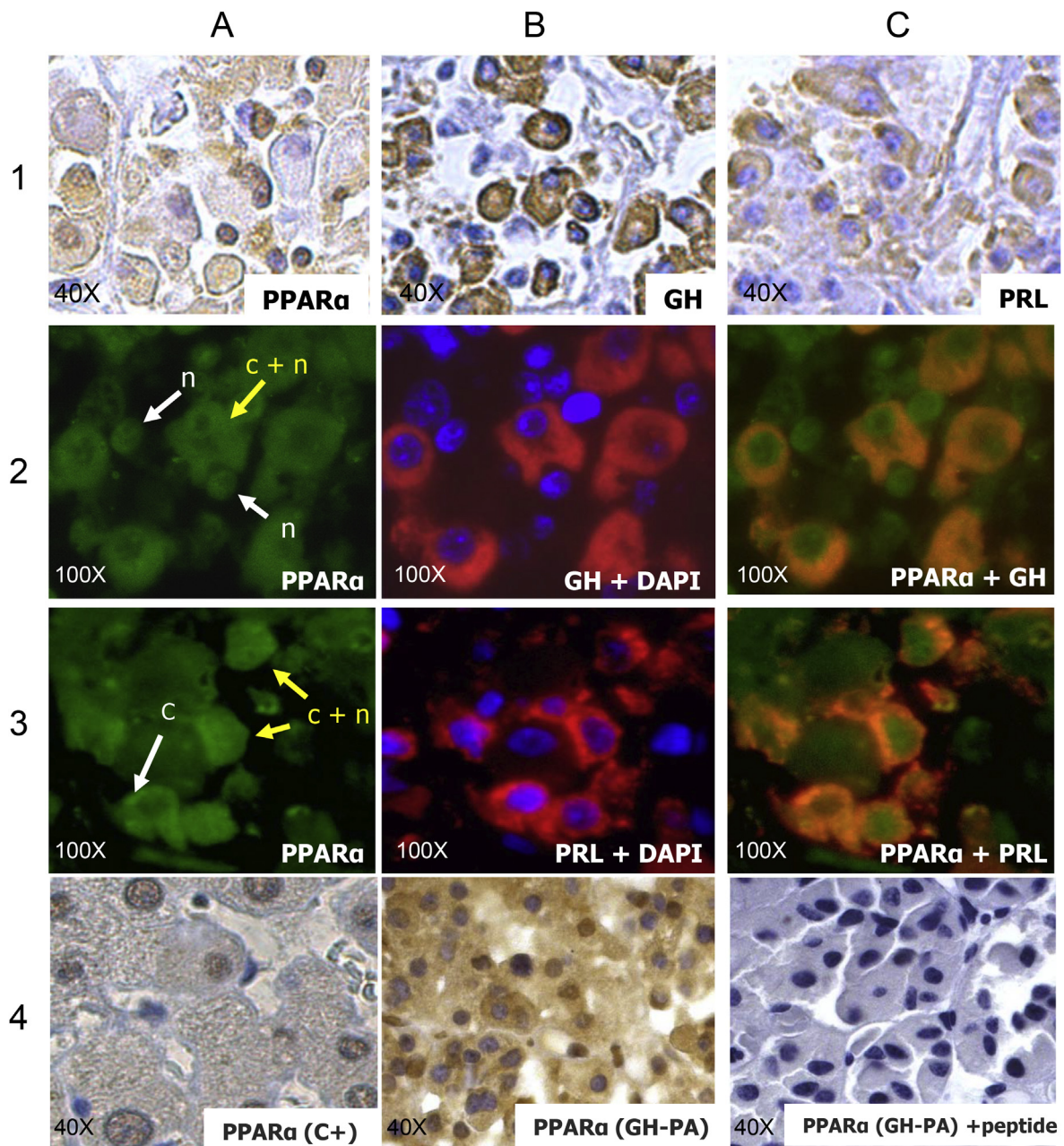


Fig. 1. PPAR α expression in the normal pituitary. Line 1 shows immunohistochemical staining for PPAR α (1A), GH (1B) and PRL (1C) in a normal post-mortem pituitary; Lines 2 and 3 show FITC immunofluorescence (green) for PPAR α (2A,3A), rhodamine immunofluorescence (red) for GH (2B) and PRL (3B) with nuclear DAPI staining (blue), merged PPAR α and GH (2C) or PRL (3C) in normal post-mortem pituitary sections; arrows in 2A and 2B indicate nuclear (n) and cytoplasmic (c) PPAR α localization; Line 4 shows immunohistochemical staining for PPAR α in a positive liver tissue control (4A) and a case of somatotropinoma displaying cytoplasmic and nuclear PPAR α immunostaining (4B), abolished by pre-incubation with a PPAR α peptide (4C).

PRL secretion in GH₃ cells (Fig. 5). After preliminary experiments revealing a significant increase in GH secretion with 25 μ M fenofibrate ($P < 0.0001$ vs control at 24 h and 48 h, P ns at 72 h), dose-dependent experiments were performed. A progressive dose-related increase in GH secretion was observed ($P < 0.0001$ at 12.5 μ M vs control, $P < 0.0001$ at 25 μ M vs 12.5 μ M, $P < 0.0001$ at 50 μ M vs 25 μ M), with no apparent effect on PRL secretion. In contrast, a bimodal effect on GH transcription was observed, with a modest but significant increase at 25 μ M ($P = 0.009$ vs control, $P = 0.004$ vs 12.5 μ M) and a significant decrease at 50 μ M ($P = 0.01$ vs control, $P = 0.0001$ vs 25 μ M), associated with a dose-dependent

decrease in PRL gene transcription ($P < 0.0001$ at 25 μ M and 50 μ M vs control, $P < 0.0001$ at 50 μ M vs 25 μ M). Similarly, the intracellular content in GH showed very slight increase and decrease at 25 μ M and 50 μ M, respectively, with a clear dose-dependent reduction in PRL content. Taken together, these findings suggest that the increase in GH secretion induced by fenofibrate is due to a dose-dependent stimulation of GH release rather than in changes in gene transcription or protein synthesis. Similarly, PRL secretion appears to be maintained through a dose-dependent increase in PRL release in front of a parallel reduction in PRL gene transcription and, presumably, protein synthesis.

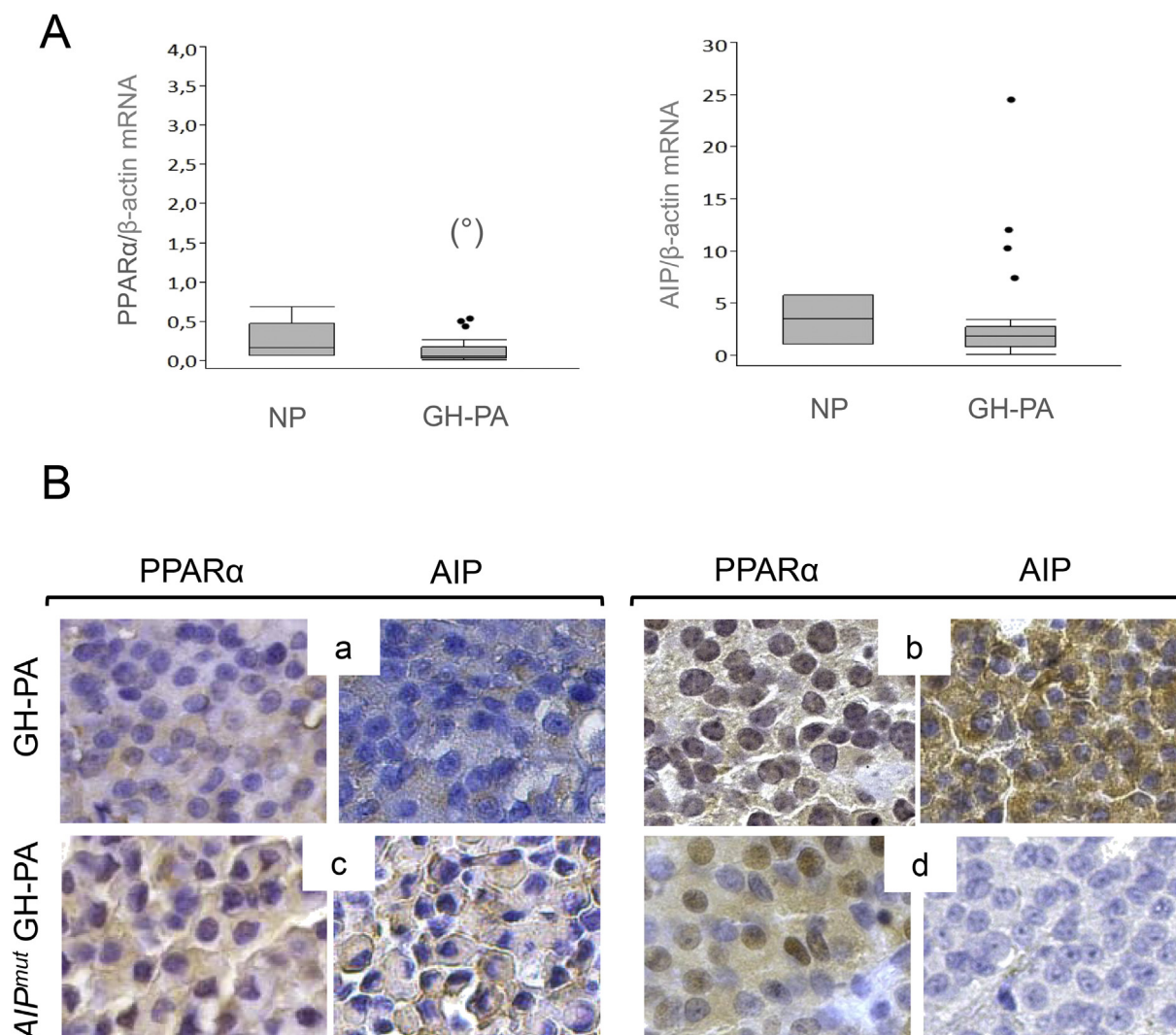


Fig. 2. Expression of PPAR α and AIP in somatotrinomas. (A) Relative expression of PPAR α / β actin mRNA (left) and AIP/ β actin mRNA (right) obtained by Real Time RT-PCR in normal pituitaries (NP) and in 26 somatotrinomas (GH-PA). A non-significant trend was observed towards transcriptional PPAR α down-regulation in somatotrinomas [(°) $P = 0.07$], differences in AIP transcripts were not significant (P ns). (B) PPAR α and AIP immunostaining in representative cases of somatotrinomas (GH-PA) (magnification $\times 40$): (a) an untreated tumour, (b) a responsive SSA-treated tumour, (c) and (d) are two AIP^{mut} cases (see Table 2, untreated case 2 and unresponsive SSA-treated case 4, respectively).

Table 1
PPAR α and AIP immunohistochemistry in somatotrinomas.

	Somatotropinomas
Number (n)	39
PPAR α immunopositivity (n)	32 (82.0%)
PPAR α score ^a	
-PPAR α (total)	3.0 (0–6)
-PPAR α (cytoplasmic)	2.0 (0–3)
-PPAR α (nuclear)	1.0 (0–3)
PPAR α (% nuclei) ^a	22.0 (0–76)
AIP immunopositivity (n)	32 (82.0%)
AIP score	4.0 (0.5–6)
PPAR α and AIP immunopositivity (n)	27 (69.2%)

^a Expressed as median (range).

3.2.3. Effect of octreotide on PPAR α expression and on GH₃ cells response to fenofibrate

In order to evaluate the potential effects of octreotide (OCT) on PPAR α expression and to further elucidate the mechanisms of hormone secretion induced by fenofibrate (FF), GH₃ cells were subsequently treated with OCT 10⁻⁸ M in the presence or in the

absence of FF 25 μ M (Fig. 6). A slight increase in PPAR α protein content was observed after OCT treatment, regardless of FF. According to immunofluorescence, PPAR α was also more diffusely expressed in OCT-treated cells than in control cells. Whereas both OCT and FF were able to significantly reduce GH₃ cell growth ($P < 0.0001$ for both), the effect of FF was significantly stronger ($P < 0.0001$ vs OCT) and no additive effect of drug combination was observed. In contrast, whereas OCT alone induced a non-significant decrease in GH secretion, it significantly inhibited the increase in GH concentration induced by FF ($P = 0.0027$ in FF vs control, $P = 0.0006$ in OCT + FF vs FF, P ns OCT + FF vs control). Similarly, although OCT alone had no significant effect on PRL concentration, a significant reduction was observed in the presence of FF ($P = 0.003$ in OCT + FF vs FF) (data not shown). Taken together, these data further suggest that the main effect of fenofibrate is to stimulate hormone release, which is inhibited by OCT.

4. Discussion

This study provides the first evidence of PPAR α expression in the

Table 2
PPAR α and AIP immunohistochemistry in *AIP*^{mut} somatotropinomas and clinical characteristics.

	AIP change ^o	Patient	Setting	Tumour	Treatment	PPAR α score (t)	PPAR α score (c)	PPAR α score (n)	AIP score
1	AIP ^{R304X}	M, 8 yrs	FIPA	Ma, Inv-	SSA+	1	1	0 (5%)	2
2	AIP ^{Q285fs}	M, 21 yrs	FIPA	Ma, Inv+	SSA-	2	1	1 (15%)	2
3	AIP ^{Q285fs}	M, 34 yrs	FIPA	Ma, Inv+	SSA+	1	1	0 (7%)	1
4	AIP ^{A277P}	M, 12 yrs	Sporadic	Ma, Inv+	SSA+	4	2	2 (44%)	1 ^a

AIP^{mut}: germline AIP mutations; ^o: AIP protein change; FIPA: Familial Isolated Pituitary Adenoma; Ma: macroadenoma; Inv-: non-invasive; Inv+: invasive; SSA+: treated with somatostatin analogues before surgery; SSA-: untreated. PPAR α scores: (t): total, (c) cytoplasmic, (n) nuclear for individual nuclear scores the percentage of immunopositive nuclei is given within brackets. Of note, none of the *AIP*^{mut} treated tumours was responsive to SSA.

^a with large AIP negative areas.

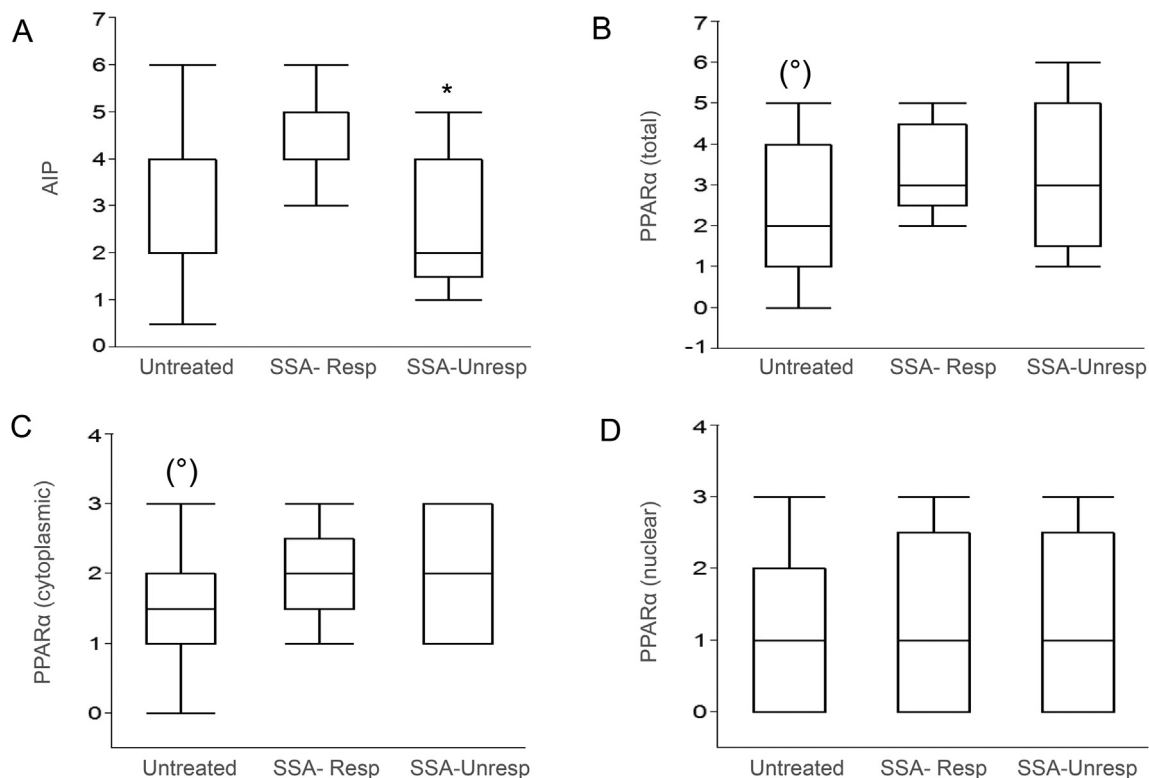


Fig. 3. Influence of pre-operative treatment with somatostatin analogues (SSA) on AIP and PPAR α immunostaining in somatotropinomas. As shown in (A), the AIP score was significantly higher in those responsive to SSA compared to unresponsive cases [$*P = 0.013$], though no significant difference in AIP expression was found between treated and untreated tumours overall. Both the total PPAR α score (B) and the cytoplasmic PPAR α score (C) tended to be higher in treated than in untreated tumours on the whole series ($P = 0.078$ and $P = 0.053$, respectively), such differences reached significance excluding *AIP*^{mut} cases [(°) $P = 0.048$ and $P = 0.043$, respectively]. The nuclear PPAR α score (D) was not influenced by SSA treatment or the response to SSA.

normal human pituitary as well as in a large majority of somatotropinomas and in GH₃ cells. We found PPAR α to be normally expressed by somatotrophs and lactotrophs, with a trend towards transcriptional down-regulation in somatotropinomas as compared to normal pituitaries. However, PPAR α mRNA and immunostaining were found to be unrelated to the macroscopic characteristics of the tumours, suggesting that, unlike AIP (Jaffrain-Rea et al., 2009; Kasuki Jomori de Pinho et al., 2011), loss of PPAR α expression is not related to tumour progression and has no prognostic value in somatotropinomas. Because AIP was reported to form a cytosolic complex with PPAR α in the rat liver (Sumanasekera et al., 2003), correlations with AIP expression or mutation status were searched for, but only a modest association between AIP and PPAR α was found. Indeed, although nearly 70% of somatotropinomas expressed both AIP and PPAR α , with a high PPAR α expression being twice more frequent in high AIP-expressing cases, no significant relationship between AIP and PPAR α scores was observed. This is in contrast with AHR, which cytoplasmic score

was strongly correlated to the AIP score (Jaffrain-Rea et al., 2009, 2013). The low PPAR α immunoscore in three *AIP*^{mut} somatotropinomas associated with a truncating mutation is reminiscent of the loss of AHR expression in such tumours (Jaffrain-Rea et al., 2009), but additional cases should be studied since a minority of somatotropinomas could express PPAR α despite very low AIP immunostaining. Additional factors are known to enhance the stability of the PPAR α protein, in particular phosphorylation (Blanquart et al., 2004), and the stability and/or translatability of PPAR α mRNA can be influenced by microRNAs, as reported in the human liver (Kida et al., 2011) and cancer cell lines (Tong et al., 2011; Wang et al., 2013). Some microRNAs have been reported to target AIP mRNA in GH-PA (Trivellin et al., 2012; Denes et al., 2015) and dysregulated microRNAs might also account for the discrepancy between PPAR α transcripts and immunostaining in these tumours. Overall, the effective role of AIP/PPAR α interactions in somatotropinomas remains uncertain. Biochemical experiments on protein-protein interactions in the presence of *AIP* mutations or

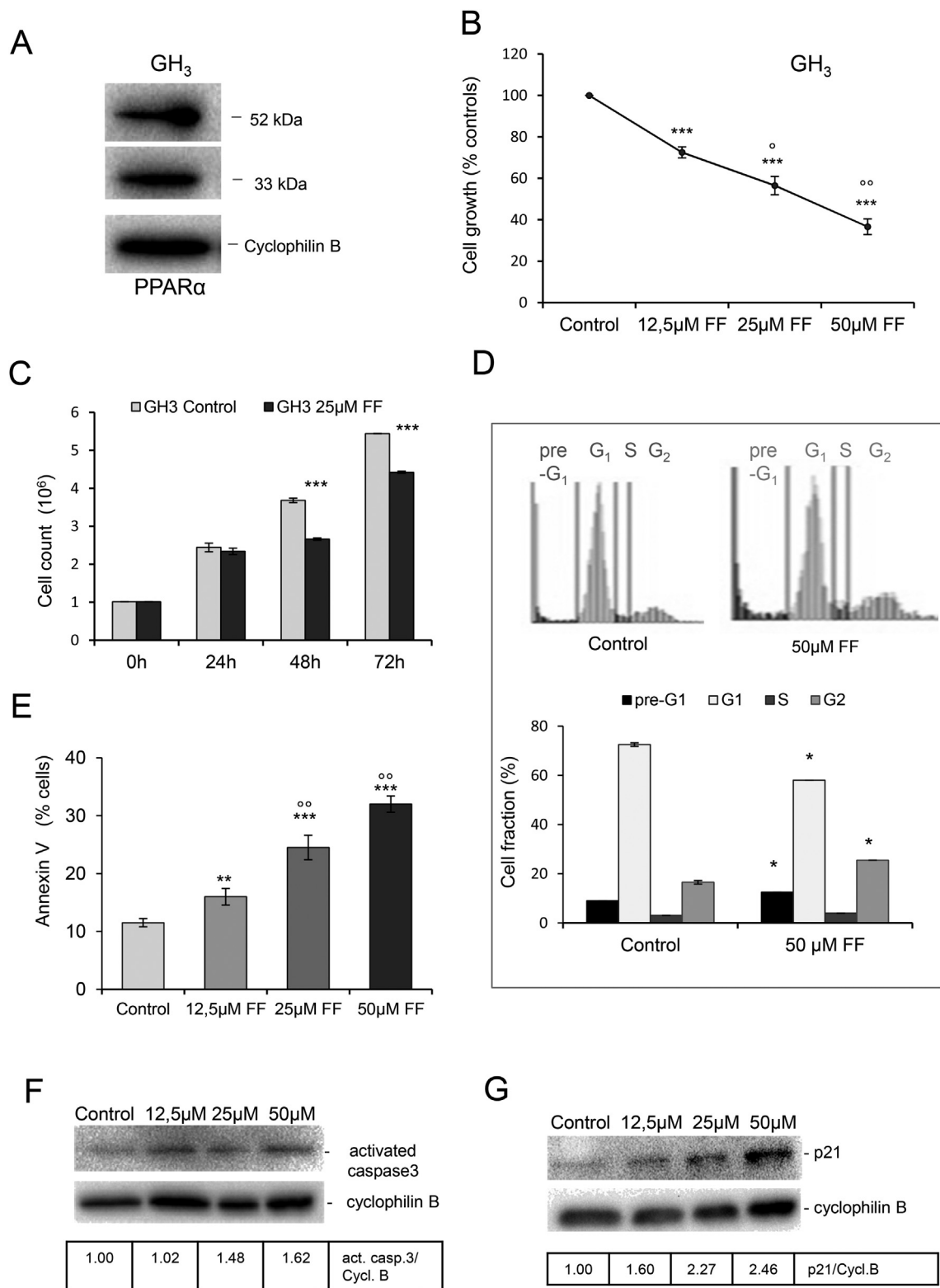


Fig. 4. PPAR α expression and the effects of fenofibrate on GH₃ cell growth. (A) Expression of PPAR α protein in GH₃ cells: a full length (52 kd) and a cleaved form (33 kd) were observed (B) Fenofibrate significantly reduced the growth of GH₃ cells in a dose-dependent manner $***P < 0.0001$ vs control cells, $^{\circ}P = 0.0019$ vs 12.5 μ M; $^{\circ\circ}P = 0.0003$ vs 25 μ M (mean \pm SD of three different experiments). (C) An example of time-dependent experiment performed with fenofibrate (FF 25 μ M) showing a significant decrease in cell growth after 48 h and 72 h of treatment. (D) Analysis of the cell cycle after 48 h of treatment with 50 μ M fenofibrate (FF) revealed a significant increase of cells in the preG₁ (apoptotic) and G₂ fractions, accompanied by a significant decrease in the G₁ fraction ($*P < 0.0025$ for each fraction vs control cells). (E) A dose-dependent increase in AnnexinV fluorescence was observed ($**P < 0.005$ and $***P < 0.0001$ vs control cells, $^{\circ\circ}P = 0.0003$ vs lower concentration). Western blot analysis indicated a parallel increase in the expression of activated caspase 3 (F) and p21 (G).

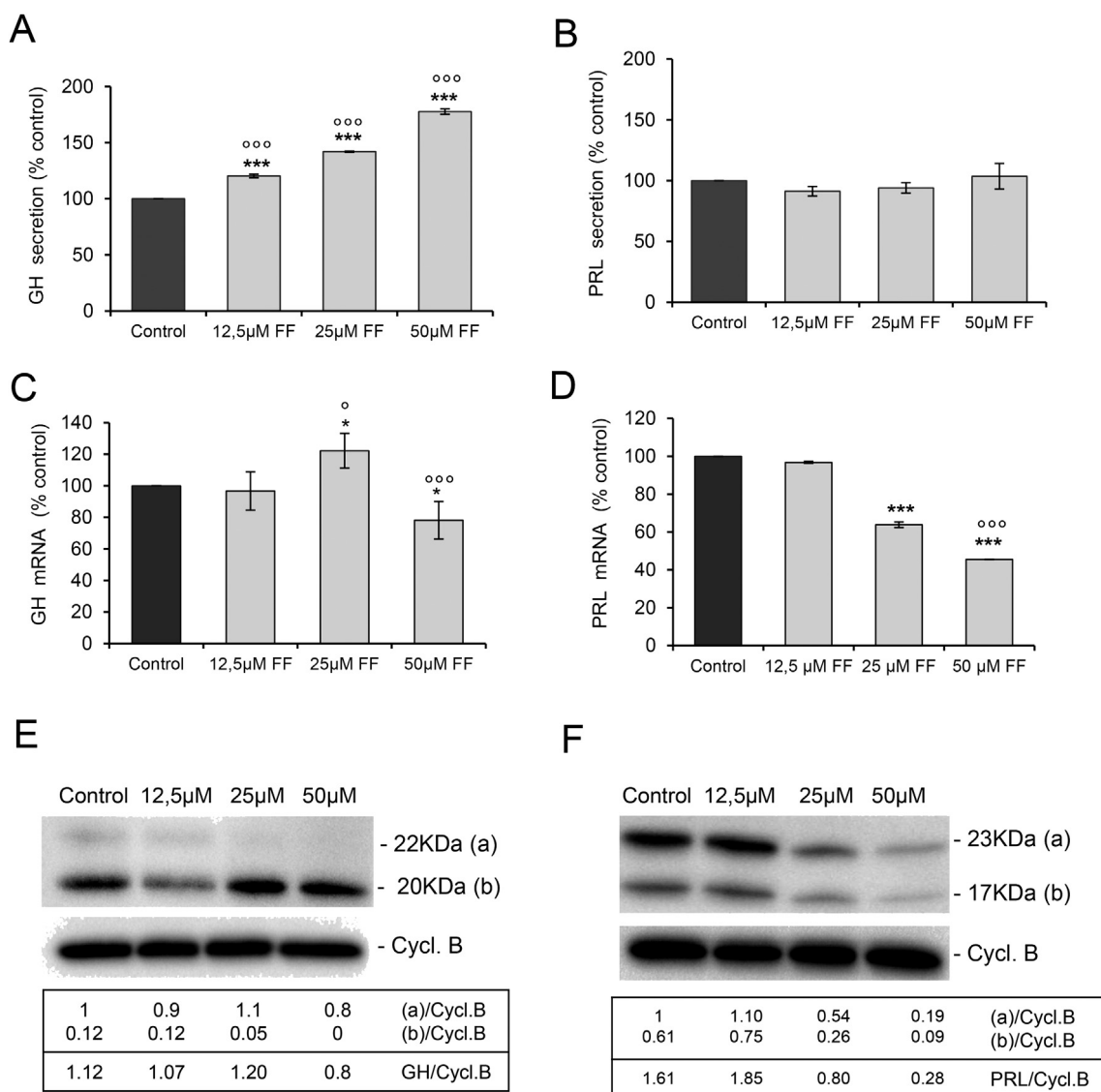


Fig. 5. Endocrine effects of fenofibrate in GH₃ cells. Fenofibrate induced a dose-dependent increase in GH (A) but not on PRL (B) secretion. In contrast, a bimodal effect was observed on GH gene transcription (C) (*P < 0.01 vs control cells, °P = 0.004 vs 12.5 µM, °°°P < 0.0001 vs 25 µM) contrasting with a progressive decrease in PRL gene transcription at both concentrations (D) (**P < 0.0001 vs control cells, °°°P < 0.0001 vs lower concentration). A similar trend was observed on intracellular GH (E) and PRL (F) content, with slight increase in GH intracellular content at 25 µM followed by a decrease at 50 µM, and a clear decrease in PRL intracellular content at 25 µM and especially at 50 µM.

studies on the effects of AIP silencing on PPAR α expression and function would be useful to address such issue. However, based on this study, PPAR α is unlikely to play a major role in AIP-related pituitary tumorigenesis.

Unexpectedly, the only factor found to significantly influence PPAR α immunostaining in somatotropinomas was pre-operative treatment with SSA. Indeed, the majority of treated tumours (62.5% vs 27.5% of untreated cases) displayed a high expression of PPAR α and a modest increase in PPAR α expression was observed after octreotide treatment in GH₃ cells. Because treated somatotropinomas also displayed a higher expression of AIP, which has been identified as a mediator of SSA in such tumours (Chahal et al., 2012, Jaffrain-Rea et al., 2013), we wondered if the expression of PPAR α was also associated with the response to pre-operative pharmacological treatment. However, only the expression of AIP was significantly higher in responsive than in unresponsive cases. The nuclear expression of PPAR α was also similar in responsive and unresponsive tumours. Thus, although increased AIP expression

may contribute to enhance the cytoplasmic stability of PPAR α in treated cases, other factors are involved and PPAR α is unlikely to be a mediator of SSA in somatotropinomas.

We subsequently tested the effects of fenofibrate on GH₃ cells. Fenofibrate was chosen because of its specific PPAR α agonist activity (Kota et al., 2005) and common pharmacological use for the treatment of dyslipidemia (Adkins and Faulds, 1997). GH₃ cells expressed PPAR α , including a cleaved form which was previously reported in rat neurons (Koch et al., 2011). Differential effects on cell growth and hormone secretion were observed. On one hand, fenofibrate was found to inhibit cell growth in a dose and time-dependent manner. This finding is consistent with the growth inhibiting properties of PPAR α ligands reported in cancer cell lines of different origin – including breast, colon, and liver cancer – although none was of endocrine origin (Pozzi and Capdevila, 2008). Accordingly, PPAR α ligands have been recently proposed as an adjunctive therapy for severe malignancies such as glioma (Binello et al., 2014), non small cell lung cancer (Skrypnik et al., 2014) or

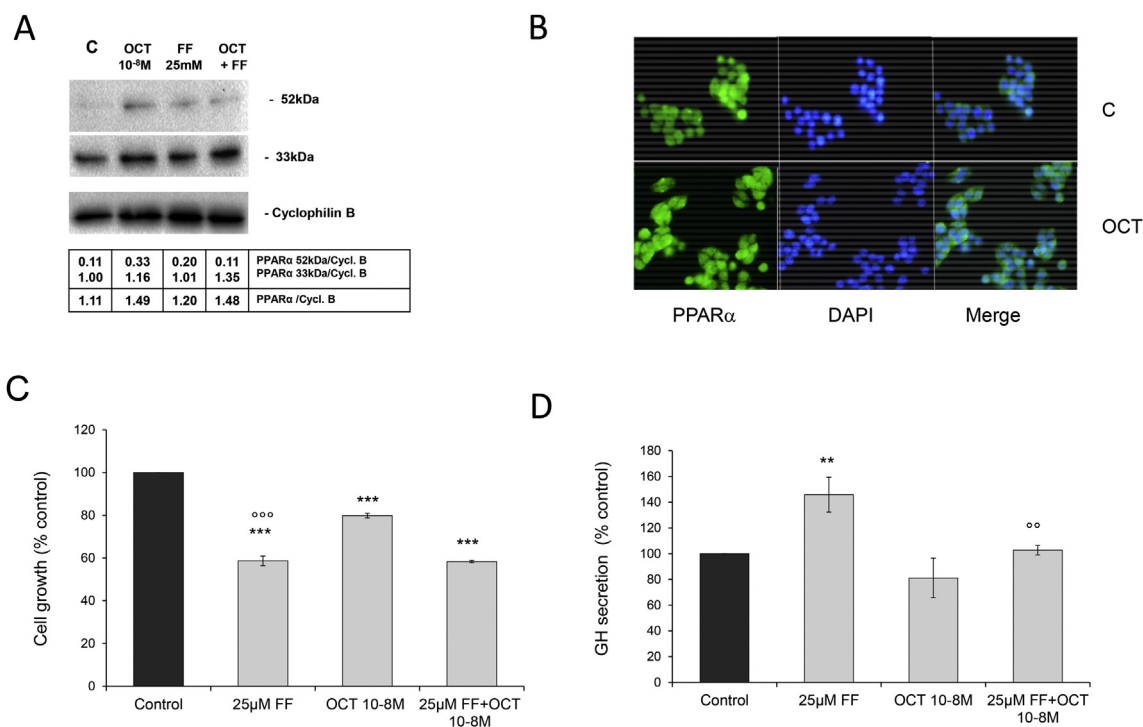


Fig. 6. Effect of octreotide on PPAR α expression and interaction with fenofibrate in GH $_3$ cells. (A) Expression of PPAR α according to pharmacological treatment with fenofibrate (FF) 25 μ M, octreotide (OCT) 10 $^{-8}$ M or both. A slight increase in PPAR α content was observed in cells treated with OCT (lane 2), which was not modified by fenofibrate co-treatment (lane 4). (B) Immunofluorescence for PPAR α according to OCT treatment (10 $^{-8}$ M). PPAR α was more diffusely expressed in OCT-treated cells than in control cells. Cell growth (C) and GH secretion (D) during pharmacological treatment with fenofibrate (FF) 25 μ M, octreotide (OCT) 10 $^{-8}$ M and both. ***P < 0.0001 vs control and °°°P < 0.0001 vs OCT (C); **P = 0.0027 vs control, °°P = 0.0006 vs FF (D).

triple-negative breast cancer (Li et al., 2014). Because anti-proliferative effects of PPAR γ agonists, thiazolidinediones, were previously reported in GH $_3$ cells (Bogazzi et al., 2004) and members of the PPAR family may share transcriptional effects through binding on common PPRE consensus sequences, similar molecular mechanisms might be involved. Indeed, both were found to exert pro-apoptotic effects on GH $_3$ cells, with an increased proportion of cells in the preG $_1$ fraction and an increased expression of annexin V and activated caspase 3 (Bogazzi et al., 2004; this study). However, in contrast with thiazolidinediones, fenofibrate was found to increase GH secretion in a dose-dependent manner, which was not sustained by a dose-dependent increase in gene transcription. Interestingly, PRL secretion was apparently unaffected despite the dose-dependent reduction in PRL gene transcription and intracellular protein content, suggesting that fenofibrate was able to maintain PRL release despite a reduction in PRL gene transcription and, presumably, protein synthesis. Although some hormone leaking from dead cells cannot be formally excluded, such effects were inhibited by octreotide. Taken together, these results support the hypothesis that the main effect of fenofibrate in GH $_3$ cells is to stimulate hormone release. This is reminiscent of data reported in a pancreatic insulinoma cell line (HIT-T15), in which fenofibrate was found to stimulate insulin release through an inhibition of K $^+$ channels, including K $_{ATP}$ and voltage-gated K v channels (Shimomura et al., 2006). A variety of factors influence hormone release through the modulation of the electrical activity of pituitary cells (Stojilkovic, 2012), in particular somatotrophs (Yang et al., 2012), and some of them are involved in their hormonal response to somatostatin and its analogues (Ben-Shlomo and Melmed, 2010). Of note, K v channels are inhibited by Ghrelin (Han et al., 2005) and activated by SSA (Yang et al., 2005). Thus, it is tempting to hypothesize that similar effects of fenofibrate may account for its

differential impact on hormone synthesis and secretion in GH $_3$ cells. An open issue is whether the acute effects on hormone release would occur during chronic treatment, since sustained inhibition of cell proliferation and intracellular hormone depletion may limit the effects on hormone secretion. Indeed, long-term fenofibrate treatment was found to inhibit glucose-induced insulin secretion in obese rats (Liu et al., 2011).

Potential non-genomic effects of fenofibrate, as reported hitherto, represent a limit of this study and suggest that additional PPAR α agonists and/or manipulation of PPAR α expression should be used to further clarify the biological significance and mechanisms of action of PPAR α in somatotroph cells, with respect to hormone secretion as well as cell proliferation (Kota et al., 2005; Roberts et al., 2002). For example, we found fenofibrate to reduce PRL gene transcription in GH $_3$ cells, which is in contrast with data reported in GH $_4$ C $_1$ cells (Tolon et al., 1998). However, these authors used high concentrations of Wy14,643, so that differences in experimental conditions and/or in cell phenotype may account for such discrepancy. Instead, we focused our experiments on the 12.5–50 μ M concentration range of fenofibrate, which is in the low-medium therapeutic range for dyslipidemia (Adkins and Faulds, 1997), after preliminary experiments with 100 μ M were found to induce some toxic effects on GH $_3$ cells (data not shown). Studies on primary cultures of human somatotropinomas would also be useful to evaluate the potential effects of fenofibrate, alone or in combination with octreotide, on such tumours. Previous experience with PPAR γ agonists indicates that despite encouraging experimental data (Bogazzi et al., 2004; Heaney et al., 2003), the use of rosiglitazone in patients with secreting pituitary adenomas was limited by their inconstant and mild efficacy and side-effects (Ambrosi et al., 2004; Bastemir et al., 2007; Kreutzler et al., 2009). Whether fenofibrate, which is a cheap drug with a high safety profile, may

exert relevant pharmacological effects on normal or tumorous pituitary hormone secretion *in vivo* or on the growth somatotropinomas is currently unknown.

In conclusion, PPAR α appears as a new player in somatotrophs, which may be down-regulated in somatotropinomas. Overall, this study argues for an ancillary role of AIP in the regulation of PPAR α expression or intracellular localization and against a significant role for PPAR α down-regulation in tumour progression, so that its potential role in tumorigenesis is uncertain. However, the tumour expression of PPAR α was enhanced by SSA. Because fenofibrate had anti-proliferative effects on GH $_3$ cells and SSA inhibited the hormone release induced by fenofibrate *in vitro*, potential interactions between PPAR α agonists and SSA may deserve further investigation.

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