



Published in final edited form as:

Antiviral Res. 2010 May ; 86(2): 137–143. doi:10.1016/j.antiviral.2010.02.004.

Effect of ritonavir and atazanavir on human subcutaneous preadipocyte proliferation and differentiation

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Abstract

Protease inhibitors (PIs) have been implicated in the development of HIV-associated lipodystrophy through a reduction in the differentiation of preadipocytes. While atazanavir (ATV) is associated with fewer clinical metabolic abnormalities in the short-term, the effects of long-term exposure are not known. ATV effects on preadipocyte replication or differentiation would indicate the potential for long-term problems. This study compared ritonavir (RTV) and ATV effects on preadipocyte replication and differentiation in human primary cultures. Preadipocytes from subcutaneous fat were studied in the presence of therapeutic concentrations of RTV and ATV for replication, differentiation, and adipokine secretion. The effects of the drugs on the expression of PPAR γ and related genes during differentiation were also assessed by real time quantitative PCR. RTV induced a significant inhibition of preadipocyte proliferation, differentiation and adiponectin secretion. ATV at concentrations within the range of therapeutic levels did not affect differentiation or adiponectin secretion, but did have inhibitory effects on preadipocyte proliferation. Inhibition of differentiation by PIs was associated with a decrease expression of PPAR γ , C/EBP α , and aP2 genes.

In summary, although ATV at therapeutic levels has a smaller impact on adipogenesis, alterations in preadipocyte proliferation suggest the potential for adverse effects with long-term use.

Keywords

Adipogenesis; adiponectin; HIV protease inhibitors; preadipocyte; PPAR γ

1. INTRODUCTION

Protease inhibitors (PIs) have been linked to the HIV-associated lipodystrophy (LD), characterized by loss of peripheral fat (lipoatrophy), dyslipidemia and insulin resistance (Jain et al., 2001; Koutkia and Grinspoon, 2004; Miller et al., 2003; Rudich et al., 2005) and in particular to a reduced ability of preadipocytes to differentiate (Dowell et al., 2000; Grigem et

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al., 2005; Jones et al., 2008; Lenhard et al., 2000). However, not all PI have the same propensity to induce metabolic complications or cause the same degree of adverse effects (Jain et al., 2001). Among the more currently used PIs, ritonavir (RTV), a very effective drug often used as a booster agent, is often associated with clinical LD. In contrast, atazanavir (ATV), a relatively newer PI, has been shown to be associated with fewer metabolic abnormalities and improve dyslipidemia and metabolic profile in patients previously treated with other PIs (Becker, 2003; Jemsek et al., 2006; Möbius et al., 2005; Sension et al., 2009). However, it is still not completely clear whether the use of ATV, in addition to reducing metabolic abnormalities, may also prevent or minimize the development of lipoatrophy. A better understanding of the effects of a PI on adipocyte metabolism is an important step in ensuring that PIs are both effective and not associated with side effects with long-term use.

At the cellular level, loss of body fat associated with PI treatment has been shown to be mainly related to inhibition of adipocyte differentiation with lower accumulation of lipids (Dowell et al., 2000; Rudich et al., 2005). Even in the absence of HIV infection, PIs have been shown to inhibit adipocyte differentiation and lipid accumulation, impair insulin signaling, and adipokine secretion (e.g. (Dowell et al., 2000; Grigem et al., 2005; Kim et al., 2006; Roche et al., 2002; Rudich et al., 2005; Zhang et al., 1999)). Most studies on the effect of PIs on adipogenesis have only addressed aspects of differentiation and have not assessed proliferation of preadipocytes. Preadipocyte proliferation is important since cells undergo clonal expansion prior to differentiation (Otto and Lane, 2005). Inhibition of preadipocyte proliferation may represent an additional mechanism by which PIs contribute to lipoatrophy, i.e. by reducing the number of precursor cells available for differentiation. Little is known about the effect of ATV on this important pathway. Therefore, this study was undertaken to compare the effects of RTV and ATV on preadipocyte replication. The study was performed in human primary cell cultures rather than rodent or human cell lines as used by others (e.g. (Dowell et al., 2000; Grigem et al., 2005; Kim et al., 2006; Lenhard et al., 2000; Roche et al., 2002; Vernochet et al., 2005; Zhang et al., 1999)) in order to reflect more nearly conditions in patients. Preadipocytes from subcutaneous fat, the depot generally affected by lipoatrophy, were studied in the presence of therapeutic concentrations of both RTV and ATV. Cells were assessed for replication, differentiation, and adipokine secretion. The molecular mechanism of the effects of PI on adipogenesis was also explored by observing changes in the expression of genes involved in the PPAR γ pathway (i.e., PPAR γ C/EBP α , SRBP-1 and aP2) during differentiation.

2. METHODS

2.1. Patients and study design

Subcutaneous fat tissue samples were obtained from healthy kidney donors undergoing nephrectomy at the Stony Brook University Medical Center. 15 Subjects (11 females, 4 males,) with an average age of 42 ± 12 y and BMI of 30 ± 5 , all HIV-seronegative, participated in the study. Subcutaneous fat was removed from the mid-abdominal wall area near the umbilicus, after induction of standard general anesthesia and immediately placed in a sterile Hank's Buffered Salt Solution (HBSS) containing antibiotics and amphotericin, pH 7.4. Preadipocytes were isolated from the adipose tissue and submitted in vitro to antiretroviral medications RTV at 0, 5 and 10 $\mu\text{mol/L}$ and ATV at 0, 10 and 30 $\mu\text{mol/L}$ on both the preadipocyte replication and preadipocyte differentiation. The effects of antiretroviral medications were compared with control samples which were preadipocytes cultured and stimulated to differentiate as described below but in the absence of antiretroviral medications.

The protocol was approved by the Stony Brook University Institutional Review Board and all subjects gave a written informed consent before enrollment in the study.

2.2. Pre-adipocyte isolation and culture

Fat biopsies were immediately transported to the lab, and processed within one hour from collection. Preadipocytes were isolated in aseptic conditions, with a modification of a previously described method (Hauner et al., 1989). Briefly, fat specimens were washed, freed from any visible blood vessel and connective tissue, and digested for 45 min at 37°C in phosphate-buffered saline (PBS) containing 3 mg/mL collagenase (Type II, Worthington, Lakewood, NJ), and 3.5 % bovine serum albumin, pH 7.4. Following digestion, stromal cells were separated from mature adipocytes by centrifugation and incubated in erythrocyte lysing buffer (154 mmol/L NH₄Cl, 10 mmol/L K₂HPO₄, 1 mmol/L EDTA, pH 7.4) for 10 min at room temperature to eliminate contaminating red blood cells. Cell suspension was then filtered through a 70 µm nylon filter to remove debris and then centrifuged. Pelleted preadipocytes were resuspended and plated in medium consisting of DMEM/F-12 (Gibco, Carlsbad, CA) supplemented with 10 % Fetal Calf Serum (FCS), 2 mmol/L glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco). After incubation at 37°C in a humidified atmosphere with 5% CO₂ for 16–18 h, attached cells were extensively washed with warm PBS, trypsinized and counted. Preadipocytes were then plated out in the same medium at a density of 5 × 10³/cm².

For the proliferation experiments, cells were incubated for 48 hours to allow an optimal attachment. After 48 hours the medium was aspirated off and replaced with fresh medium containing RTV and ATV at varying concentrations. Cell growth (see below) was then assessed after 24 and 72 hours.

For the assessment of differentiation and gene expression, cells were grown in medium until reaching confluence. After reaching confluence, differentiation was induced by replacing the basal medium with a chemically defined serum-free medium containing varying concentrations of RTV (0, 5 and 10 µmol/L) and ATV (0, 10 and 30 µmol/L). The differentiation medium consisted of DMEM/F-12 medium supplemented with 15 mmol/L HEPES, 10 mg/L transferrin, 33 µmol/L biotin, 17 µmol/L pantothenate, 0.5 µmol/L insulin, 0.1 µmol/L dexamethasone, 0.5 nmol/L triiodothyronine (T3), 0.5 µmol/L rosiglitazone, 540 µmol/L 3-isobutyl-1-methylxanthine (IBMX) (Tchkonina et al., 2002). In all experiments medium was replaced every 2–3 days and differentiation assessed after 12 days.

Since RTV and ATV were dissolved in DMSO, all control cultures contained a comparable amount of vehicle alone (< 0.1 %).

2.3. Assessment of preadipocyte proliferation

The effect of antiretroviral drugs RTV and ATV on preadipocyte cell number was assessed with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) based assay, as we have previously described (Caso et al., 2004). After addition of a 5 mg/mL MTT solution (Sigma, St. Louis, MO), cells were incubated for 3 hours at 37°C, 5% CO₂. Insoluble formazan crystals from the conversion of MTT by mitochondrial dehydrogenases of viable cells were then dissolved in DMSO and measured spectrophotometrically at 570 nm with background subtraction at 690 nm (Carmichael et al., 1987).

Preliminary experiments demonstrated the validity of the MTT assay to assess cell number. Preadipocytes plated at densities of 1, 2, 4, 6, 8, 12, 16, 32 × 10³/0.3 cm² for 24 hours indicated a linear relationship between absorbance and cell density (R = 0.996).

Since the cultures were all plated at the same initial cell density, the cell number after 24–72 hours is taken as a measure of cell replication, with a reduction in cell number taken to represent a decrease in the rate at which cells were proliferating.

2.4. Assessment of preadipocyte differentiation

Adipocyte differentiation was quantified after 12 day by assessing the activity of the lipogenic marker enzyme glycerol-3-phosphate dehydrogenase (GPDH), by measuring the intracellular lipid accumulation after Oil Red O staining and by directly counting the proportion of differentiated cells.

GPDH assay—GPDH activity was determined spectrophotometrically by monitoring the oxidation of NADH, as previously described (Wise and Green, 1979). Cells were harvested in cold 50 mmol/L TRIS buffer (pH 7.5) containing 1 mmol/L EDTA and 1 mmol/L 2-mercaptoethanol. The enzymatic assay mixture consisted of 50 mmol/L triethanolamine, 0.16 mmol/L NADH, 5 mmol/L EDTA and 0.1 mmol/L 2-mercaptoethanol, pH 7.5. The reaction was started by addition of 0.2 mmol/L dihydroxyacetone phosphate and the oxidation of NADH was assessed from the change in absorbance at 340 nm using a Spectra Max M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). GPDH activity was standardized to the amount of cell protein and expressed as mU/ml/mg of protein, with 1 mU corresponding to the amount of enzyme oxidizing 1 nmol of NADH/min.

Oil Red O staining—After fixing the cells in a 10 % formaldehyde solution in PBS, neutral lipids were stained with Oil Red O, as described previously (Janderová et al., 2003). The intracellular lipid accumulation was then quantified by extracting the dye in isopropanol and assessing the absorbance spectrophotometrically at 500 nm.

Cell counting—Adipocyte differentiation was also assessed by directly counting the number of differentiated and undifferentiated cells. Cultures stained with Oil Red O were counterstained with hematoxylin to visualize nuclei/cell contours. Differentiated cells were easily discriminated because of the characteristic multiple cytoplasmic lipid droplets, which stained bright red with Oil Red O. Five areas from each double-stained culture were randomly selected and photographed at a 40× magnification. All the differentiated and un-differentiated cells in each picture (≥ 200 cells per picture, ≥ 1000 cells in total) were counted by a person blinded to the treatment groups. The percent of differentiated cells in each treatment was obtained by averaging the results of the 5 replicates. The average coefficient of variation for the 5 replicates from a sample was approximately 12 %.

Assessment of gene expression—Expression of peroxisome proliferator-activated receptor γ (PPAR γ), CCAT-enhancer binding protein α (C/EBP α), the sterol-regulatory-element-binding protein-1 (SREBP1c), the adipocyte fatty acid binding protein (aP2), and interleukin 6 (IL-6) mRNA was assessed before and 2, 4, 8 and 12 days after addition of the differentiation medium by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). Total cell RNA was extracted with RNeasy lipid tissue mini kit (Qiagen, Inc., Valencia, CA), according to the Manufacturer's instructions. cDNA was prepared using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). All PCR reactions were carried out using Tagman Master Mix and commercially available primers (all from Applied Biosystems) with an Opticon Real Time Detection System (Bio-Rad Laboratories, Hercules, CA). The expression of multiple genes in multiple cultures from the same individual were calculated with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as internal control. Data are presented as the fold change in expression relative to expression of the target gene in undifferentiated pre-adipocytes.

Adiponectin production—The ability of cultures to secrete the adipokine, adiponectin, was determined after 12 days in differentiation medium containing varying concentrations of RTV or ATV. After incubation for 2 hours in fresh medium, the amount of adiponectin released in

the incubation medium was measured by ELISA, with a commercially available kit (Linco Research, St. Charles, MO). Adiponectin production was normalized to the cell protein and expressed as ng/mg protein.

2.5. Statistics

Results are expressed as means \pm SD. Differences between control and treated cultures were analyzed using a randomized block ANOVA with post-hoc Bonferroni's multiple comparison test. P values \leq 0.05 were considered statistically significant.

3. RESULTS

3.1. Effect of PIs on preadipocyte proliferation

Both RTV and ATV inhibited preadipocyte proliferation in a concentration-dependent manner. As shown in Figure 1, the effect of RTV was evident at drug concentrations \geq 5 μ mol/L. In cultures with 5 μ mol/L RTV, cell number was reduced by 11 % at 24 hours ($P < 0.01$) and by 15 % at 72 hours ($P = 0.05$) compared to controls. Inhibition of replication increased progressively with increasing concentrations of RTV. In the presence of 30 μ mol/L RTV, the cell number was reduced by 43 % at 24 hours ($P < 0.001$) and 46 % at 72 hours ($P < 0.001$) compared to controls (Fig. 1). ATV also depressed preadipocyte proliferation, although at concentrations higher than RTV. At a concentration of 5 μ mol/L, ATV did not have a detectable effect on cell number. The cell number of cultures incubated with 10 μ mol/L ATV was comparable to that of controls at 24 hours, but significantly lower after 72 hours (-13% , $P < 0.005$). Increasing ATV concentrations to 30 μ mol/L resulted in a depression of cell number by 14 % at 24 hours and 24 % ($P < 0.001$) at 72 hours (Fig. 1).

3.2. Effect of PIs on preadipocyte differentiation

Assessment of GPDH enzyme activity showed a significant reduction of preadipocyte differentiation by RTV at both 5 and 10 μ mol/L (Fig. 2A). GPDH activity was reduced by 14 % ($P < 0.005$) in the presence of 5 μ mol/L RTV. Inhibition of differentiation was more prominent when RTV concentration was increased to 10 μ mol/L (-32% , $P < 0.001$; Fig. 2A). ATV showed no effect on GPDH activity at a concentration of 10 μ mol/L. However, a significant reduction of GPDH activity was observed when ATV concentration was increased to 30 μ mol/L (-28% , $P < 0.05$; Fig. 2A).

Measurement of lipid accumulation by quantification of eluted Oil Red O stain also revealed an inhibition of differentiation by RTV. RTV at concentration of 5 and 10 μ mol/L inhibited lipid accumulation by 18 ($P = 0.05$) and 44 % ($P < 0.001$) respectively (Fig. 2B). Similarly to the assessment of GPDH activity, ATV only affected lipid accumulation at a concentration of 30 μ mol/L (-20% , $P < 0.005$) (Fig. 2B).

Direct observation of the cell cultures confirmed that RTV not only inhibited lipid accumulation but also reduced the number of differentiating cells. The percent of differentiated cells in cultures with 5 and 10 μ mol/L RTV were respectively 12 % ($P = 0.05$) and 36 % ($P = 0.001$) lower than controls (Fig. 2C). Similar concentrations of ATV had no effect on the proportion of differentiated cells (Fig. 2C). However, a 20 % reduction in the proportion of differentiated cells was observed at 30 μ mol/L ATV concentrations ($P < 0.001$) (Fig. 2C).

3.3. Effect of PIs on adiponectin secretion

A significant depression in adiponectin secretion was observed in the presence of RTV. Adiponectin secretion was reduced by 25 % ($P < 0.01$) at 5 μ mol/L RTV and by 59 % ($P < 0.001$) at 10 μ mol/L RTV (Fig. 3). A reduction in the secretion of adiponectin was also detected in the presence of ATV, but only at a concentration of 30 μ mol/L (-43% , $P < 0.05$; Fig. 3).

3.4. Gene expression during preadipocyte differentiation in the presence of PIs

The expression of SREBP1c, PPAR γ , C/EBP1 α , and aP2 genes progressively increased over the differentiation period (Fig. 4, Control). The expression of C/EBP1 α was maximal on day 8, while the expression of the other 3 genes was highest on day 12 (Fig. 4). Although the expression of SREBP1c also increased with length of exposure to the differentiation medium, SREBP1c expression was higher at day 2 (43% of the final value) than expression of PPAR γ , C/EBP1 α , and aP2. Incubation with 5 and 10 $\mu\text{mol/L}$ RTV progressively inhibited the increase of PPAR γ , C/EBP1 α , and aP2 gene expression on day 8 and 12. ATV also showed a similar effect, but only at 30 $\mu\text{mol/L}$ concentration (Fig. 4). There was no evident effect of both PIs on the expression of the SREBP1c gene.

The expression of the IL-6 gene did not show any change over the differentiation period, and was not affected by either PI (data not shown).

4. DISCUSSION

In assessing the potential for the PIs RTV and ATV to affect adipocyte phenotype, there are three possible ways in which to reduce the formation of mature adipose tissue; one, is to reduce the number of cells available for differentiation (reduced preadipocyte proliferation, i.e. MTT assay), a second is to reduce the proportion of preadipocytes which differentiate (i.e. cell counting), and a third is to reduce the amount of lipid which accumulates per differentiated adipocyte (i.e. Oil Red O staining). In fact, in this study there is evidence that both protease inhibitors RTV and ATV exhibit all three mechanisms. There was an inhibition of proliferation and both aspects of differentiation in primary cultures of human preadipocytes treated with RTV and ATV. Although both drugs showed a concentration-dependent effect on both proliferation and differentiation, higher concentrations of ATV were needed than the concentrations of RTV eliciting negative effects. The inhibition of preadipocyte differentiation by either PI was associated with an inhibition of the production of the adipokine, adiponectin, and with lower expression of genes controlling the differentiation process (i.e. PPAR γ and C/EBP1 α) or coding for markers of the mature adipocyte phenotype (i.e. aP2).

RTV depressed both proliferation of preadipocytes and preadipocyte differentiation at concentrations within the range of those observed in the plasma of HIV patients on HAART (Gatti et al., 1999). At a concentration of 10 $\mu\text{mol/L}$, which is approximately 3–4 fold lower than the maximal plasma concentration of the drug (i.e. C_{max}) (Gatti et al., 1999), RTV inhibited lipid accumulation by 46% (Fig. 2B). The Negative effects of RTV on total adipocyte formation resulted from both a reduction of the number of cells differentiating into mature adipocytes (Fig. 2C) and the amount of fat synthesized in each cell (Fig. 2B). At the same concentrations inhibiting differentiation, RTV also showed a depressive effect on preadipocyte proliferation (Fig. 1).

Incubation of preadipocytes with ATV within the normal therapeutic range inhibited preadipocyte replication (10 $\mu\text{mol/L}$, Fig. 1B). A decrease in the proliferative capacity of preadipocytes could potentially affect lipogenesis by resulting in a smaller pool of cells with ability to differentiate to mature adipocytes. However, the inhibitory effect of ATV on preadipocyte differentiation was only observed at 30 $\mu\text{mol/L}$ concentration, which is generally higher than maximal plasma levels observed during HAART (i.e. $C_{\text{max}} \sim 10 \mu\text{mol/L}$) (Solas et al., 2008). The lack of effects of ATV on adipogenesis in the range of concentrations observed in the plasma of treated HIV patients is in line with the clinical findings that treatment with ATV is associated with fewer lipid abnormalities and alterations in body fat composition generally associated with lipodystrophy (Haerter et al., 2004; Jemsek et al., 2006; Möbius et al., 2005; Sension et al., 2009), and in keeping with in vitro data showing that differentiation of preadipocytes was significantly inhibited by RTV, but not by ATV a concentration of 10 $\mu\text{mol/L}$.

L (Jones et al., 2008). However, although ATV may overall have less negative impact on fat differentiation than RTV, the effect on preadipocyte replication suggests the potential for a reduction in overall adipose tissue formation.

The mechanisms responsible for the depression of adipocyte differentiation by PI are not completely clear, although these may include a disturbance in the function of PPAR γ -dependent pathways (Caron et al., 2009), perhaps mediated by SREBP1c (Caron et al., 2001; Caron et al., 2009; Fajas et al., 1999). In the present study, the expression of SREBP1c, PPAR γ and C/EBP1 α genes progressively increased during differentiation, as did that of the PPAR γ -dependent and adipocyte specific gene, aP2, but were depressed in the culture treated with 5 and 10 μ mol/L RTV and 30 μ mol/L ATV (Fig. 4). The depressive effects of PI on PPAR γ and C/EBP1 α gene expression are in line with evidence of reduced expression of PPAR γ and C/EBP1 α mRNA in fat samples of lipodystrophic HIV-infected patients treated with PIs (Bastard et al., 2002) and support the view that alterations of the PPAR γ pathway may play a primary role in the pathogenesis of the disturbances of adipocyte differentiation caused by PIs (Caron et al., 2009; Dowell et al., 2000). Interestingly, the blunting effect of PIs on PPAR γ and C/EBP1 α gene expressions was not evident early in the differentiation process, but was only observed after 4 d (Fig. 4), suggesting that the effects of PIs on the PPAR γ pathway may not be part of the early events of differentiation. Differently from PPAR γ and C/EBP1 α , SREBP1c gene expression was not affected by RTV and ATV during differentiation (Fig. 4).

Several alterations in the production of cytokines and adipokines have been observed in HIV-treated patients. In particular, increased plasma levels of pro-inflammatory cytokines and reduced production of adiponectin by the fat tissue have been related to alterations in glucose metabolism and the development of insulin resistance and other metabolic abnormalities associated with lipodystrophy (Jan et al., 2004; Lagathu et al., 2005; Lihn et al., 2003; Mynarcik et al., 2002; Sutinen et al., 2003). Previous studies using murine and human preadipocyte cell lines have shown that PIs can directly stimulate IL-6 and suppress adiponectin gene expressions (Grigem et al., 2005; Kim et al., 2006; Lagathu et al., 2004), suggesting that the inhibitory effect of PIs on adiponectin may be mediated by the induction of IL-6 (Grigem et al., 2005). Unlike studies using human preadipocyte cell lines (Grigem et al., 2005; Kim et al., 2006), the present study did not find an alteration in IL-6 gene expression with PIs. An inhibition of adiponectin production was observed in the primary cultures in the present study with 5 and 10 μ mol/L RTV and 30 μ mol/L ATV, findings in contrast to Kim et al. (Kim et al., 2006), who did not observe inhibition of adiponectin production in a differentiated human adipocyte cell line treated with 50 μ mol/L ATV for 18 h. The current study would suggest that the decrease in adiponectin production with PIs is not mediated by IL-6.

In summary, although the study is limited by being an in vitro assessment, it does suggest that PIs may contribute to lipoatrophy by affecting fat cell differentiation and metabolism. In particular, RTV within therapeutic plasma concentrations inhibited both preadipocyte proliferation and ability of preadipocytes to differentiate into mature adipocytes, lipid accumulation and adiponectin secretion. In contrast, ATV at concentrations close to those observed in treated patients did not affect differentiation or adiponectin secretion but showed an inhibitory effect on preadipocyte proliferation. Although the clinical observations with ATV suggest fewer metabolic and fat distribution abnormalities, the finding that ATV may hinder preadipocyte replication suggests that there is still potential for deleterious effects on the accumulation of adipose tissue especially with long term exposure to the drug.

Acknowledgments

The authors would like to thank Mr. Stephen Knapik for his invaluable help in recruiting, Dr. Louise Stuart for her assistance in cell counting, and all the patients who kindly agreed to participate in the study.

The study was supported in part by NIH grant DK049316, NIH General Clinical Research Grant 5-MO1-RR-10710, and New York Empire Clinical Research Investigator Program (ECRIP) fellowship to GC.

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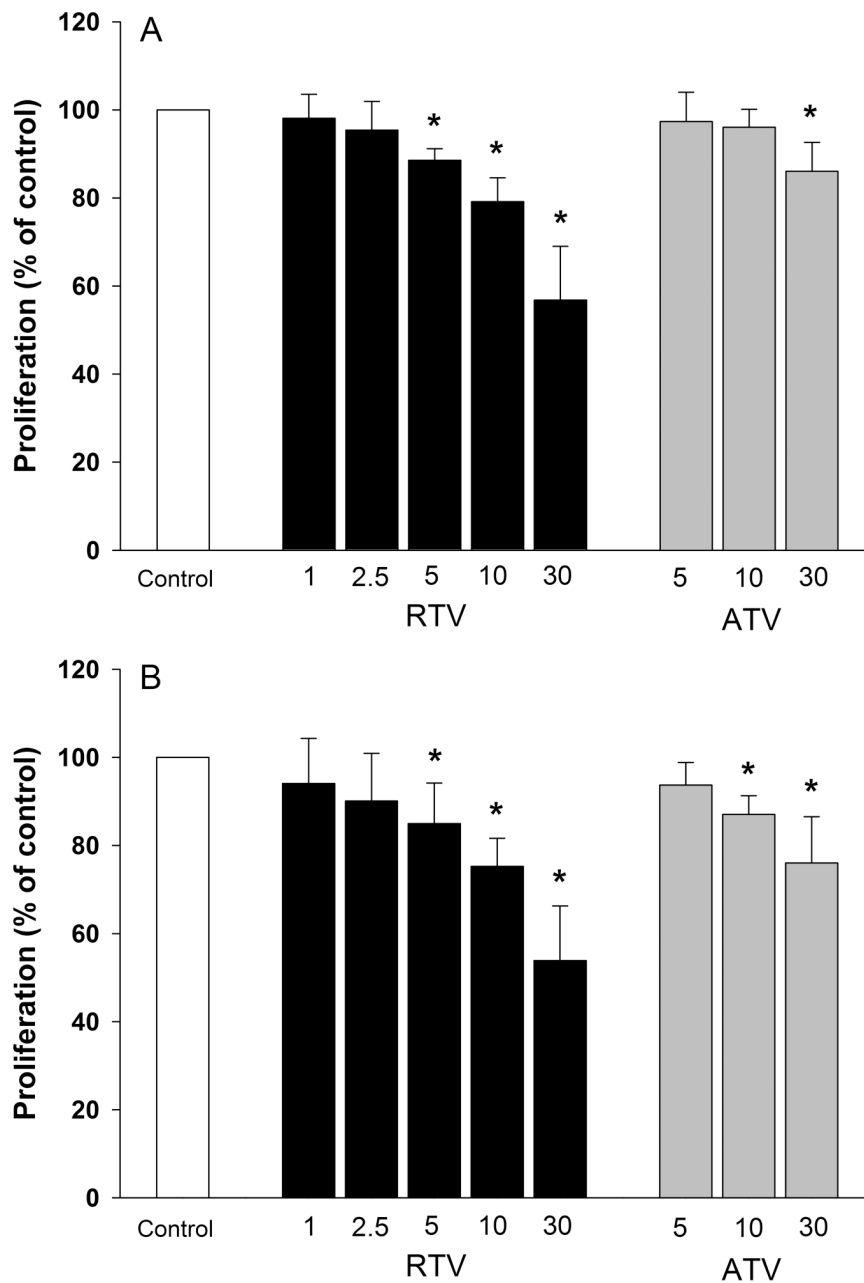


Figure 1. Pre-adipocyte proliferation in the presence of varying concentrations ($\mu\text{mol/L}$) of ATV and RTV for 24 (Panel A) and 72 hours (Panel B) (MTT test)
 The results are expressed as percent values of control cultures (mean \pm SD, n = 6). * $P \leq 0.05$.

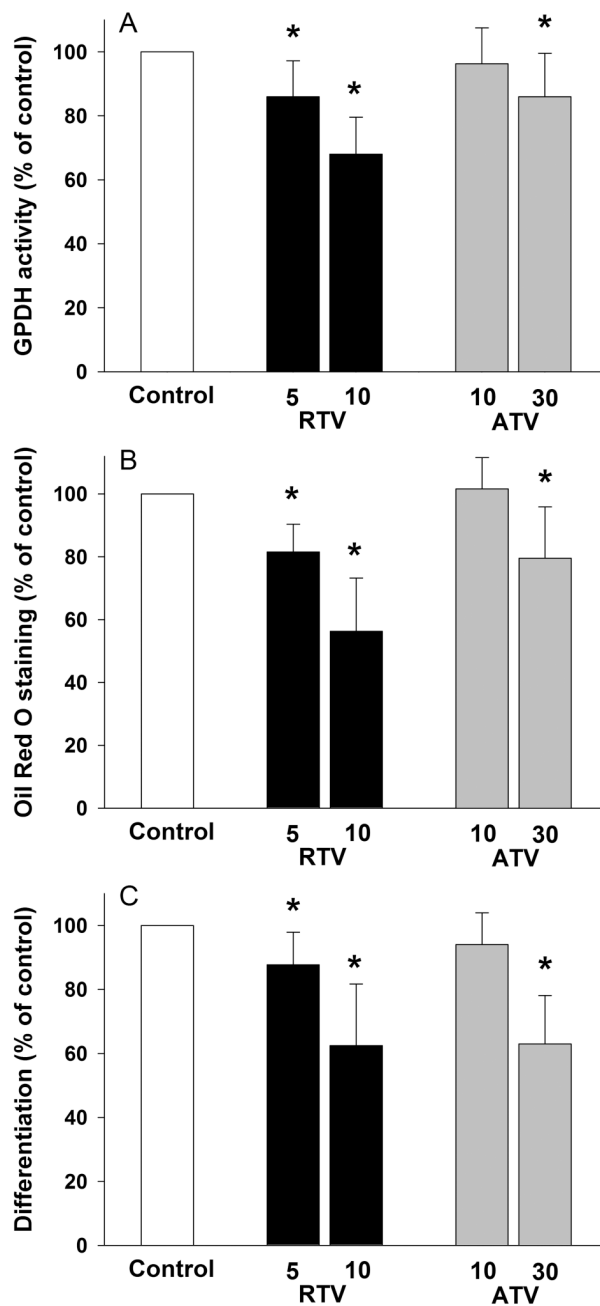


Figure 2. Effect of RTV (5 and 10 μmol/L) and ATV (10 and 30 μmol/L) on differentiation of human preadipocytes

Differentiation was assessed after 12 days by measuring the GPDH activity (panel A), Oil Red O staining (Panel B) and by direct count of the proportion of differentiated cells (Panel C). All results are expressed as percent values of control cultures. mean ± SD, (RTV groups, n = 10; ATV groups n = 11). * P ≤ 0.05.

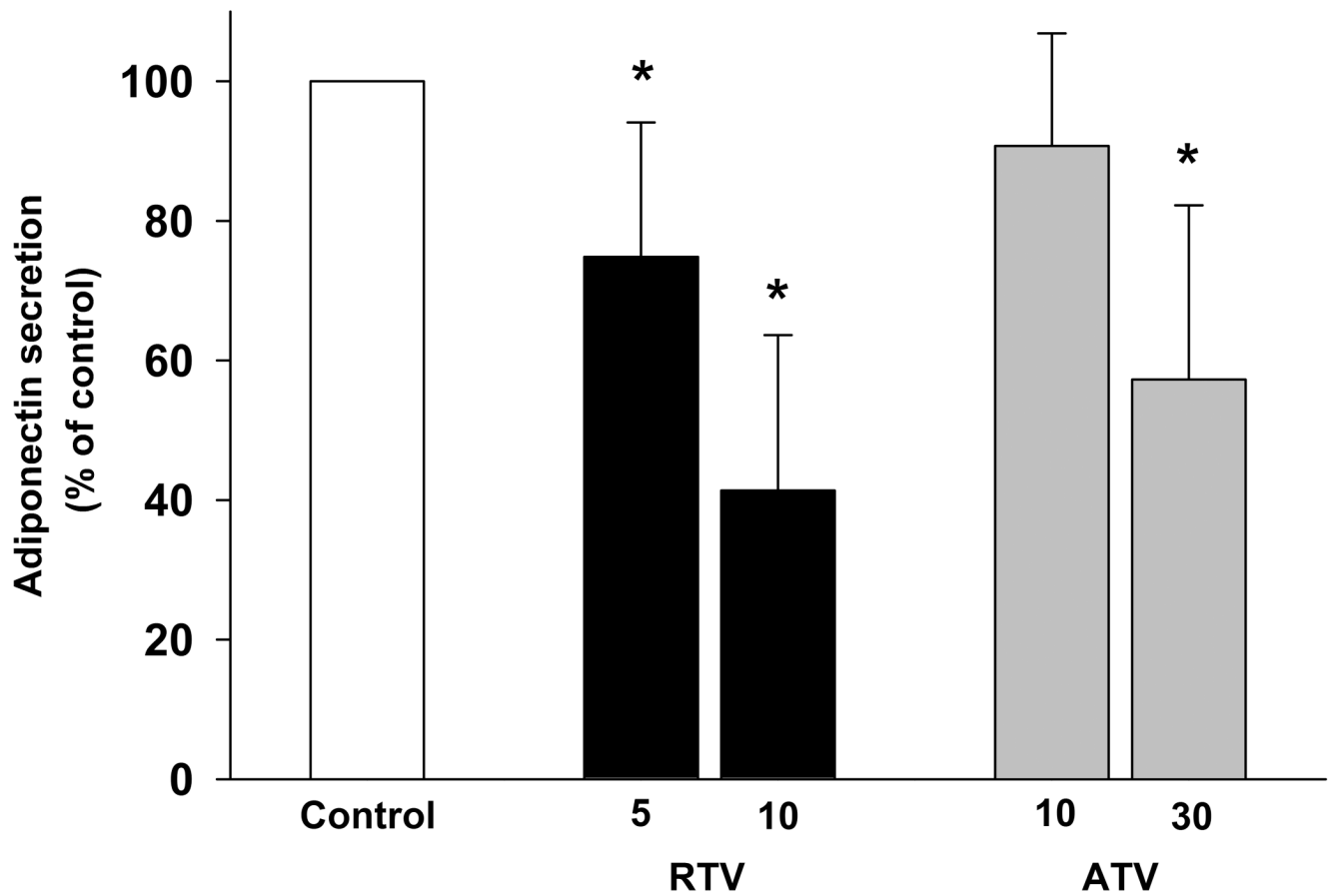


Figure 3. Effect of RTV (5 and 10 $\mu\text{mol/L}$) and ATV (10 and 30 $\mu\text{mol/L}$) on adiponectin secretion Adiponectin secretion was assessed after 12 days differentiation. Results were calculated as $\text{ng}/\mu\text{g}$ of cell protein and expressed as percent values of control cultures. mean \pm SD, (RTV group, n = 9; ATV group n = 10). * P < 0.05.

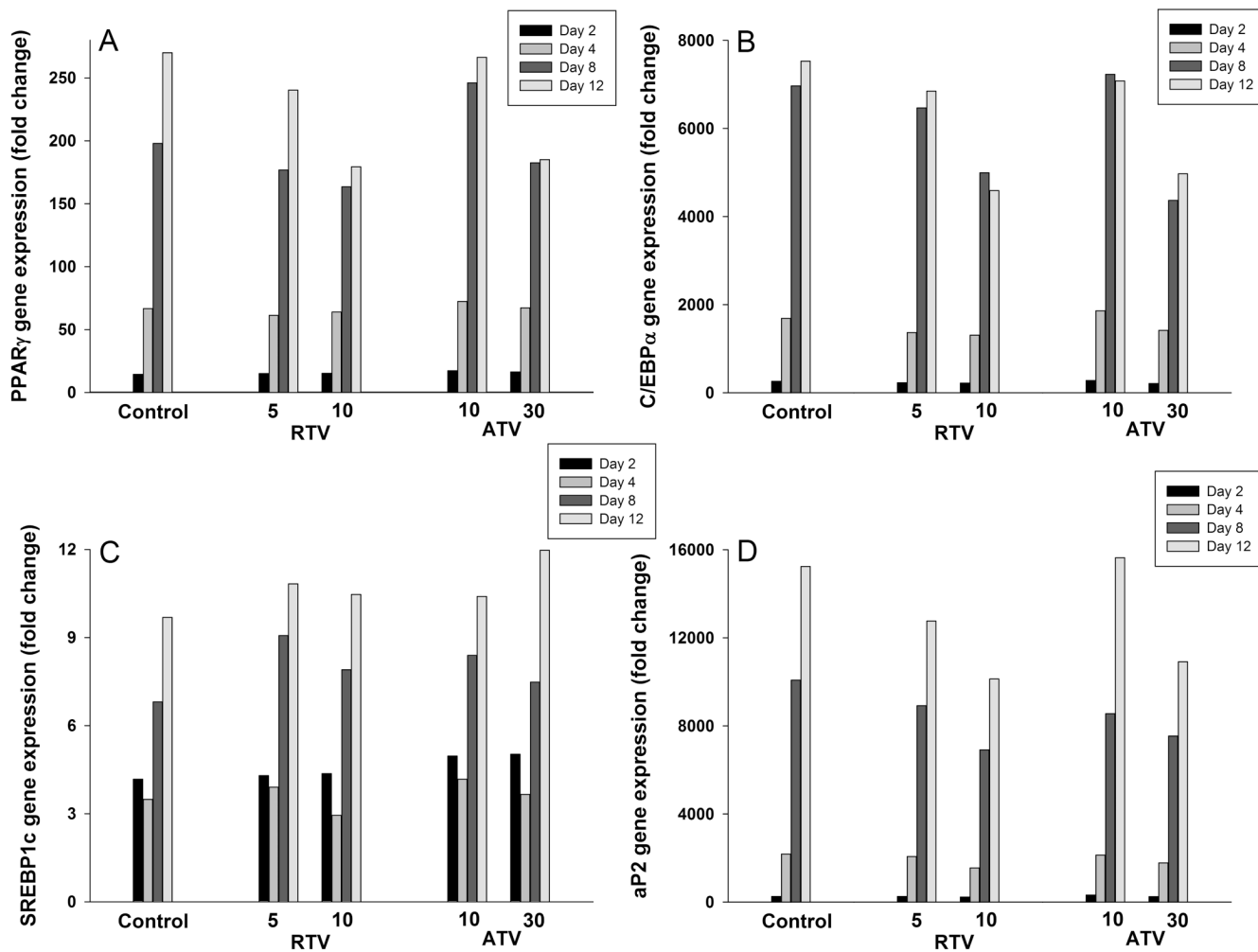


Figure 4. Effect of RTV (5 and 10 $\mu\text{mol/L}$) and ATV (10 and 30 $\mu\text{mol/L}$) on the time course expression of PPAR γ (Panel A), C/EBP α (Panel B), SREBP1c (Panel C), and a2P (Panel D) genes during differentiation

Gene expression was assessed by qRT-PCR in control and treated cultures on day 2, 4, 8 and 12. Data are expressed as fold changes from undifferentiated preadipocytes and normalized to GAPDH mRNA expression.