

HIV Protease Inhibitor-Specific Alterations in Human Adipocyte Differentiation and Metabolism

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Abstract

KIM, ROY J., CAMELLA G. WILSON, MARTIN WABITSCH, MITCHELL A. LAZAR, AND CLAIRE M. STEPPAN. HIV protease inhibitor-specific alterations in human adipocyte differentiation and metabolism. *Obesity*. 2006;14:994–1002.

Objective: Human immunodeficiency virus (HIV) patients on antiretroviral regimens frequently develop a syndrome of abnormal fat distribution, insulin resistance, and dyslipidemia. This lipodystrophic syndrome has been most closely linked to the use of HIV protease inhibitors (PIs). Several mechanisms have been postulated to explain these adverse effects of PIs, based largely on studies of rodent adipocytes. Intriguingly, atazanavir, a newer PI equally effective against HIV, is associated with fewer signs of lipodystrophy. We hypothesized that the less deleterious clinical effects of atazanavir would be reflected in physiological differences observed in PI-treated adipocytes.

Research Methods and Procedures: We compared the effects of atazanavir and an older PI associated with lipodystrophy, ritonavir, on differentiation, gene expression, adipocytokine secretion, and insulin signaling in a human adipocyte cell line.

Results: Ritonavir inhibited human adipocyte differentiation and induced apoptosis to a greater extent than atazanavir. Treatment of mature adipocytes with ritonavir, but not

atazanavir, also selectively decreased insulin signaling. Moreover, ritonavir also selectively decreased expression of adiponectin, an insulin-sensitizing adipocytokine, while inducing interleukin-6, a proinflammatory cytokine implicated in insulin resistance.

Discussion: These data suggest that the distinct metabolic side effect profiles of these PIs could be a consequence of their differential effects on adipocyte physiology.

Key words: lipodystrophy, adipocyte, insulin resistance, human immunodeficiency virus

Introduction

Highly active antiretroviral therapy has prolonged survival and reduced opportunistic infections in human immunodeficiency virus (HIV)¹-infected individuals. Complicating treatment of HIV has been the development of a syndrome of abnormal fat distribution, dyslipidemia, and insulin resistance (1). These metabolic derangements increase the risk of cardiovascular disease in people treated for HIV (2–5). The development of this lipodystrophy syndrome has been most closely linked with the protease inhibitor (PI) class of anti-HIV agents, although the nucleoside reverse transcriptase inhibitor, d4T, has also been implicated. As effective antiretroviral drugs become more globally available, the prevalence of this syndrome will likely increase. A greater understanding of the mechanisms underlying the development of this syndrome could lead to safer antiretrovirals and could also inform our understanding of metabolic abnormalities in non-HIV-infected individuals such as those with the metabolic syndrome, obesity, or type 2 diabetes.

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¹ Nonstandard abbreviations: HIV, human immunodeficiency virus; PI, protease inhibitor; PPAR- γ , peroxisome proliferator-activated receptor- γ ; SGBS, Simpson Golabi Behmel Syndrome; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; IRS-1, insulin receptor substrate-1; IR β , insulin receptor β subunit; ELISA, enzyme-linked immunosorbent assay; RTqPCR, real-time quantitative polymerase chain reaction; PI3K, phosphoinositide-3-kinase; HPRT, hypoxanthine ribosyl transferase; IL, interleukin.

Several mechanisms have been proposed to explain how PIs cause the metabolic abnormalities of the lipodystrophy syndrome. In murine-derived adipocytes, impairments of insulin signaling at several points along the insulin-signaling pathway have been described (6–12). PIs have been shown to inhibit murine adipocyte differentiation and triglyceride accumulation and increase lipolysis (7,8,13,14). In subcutaneous fat from patients with HIV-associated lipodystrophy, increased apoptosis and decreased expression of sterol regulatory element binding protein 1, CAAT enhancer binding protein α , and peroxisome proliferator-activated receptor- γ (PPAR- γ) (15,16) have been described.

We used a human adipocyte cell line to study the effects of PIs on human adipocyte differentiation and function. We compared the effects of ritonavir and atazanavir, two PIs efficacious as antiretrovirals, yet differing widely in their propensity to induce clinical lipodystrophy. Here we show that ritonavir, a PI associated with clinical lipodystrophy, has profound effects on adipocyte differentiation and function. Atazanavir, associated with fewer metabolic abnormalities than other PIs (17–21), has remarkably attenuated effects on human adipogenesis, insulin sensitivity, and adipokine secretion compared with the more lipodystrophic PI. These data suggest that the distinct metabolic side effect profiles of these agents could be a consequence of their differential molecular effects within the adipocyte.

Research Methods and Procedures

Simpson Golabi Behmel Syndrome Cells

Simpson Golabi Behmel Syndrome (SGBS) human adipocytes were used for all experiments. These were originally derived from an individual affected with the Simpson Golabi Bemel syndrome as described previously (22). The preadipocytes were grown in growth medium containing Dulbecco's modified Eagle's medium (DMEM) F-12 (Invitrogen, Carlsbad, CA), 8 mg/liter biotin (Sigma, St. Louis, MO), 4 mg/liter pantothenic acid (Sigma), 1% penicillin-streptomycin (Invitrogen), and 10% bovine calf serum (Sigma). Media were changed twice a week. On reaching confluence, differentiation into mature adipocytes was induced using medium containing DMEM F-12, biotin, pantothenic acid, penicillin-streptomycin, 500 μ M isobutylmethylxanthine (Sigma), 20 nM dexamethasone (Sigma), 10 μ M cortisol (Sigma), 0.2 nM triiodothyronine (Sigma), 10 mg/liter apo-transferrin (Sigma), 20 nM recombinant human insulin (Sigma), and 2 μ M rosiglitazone. The cells were maintained in the induction medium for 7 days and maintained for an additional 9 to 11 days in adipogenic medium containing DMEM F-12, biotin, pantothenic acid, penicillin-streptomycin, cortisol, triiodothyronine, apo-transferrin, and insulin. All experiments were conducted on mature adipocytes at Days 16–18 of differentiation.

Adipogenesis

Adipocytes treated with vehicle, ritonavir, or atazanavir were stained for neutral lipid with Oil-red-O at Day 14 of differentiation and counterstained for nuclei with 4',6-diamidino-2-phenylindole. To determine cell adherence at 14 days, the number of nuclei per high-powered field was counted manually from four randomly chosen fields. Apoptosis at Day 14 was determined using the Cell Death Detection Assay (Roche, Basel, Switzerland) according to the manufacturer's instructions. PIs were obtained from the NIH AIDS Reagent Program. The vehicle for ritonavir was ethanol and for atazanavir was dimethyl sulfoxide. In all experiments, all treated cells received one or both vehicles consisting of 0.1% ethanol and/or 0.1% dimethyl sulfoxide as appropriate.

2-Deoxyglucose Uptake

Cells were incubated for 18 hours in PI or vehicle in medium containing 5 mM 2-deoxyglucose. Cells were washed twice and incubated in a buffered solution of 130 mM NaCl, 4.6 mM KCl, 2.5 mM NaH₂PO₄, 10 mM MgSO₄, 2.5 mM CaCl₂, and 10 mM HEPES, with insulin or phosphate-buffered saline (PBS) for 10 minutes. ³H labeled 2-deoxyglucose was added, and the cells were incubated for a further 60 minutes. The reaction was halted, and cells were washed in ice cold PBS. The cells were solubilized in PBS with 10% sodium dodecyl sulfate (SDS), and radioactivity was determined. Results are expressed as counts per minute normalized to protein content and duration of insulin stimulation.

Immunoprecipitation

Mature adipocytes were incubated for 18 hours in 5 mM glucose medium containing ritonavir, atazanavir, or vehicle. Co-incubation with 100 μ M sodium orthovanadate was done in some experiments. Cells were harvested after 10 minutes of insulin (1 nM) or PBS treatment. Cells were washed in cold PBS and lysed in a buffered solution of 150 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA (Invitrogen), a complete PI tablet (Roche), 4 mM sodium orthovanadate (Sigma), 10% glycerol, 200 mM sodium fluoride (Sigma), and 1% Triton x-100 (Sigma). The lysate was centrifuged at 14,000 rpm for 15 minutes, and the lipid phase was removed. Protein content was quantified with the BCA Protein Assay (Pierce Biotechnology, Inc., Rockford, IL). Peptides of interest were immunoprecipitated using 200 μ g of cell lysate per 2 μ g of antibody to insulin receptor substrate-1 (IRS-1; Upstate, Charlottesville, VA) or insulin receptor β subunit (IR β ; BD Biosciences, San Jose, CA) and mixed at 4 °C for 2 hours. Fifty microliters of a 50% slurry of protein A agarose (Invitrogen) was added, and the reaction was mixed for an additional 2 hours at 4 °C. The proteins were separated by SDS-polyacrylamide gel electrophoresis before immunoblotting.

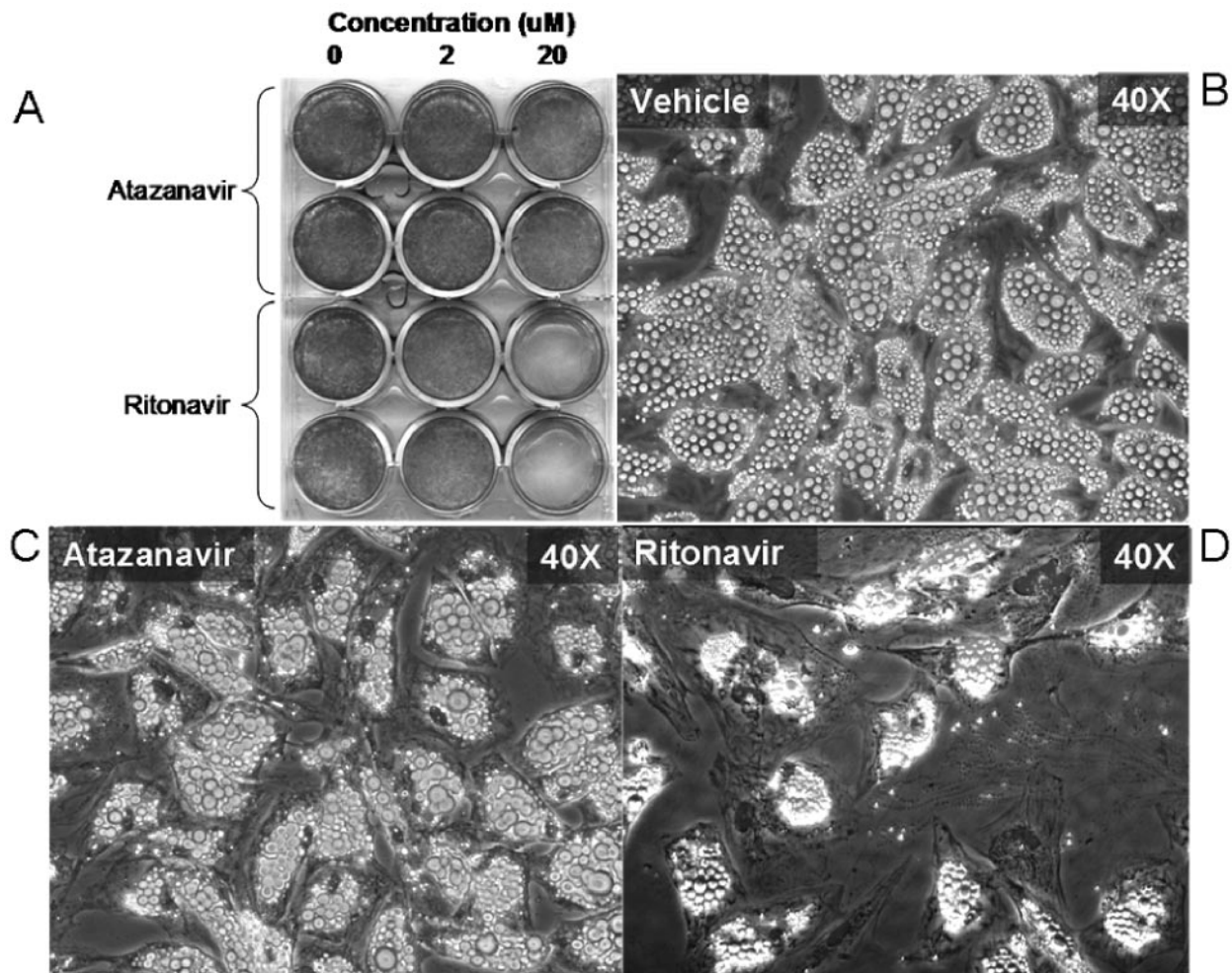


Figure 1: The effect of PIs on differentiation of SGBS human adipocytes. SGBS cells grown to confluence were treated with differentiation medium and vehicle, atazanavir, or ritonavir for 14 days at the concentrations shown. At Day 14, cells were stained with Oil-red-O (A). Cells treated with vehicle (B), 20 μ M atazanavir (C), or 20 μ M ritonavir (D) were stained with both Oil-red-O and 4',6-diamidino-2-phenylindole.

Protein Analysis

Proteins were transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA) in a buffer containing 50 mM Tris, 380 mM glycine, 10% SDS, and 20% methanol. Membranes were blocked and blotted according to the instructions from each antibody manufacturer. The antibodies used were for phosphotyrosine (Upstate and Zymed, Carlsbad, CA), phosphoinositide-3-kinase (PI3Kp85; Upstate), IRS-1, and IR β . Adiponectin (also called acrp30) secretion was assessed by enzyme-linked immunosorbent assay (ELISA) (B-Bridge International, Inc., Sunnyvale, CA).

Real-time Quantitative Polymerase Chain Reaction

RNA was purified from adipocytes with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). cDNA was generated using the First Strand Synthesis System for RT-

PCR (Invitrogen). Primers and probes for real-time quantitative polymerase chain reaction (RTqPCR) gene expression studies were proprietary oligonucleotides available commercially (Applied Biosystems, Foster City, CA). Gene expression results were normalized to the endogenous control gene *hypoxanthine ribosyl transferase (HPRT)*, which, through our testing, was found to be an abundant gene product that did not change in our experimental conditions (data not shown). All PCR reactions were carried out using Taqman Universal Polymerase Master Mix (Applied Biosystems) and the PRISM 7900 instrument (Applied Biosystems). Changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method (23). The results of RTqPCR and the ELISA assays were compared using a two-sample Student's *t* test, with $p < 0.5$ used to determine statistical significance.

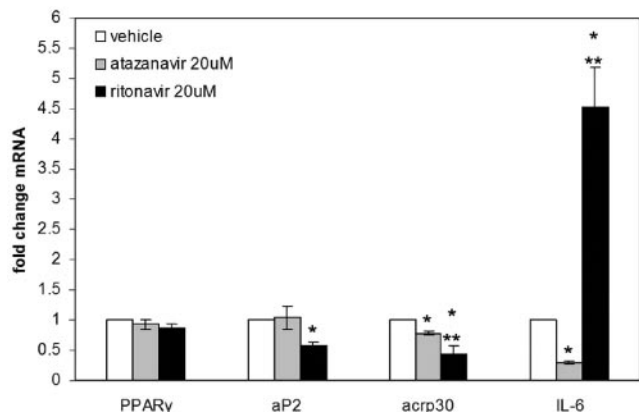


Figure 2: The effect of PIs on gene expression during adipogenesis. Confluent human preadipocytes were treated with differentiation medium supplemented with 20 μ M atazanavir, 20 μ M ritonavir, or vehicle. Gene expression was determined by RTqPCR. Results shown are after correction for levels of the endogenous housekeeping gene *HPRT* and normalized to vehicle-treated cells. * $p < 0.03$ vs. vehicle; ** $p < 0.05$ vs. atazanavir. Results from four experiments are shown.

Results

PI-specific Effects on Human Adipogenesis

Previous studies of PI effects on adipocytes have relied mainly on rodent models. We studied the effects of ritonavir and atazanavir in the novel SGBS cell model of human adipogenesis (22). SGBS cells can be readily differentiated to multilocular adipocytes in vitro (22) (Figure 1B). Ritonavir inhibited lipid accumulation at concentrations of 2 and 20 μ M (Figure 1A and B), both of which are concentrations relevant to the therapeutic levels observed in patients. For example, in one study of ritonavir kinetics (generally dosed at 600 mg twice daily), the range of peak and trough plasma ritonavir concentrations were 22 to 37 and 11 to 18 μ M, respectively (24). The common current practice of using ritonavir as a boosting agent suggests that many patients are still chronically exposed to lower levels of this drug. In contrast, atazanavir only mildly inhibited lipid accumulation and only at a dose higher than what is generally observed in clinical practice ($C_{max} = 4.5 \mu$ M) (25). The reduction in lipid accumulation by ritonavir correlated with a 50% reduction in gene expression of adipocyte-specific genes aP2 and adiponectin (Figure 2). Surprisingly, expression of PPAR- γ was not reduced by ritonavir, suggesting that the effect of this PI may occur relatively late in adipocyte differentiation (26).

PI-specific Proapoptotic Effects

In addition to the reduced lipid accumulation per cell, ritonavir treatment significantly reduced cell number in a manner that was not observed with atazanavir (Figures 1

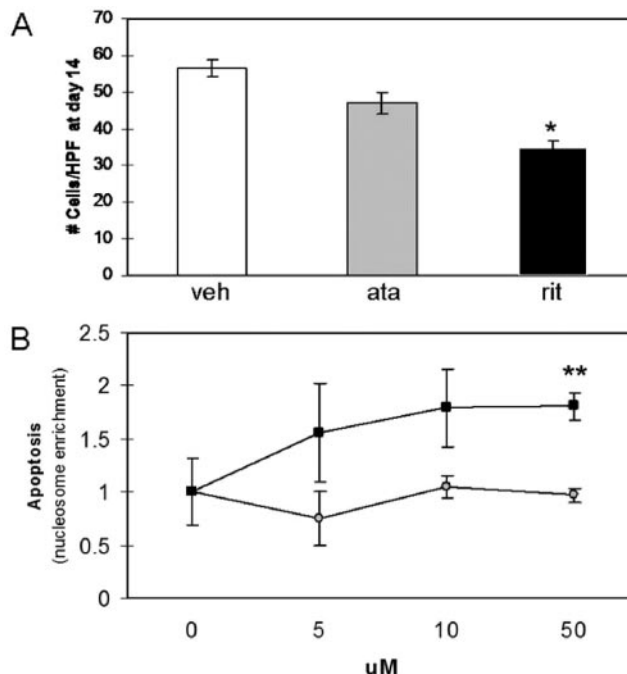


Figure 3: Effect of PIs on cell viability. (A) Human adipocytes were treated with vehicle (veh) or 20 μ M of atazanavir (ata) or ritonavir (rit) during differentiation. Cells per high-powered field (HPF) were counted to grossly assess toxicity of the agents. Ritonavir, but not atazanavir, caused a significant decrease in cell number. (B) Mature adipocytes were incubated for 18 hours in ritonavir (■) or atazanavir (○) at the indicated doses. Apoptosis was assessed by quantification of nucleosome-associated DNA fragments and normalized to vehicle-treated cells. Results from three experiments are shown. * $p = 0.0004$; ** $p = 0.01$

and 3A). The reduced cell number suggested that ritonavir might be pro-apoptotic in adipocytes. Indeed, ritonavir, but not atazanavir, induced apoptosis in mature adipocytes at a concentration of 50 μ M (Figure 3B).

PI-specific Effects on Insulin Action in Mature Human Adipocytes

The above studies focused on effects of PIs on adipocyte differentiation and lipid accumulation. We next studied the effect of PIs on mature adipocytes. Ritonavir blunted insulin-stimulated 2-deoxyglucose uptake into cultured human adipocytes (Figure 4), similar to earlier observations in PI-treated murine adipocytes (7–9,27). Atazanavir, however, did not inhibit insulin-stimulated glucose uptake in human adipocytes (Figure 4), an observation similar to one made recently in mouse adipocytes (28).

To further study the effect of the PIs on insulin-stimulated glucose uptake, we examined insulin signaling events in human adipocytes. Ritonavir had little or no effect on insulin-stimulated IR β autophosphorylation at either a low (5 μ M) or high (50 μ M) concentration of drug (Figure 5A).

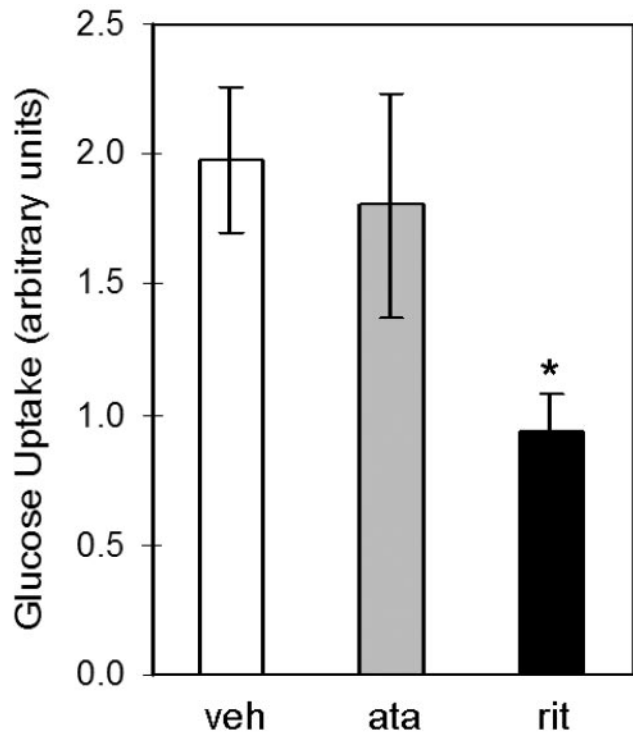


Figure 4: Ritonavir inhibits insulin-stimulated glucose uptake in human adipocytes. Insulin stimulation of 2-deoxyglucose uptake was lower in 50 μM ritonavir-treated cells (0.93 ± 0.15 units) than in vehicle (open bar; 1.98 ± 0.28 units) or 50 μM atazanavir-treated cells (gray bar; 1.8 ± 0.43 units). Results from five experiments are shown. * Ritonavir vs. vehicle: $p = 0.003$; ritonavir vs. atazanavir: $p = 0.05$.

However, ritonavir treatment did cause a decrease in insulin-stimulated tyrosine phosphorylation of IRS-1 (Figure 5B). In contrast, at the same doses, atazanavir had minimal effects on IRS-1 phosphorylation. The inhibitory effect of ritonavir on insulin-stimulated IRS-1 phosphorylation was also seen in adipocytes treated with the tyrosine phosphatase inhibitor sodium orthovanadate (Figure 5C), suggesting that ritonavir did not activate a protein tyrosine phosphatase (29). Because serine phosphorylation of IRS-1 can inhibit its own insulin-stimulated tyrosine phosphorylation, we also found that ritonavir did not alter phosphorylation of serines 312, 616, and 636/639 (data not shown). However, consistent with the effect of ritonavir being at the level of IRS-1 phosphorylation, we observed a similar decrease in association of the PI3K p85 subunit with IRS-1 (Figure 5D) in the cells treated with ritonavir but much less markedly in those treated with atazanavir.

PI-specific Effects on Adipocytokine Secretion

Patients with HIV lipodystrophy syndrome have increased circulating levels of inflammatory cytokines such as interleukin (IL)-6 (30,31), which have been implicated in insulin resistance, and decreased levels of the insulin-sensitizing adipocytokine adiponectin (31–34). Both IL-6 (35–37) and adiponectin (38) have been shown to modulate insulin signaling and glucose homeostasis. Long-term treatment of adipocytes with 20 μM ritonavir dramatically induced IL-6 gene expression, whereas atazanavir reduced expression (Figure 2). Treatment for 18 hours with 50 μM ritonavir caused a 3-fold increase in IL-6 expression ($p = 0.01$), whereas there was no change with atazanavir (data

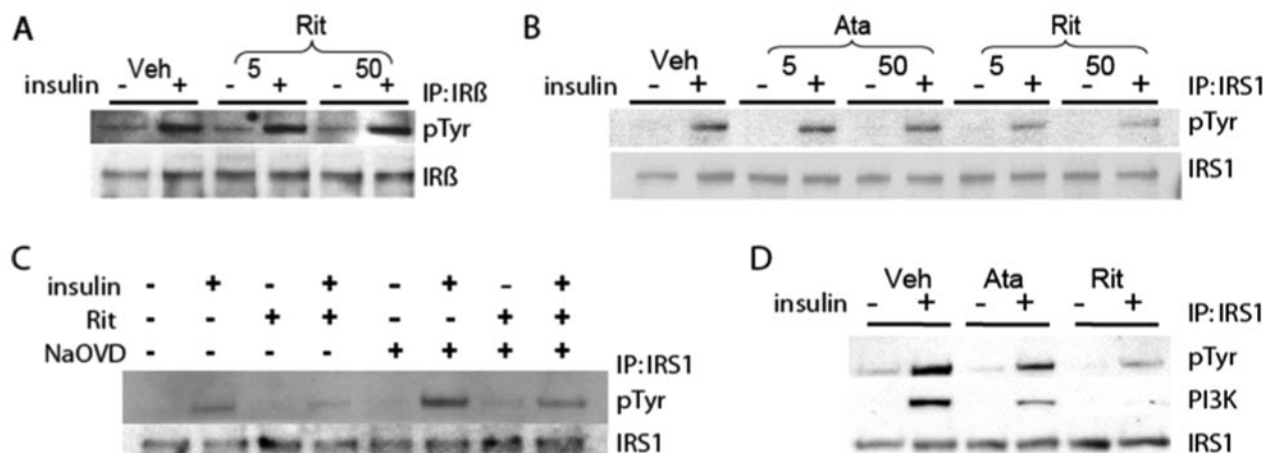


Figure 5: Ritonavir inhibits early steps in insulin signaling to a greater extent than atazanavir. Mature adipocytes were treated with vehicle, ritonavir, or atazanavir at the indicated concentrations. Cells were stimulated with insulin 1 nM for 10 minutes before immunoprecipitation and immunoblotting as indicated. (A) No effect of ritonavir on insulin-stimulated tyrosine phosphorylation of IR β . (B) Ritonavir (5 and 50 μM) inhibits insulin-stimulated tyrosine phosphorylation of IRS1. (C) The presence of a tyrosine phosphatase inhibitor does not ameliorate inhibition of insulin signaling by ritonavir 50 μM . (D) Ritonavir 50 μM also inhibits PI3Kp85 association with IRS1. Representative blots from at least three experiments are shown.

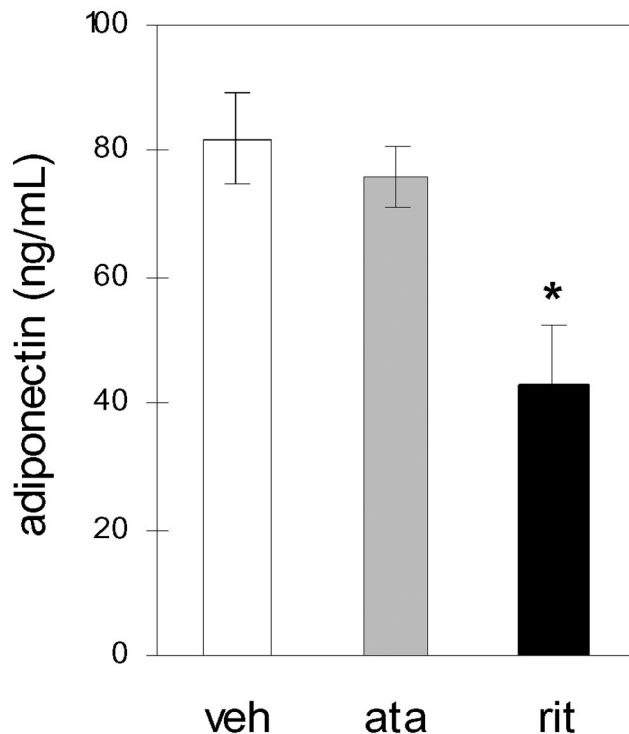


Figure 6: Ritonavir inhibits adiponectin secretion in human adipocytes. Cells were incubated for 18 hours in vehicle (open bar), 50 μ M atazanavir (gray bar), or 50 μ M ritonavir (black bar). Adiponectin secretion into the media was assessed by ELISA. Results from three experiments are shown. * $p = 0.017$.

not shown). Tumor necrosis factor- α was not detectable by RTqPCR analysis in these human adipocytes before or after PI treatment (data not shown). However, ritonavir reduced adiponectin gene expression (Figure 2) and secretion (Figure 6). In contrast, atazanavir had minimal effect on adiponectin expression or secretion (Figures 2 and 6).

Discussion

Atazanavir use has been associated with a decrease in hyperlipidemia (20,21), less insulin resistance (18,39), and reversal of lipodystrophy (19). Given the increased risk of cardiovascular morbidity and mortality with PI-inclusive anti-retroviral regimens (2–5), these advantages could make atazanavir useful for treating HIV patients with the highest burden of cardiovascular risk factors. We compared atazanavir and ritonavir in vitro to better understand the molecular basis for the favorable metabolic side effect profile associated with the former.

The lipodystrophy syndrome associated with anti-HIV PI use is characterized by lipoatrophy of subcutaneous adipose stores and accumulation of fat in the visceral compartment (40–42). This pattern of fat redistribution is associated with low adiponectin, insulin resistance, and dyslipidemia

(32,33,42,43). We found that ritonavir inhibited the expression of the PPAR- γ target gene *aP2* and markedly decreased lipid accumulation into differentiating adipocytes. Others have found similar results using different concentrations of ritonavir, different protease inhibitors, and in other cell systems (13,14,44,45). In contrast to ritonavir, we found that atazanavir had a much weaker inhibitory effect on lipid accumulation in differentiating human adipocytes and did not block *aP2* expression.

In addition to blocking adipogenesis, ritonavir also induced apoptosis in mature adipocytes, whereas atazanavir had no effect. While not all studies have indicated a role for apoptosis in HIV lipodystrophy (46), there is evidence supporting fat apoptosis as a mediating step in the pathogenesis of HIV lipodystrophy (16,47,48).

We have shown that ritonavir inhibits insulin-stimulated tyrosine phosphorylation of IRS-1 and, more distally, the recruitment of the PI3Kp85 subunit to IRS-1, as well as insulin-stimulated glucose uptake. In contrast, atazanavir had only minimal effects on proximal insulin signaling events and no discernible impact on glucose uptake. Interestingly, tyrosine autophosphorylation of IR β was not affected by ritonavir, suggesting that the disturbance of insulin signaling occurs at the level of IRS-1 tyrosine phosphorylation. The inhibition of IRS-1 phosphorylation was not mediated by increased tyrosine phosphatase activity or increased serine phosphorylation of IRS-1. This differential effect on insulin signaling could contribute to the propensity of ritonavir but not atazanavir to cause adipose-specific insulin resistance, which could accelerate depletion of subcutaneous fat stores (49).

Previous studies of insulin resistance in HIV-associated lipodystrophy syndrome have posited an interference with glucose transporter type 4 (8,9,11,50) or disrupted activation of Akt (51) as potential causes of insulin resistance in protease inhibitor-treated fat cells. Tyrosine phosphorylation of IRS-1 has been reported to be unaffected by PIs (7,9). In contrast, we found a clear decrease in early insulin signaling events beginning with IRS-1 tyrosine phosphorylation. The contrast with our results could be caused by differences in experimental conditions, including our use of adipocytes of human rather than murine origin and the use of lower concentrations of both PIs and insulin. It is also possible that PIs incite multiple disruptions in adipocytes. It has been shown, however, that PIs can accumulate within adipocytes and alter intracellular signaling and gene expression (52).

The inhibition of insulin signaling by PIs could be mediated by IL-6. We showed that expression of IL-6 is induced by ritonavir but not atazanavir. This parallels the increased IL-6 expression observed within the fat of HIV patients with lipodystrophy syndrome (16,53,54) and in murine adipocytes treated with PIs (47,54). IL-6 can block insulin signaling in adipocytes (35,36), potentially through a

decrease in insulin-stimulated IRS-1 tyrosine phosphorylation. The role of IL-6 as a mediator of insulin resistance in the HIV-associated lipodystrophy syndrome is an area requiring further study.

Adiponectin secretion was decreased by ritonavir but was unaffected by atazanavir. To our knowledge, a differential effect of atazanavir on adiponectin secretion has not been reported in the published literature. Low adiponectin levels may contribute to the systemic insulin resistance, inflammatory activation, and increased atherosclerotic disease (55) observed in patients with HIV-associated lipodystrophy. It has also been reported that adiponectin may act in an autocrine manner, enhancing insulin signaling, reducing synthesis of inflammatory cytokines, and promoting adipogenesis and lipid accumulation (56,57). Our findings raise the possibility that atazanavir's clinical benefits could be mediated by higher levels of adiponectin acting in a systemic and autocrine fashion.

In summary, we have used a newer HIV protease inhibitor, atazanavir, to delineate mechanisms by which protease inhibitors alter adipocyte metabolism. We have shown that atazanavir, compared with ritonavir, causes less inhibition of adipocyte differentiation and insulin-stimulated glucose uptake, does not induce apoptosis or an inflammatory response, and does not impair adiponectin secretion in mature adipocytes. These properties could underlie the favorable metabolic side effect profile of atazanavir observed in its clinical use.

Acknowledgments

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