Short communication

Effects of ritonavir-boosted darunavir, atazanavir and lopinavir on adipose functions and insulin sensitivity in murine and human adipocytes

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Background: Ritonavir-boosted protease inhibitors (PIs) could adversely affect metabolism and adipose tissue to different extents, depending on the molecule. Using drugs with minimal adverse metabolic effects is an important consideration in at-risk HIV-infected patients. In vitro adipocyte models can be useful for comparing the effects of different PIs.

Methods: We compared the effects of darunavir, darunavir/ritonavir, atazanavir/ritonavir and lopinavir/ritonavir in murine and human adipocytes on differentiation, mitochondrial function, reactive oxygen species (ROS) production and insulin sensitivity.

Results: In human and murine adipocytes, differentiation evaluated by lipid content and protein expression of adipogenic markers, mitochondrial function evaluated by aggregation of the cationic dye JC-1 and by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide lysis, and mitochondrial mass evaluated by MitoTracker fluorescence and the expression of mitochondrial proteins were unaffected by darunavir, mildly affected by darunavir/ritonavir and further altered by atazanavir/ritonavir and lopinavir/ritonavir. ROS production was unaltered by darunavir and darunavir/ritonavir but was increased by lopinavir/ritonavir and atazanavir/ritonavir. Regarding insulin sensitivity, darunavir and darunavir/ritonavir had no significant effect on insulin activation of protein kinase B (Akt/PKB) and MAP kinase and of glucose transport, whereas lopinavir/ritonavir and atazanavir/ritonavir partly impaired the effect of insulin. The effect of atazanavir/ritonavir was generally milder than that of lopinavir/ritonavir.

Conclusions: The various PIs differentially modified adipocyte functions. Darunavir alone did not affect adipocyte functions and only modestly altered differentiation and mitochondrial function when associated with ritonavir. Lopinavir/ritonavir adversely affected differentiation and lipid content, mitochondrial function, ROS production and insulin sensitivity, and the effect of atazanavir/ritonavir was intermediate. Thus, in vitro, darunavir/ritonavir presented a safer metabolic profile on adipocytes than atazanavir/ritonavir and lopinavir/ritonavir.

Introduction

Protease inhibitors (PIs) are still widely used to control HIV infection. Because of the inhibitory effect of ritonavir (RTV) on cytochrome CYP3A4 activity, most PIs are used in combination with a boosting concentration of RTV. Although first-generation PIs, including full-dose ritonavir, exerted major adverse effects on insulin sensitivity, lipid parameters and probably on adipose tissue function and repartition, the effect of currently used PIs is less obvious. Several PIs have, however, been associated with an increased dyslipidaemic risk, only partly explained by their dyslipidaemic effect [1,2].

The PI effects on lipid parameters are markedly dependent upon the PI used [3]. In healthy volunteers, RTV-boosted lopinavir (LPV/r) increased low-density lipoprotein (LDL) cholesterol and triglyceride levels after 5 days, whereas atazanavir (ATV) had no effect [4]. In HIV-infected patients, RTV-boosted atazanavir (ATV/r) and darunavir (DRV/r) had minimal effects on lipid parameters [3]. In healthy volunteers, ATV/r and DRV/r only slightly and similarly increased LDL cholesterol and triglycerides and decreased high-density lipoprotein (HDL) cholesterol, these effects being mainly related to the RTV boost [5]. Importantly, a once-daily dosing of
Adipose cell differentiation

Adipose cell differentiation was evaluated by cell lipid accumulation and by the protein expression of adipose cell differentiation markers. Briefly, cells cultured in 12-well plates were fixed in 10% neutral buffered formalin solution and stained with Oil red O. Adipocyte differentiation markers were identified by antibodies directed against CCAAT/enhancer binding protein-α (C/EBPα; antibody sc-61), peroxisome proliferator-activated receptor-γ (PPARγ; antibody sc-7196) and adipocyte protein-2 (aP2; antibody sc-18661) all from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), and fatty acid synthase (FAS; antibody 3180) from Cell Signaling Technology, Inc. (Danvers, MA, USA); β-actin (A5441; Sigma-Aldrich, Saint Quentin-Fallavier, France) was used as a control of protein loading.

Lipolysis

Lipolysis was evaluated at D0 and D5 of the PI treatment by the release of glycerol into the 3-day culture medium by using the free glycerol reagent kit (F6428; Sigma-Aldrich). The staining was measured at 540 nm and the results were expressed as nmol glycerol secreted per μg of cellular protein and normalized to Oil red O staining.

Mitochondrial function

Mitochondrial function was estimated by the aggregation of the cationic dye JC-1 (T-3168; Molecular Probes, Eugene, OR, USA), an indicator of mitochondrial membrane potential [15] and by protein expression on western blot of cytochrome oxidase-2 (COX-2; antibody sc-23983, Santa Cruz Biotechnologies). The reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; Sigma-Aldrich; 250 μg/ml) was also used as an index of mitochondrial function. After 1 h at 37°C, MTT staining was solubilized in DMSO and quantified at 540 nm. Mitochondrial mass was measured by the fluorescent labelling of the MitoTracker Red 580 probe (MTR; M-22425, Molecular Probes) normalized to the DNA content [16] and by the protein expression of COX-4 (antibody MS-408; Mitosciences, Eugene, OR, USA) and porin (PC548; Calbiochem, Merck KGaA, Darmstadt, C-6827 Germany); β-actin was used as an index of protein loading.

Reactive oxygen species production

Reactive oxygen species (ROS) production was assessed by the oxidation of the CM-H2DCFDA derivatives (5-[and 6]-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; Molecular Probes) and by the reduction of nitroblue tetrazolium (NBT) [11].

Insulin sensitivity

The effect of insulin on protein kinase B (Akt/ PKB) or extracellular-regulated kinase (ERK) activation was determined by western blot on whole-cell lysates from FCS-depleted cells stimulated for 10 min with insulin (100 nmol/l) using antibodies directed against Akt and p-Akt (sc-8313 and sc-7985-R; Santa Cruz Biotechnology) or ERK 1 and p-ERK (sc-93 and sc-7383; Santa Cruz Biotechnology).

Glucose transport was measured by incubating the cells for 2 h in glucose transport solution (pH 7.6) containing HEPES (12.5 mmol/l), NaCl (120 mmol/l), KCl
(5 mmol/l), MgSO₄ (1.2 mmol/l), CaCl₂ (1 mmol/l), NaHPO₄ (1 mmol/l), sodium pyruvate (2 mmol/l) and 2% BSA, with or without cytochalasin B (5 μmol/l). Insulin (100 nmol/l) and a mix containing 0.2 mmol/l 2-deoxy-glucose and 9.25 kBg 2-deoxy-[1-14C]glucose (2.2 G bq/mmol; GE-Healthcare, Saclay, France) were added successively for 30 and 5 min. Cells were washed with PBS, solubilized for 30 min with 0.1% SDS and counted. Insulin stimulation was expressed as a percentage of the basal value.

Statistical analysis
The experiments were generally repeated 3–5 times. Results are expressed as mean ±SEM relative to the protein or DNA content (MTR and ROS production). Statistical significance was determined using ANOVA and the Kruskal–Wallis non-parametric test, followed by a Fisher protected least significant difference test for pairwise differences. P-values of <0.05 were considered significant.

Results
Effect of PIs on adipocyte differentiation and lipid content
The adipocyte differentiation markers C/EBPα and PPARγ were increased in untreated murine (Figure 1A) and human (data not shown) adipocytes between D0 and D5 of the PI-treatment period, whereas the level of adipocyte markers FAS and aP2 were already maximal. The lipid content, as evaluated by Oil red O staining, increased by 1.5- and 2.2-fold in untreated murine and human adipocytes, respectively (Figure 1B).

At D5 of PI treatment, DRV did not alter the protein expression of the adipogenic markers, whereas ritonavir-boosted PIs decreased C/EBPα and PPARγ but not FAS and aP2 (Figure 1A). Lipid accumulation was unaffected by a 5-day treatment with DRV and DRV/r, whereas a 20–35% decrease was observed in murine and human adipocytes treated with ATV/r and LPV/r, respectively (Figure 1B). PI treatment did not alter the cellular protein level in murine or human adipocytes (Figure 1C), suggesting the absence of cell toxicity.

The level of glycerol released from the cells increased by 1.5-fold in the untreated control from D0 to D5. It was not increased by treatment with any PI as compared with the untreated control at D5 (data not shown), suggesting there was no involvement of lipolysis in the PI-induced decrease in lipid content.

Effect of PIs on mitochondrial functions
Mitochondrial function was evaluated by JC-1 aggregation (murine adipocytes), protein expression of the mitochondrial DNA encoded protein COX-2 [16] and MTT lysis. The mitochondrial mass was evaluated by MTR fluorescence and the expression of two mitochondrial constitutive proteins encoded by nuclear-DNA, COX-4 and porin.

DRV did not alter JC-1 aggregation, COX-2 expression, MTT lysis, MTR fluorescence or porin and COX-4 protein expression (Figure 2A–2D). DRV/r decreased MTT lysis by 30% but did not affect the other parameters. Conversely, LPV/r or ATV/r decreased JC-1 aggregation in murine adipocytes (by 44% and 38%, respectively), decreased MTT lysis by 50% and 35% (murine cells), and 35% and 27% (human cells), and increased porin and COX-4 expression and MTR fluorescence in murine and human adipocytes (Figure 2A–2D). These results indicated that ATV/r and LPV/r, but not DRV or DRV/r, induced mitochondrial dysfunction and a compensatory increase in mitochondria.

We then tested the ability of the PIs to increased ROS production by measuring the oxidation of CM-H₂DCFDA and the reduction of NBT. DRV or DRV/r did not modify ROS production, whereas LPV/r or ATV/r increased CM-H₂DCFDA oxidation and NBT reduction in murine and human adipocytes (Figure 2E and 2F).

Effect of PIs on insulin sensitivity
Insulin activation of the metabolic Akt/PKB and mitogenic MAP-kinase pathways was not significantly altered by DRV alone or in association with RTV. The other PI combinations exerted a mild effect on these insulin-signalling pathways (Figure 3A and 3B). LPV/r or ATV/r markedly impaired insulin activation of glucose transport whereas DRV or DRV/r had no effect, indicating that this test had a higher sensitivity to detect insulin resistance than Akt/PKB phosphorylation (Figure 3C).

Discussion
We show here that DRV/r exerted less adverse effects on cultured adipocytes than did ATV/r and LPV/r.

This differential effect could be related in part to differences in RTV concentrations. Indeed, in accordance with pharmacokinetic [13,14] and clinical data [5,12], the circulating concentration of RTV when associated with DRV was lower than with ATV or LPV (0.8, 1.3 and 1.4 μmol/l, respectively). Accordingly, healthy volunteers treated with DRV/r had lower lipid disorders than those treated with ATV/r [5]. In contrast, the daily dosage of 200 mg RTV is probably involved in the dyslipidaemic profile of LPV/r-treated patients.

It is important to consider the impact of recently marketed PIs on adipose tissue. Lipodystrophy remains a matter of concern in HIV-infected patients even if severe lipoatrophy phenotypes are less prevalent. In the ACTG5224s metabolic substudy of the
The ACTG5202 study, in which antiretroviral therapy (ART)-naive patients were initiated with ATV/r or efavirenz together with tenofovir/emtricitabine (TDF/FTC) or abacavir/lamivudine (ABC/3TC), 14–20% of patients lost >10% limb fat after 96 weeks, whatever the treatment [9]. In a French observational study evaluating 2,139 patients in 2009, peripheral lipatrophy was diagnosed in 26% and fat hypertrophy in 48% of patients who had been initiated into ART for <5 years [17]. Fat hypertrophy could result from

Figure 1. Effects of PIs on adipocyte differentiation and lipid content

Murine or human adipocytes taken 3 or 8 days after the induction of differentiation (D0) were incubated with the indicated protease inhibitor(s) (PIs) for 5 days (D5). Data were collected at D0 and D5. (A) Adipocyte differentiation was assessed by the protein expression of the transcription factors CCAAT/enhancer binding protein-\( \alpha \) (C/EBP\( \alpha \)) and peroxisome proliferator-activated receptor-\( \gamma \) (PPAR\( \gamma \)) and the adipocyte markers fatty acid synthase (FAS) and adipocyte protein 2 (aP2) in murine adipocytes. \( \beta \)-Actin was used as a control of protein loading. (B) PI-treated or -untreated cells were stained with Oil red O. The staining was assessed at 540 nm and normalized to the protein content evaluated in parallel. (C) Total cell protein level was assessed in 12-well plates. Experiments were repeated 3–5 times. Representative blots are shown. Results are means ±SEM. *\( p < 0.05 \) versus control untreated cells. ATV/r, atazanavir boosted with ritonavir; DMSO, dimethyl sulfoxide; DRV, darunavir; DRV/r, darunavir boosted with ritonavir; LPV/r, lopinavir boosted with ritonavir; OD, optical density.
Figure 2. Effects of PIs on mitochondrial function and ROS production

Murine or human adipocytes were incubated with the indicated protease inhibitors (PIs) for 5 days. (A) Mitochondrial membrane potential was evaluated by the aggregate/monomer ratio of JC-1 (a cationic dye) fluorescence in murine adipocytes. (B) Mitochondrial markers were evaluated by western blot. Representative blots are shown. β-Actin was used as a control for protein loading. (C) 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) reduction was performed as indicated in Methods. Results are normalized to the cellular protein content. (D) The mitochondrial mass was estimated by the fluorescent labelling of MitoTracker Red 580 probe (MTR) normalized to the DNA content. (E&F) Reactive oxygen species (ROS) production was assessed by the oxidation of 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) derivatives and the reduction of nitroblue tetrazolium (NBT) and normalized to the DNA and the protein content, respectively. Experiments were repeated 3–5 times. Results are means ± SEM. *P<0.05 versus control untreated cells. ATV/r, atazanavir boosted with ritonavir; COX-2, cytochrome oxidase-2; COX-4, cytochrome oxidase-4; DMSO, dimethyl sulfoxide; DRV, darunavir; DRV/r, darunavir boosted with ritonavir; LPV/r, lopinavir boosted with ritonavir; OD, optical density.
the effect of some PIs able to induce mitochondrial dysfunction [8]. Indeed, in situations of human and murine obesity, adipocyte hypertrophy has been linked to decreased mitochondrial function and/or mtDNA level [8] suggesting that mild mitochondrial dysfunction and increased oxidative stress could result in increased fat amount. PIs can adversely affect mitochondrial function [18]. Importantly, in ART-naive patients, ATV and efavirenz increased visceral abdominal fat (VAT) and total abdominal fat (TAT). However, the mean percentage increases from baseline in VAT, subcutaneous abdominal adipose tissue (SAT) and TAT were higher in the ATV than the efavirenz group [19]. Accordingly, in the ACTG5224s substudy, patients given ATV/r presented a greater gain in trunk fat than those receiving efavirenz [9]. In the MONOI and MONET studies, controlled HIV-infected patients switched to DRV/r presented an increased amount of trunk fat in the two arms after 48 or 96 weeks [10,20]. In the METABOLIK

Figure 3. Effects of PIs on insulin signalling

Murine or human adipocytes were incubated with the indicated protease inhibitor (PI) for 5 days. (A&B) Fetal calf serum (FCS)-depleted cells were incubated with insulin (100 nmol/l) for 10 min. Insulin stimulation of protein kinase B (Akt/PKB) and extracellular-regulated kinase (ERK) was measured on whole cell lysates by western blot. Representative experiments are shown. (C) Insulin-stimulated glucose transport in murine and human adipocytes. Experiments were repeated 3–4 times. Results are means ±SEM. *P<0.05 versus control untreated cells. ATV/r, atazanavir boosted with ritonavir; DMSO, dimethyl sulfoxide; DRV, darunavir; DRV/r, darunavir boosted with ritonavir; LPV/r, lopinavir boosted with ritonavir.
study, ART-naive patients receiving DRV/r or ATV/r together with TDF/FTC presented increased TAT and SAT but peripheral adipose tissue did not increase in the ATV/r arm, indicating a differential effect of the two combinations on adipose tissue [21]. These data support a milder and differential effect of DRV/r versus ATV/r on fat.

In vitro models are useful for deciphering the potential impact of drugs. Because even recently marketed PIs can modify adipose tissue, we comparatively evaluated the effects of these drugs on routinely used murine 3T3-L1 adipocytes but also on human adipocytes in primary culture, more relevant to the physiological situation. The results we obtained with the two cell systems are largely comparable and reveal the ability of LPV/r and, to a lesser extent, ATV/r to affect adipocyte differentiation and insulin sensitivity, and induce mitochondrial dysfunction and ROS production, whereas DRV and DRV/r have no or minimal effects.

In vitro studies have previously evaluated the ability of PIs to alter adipocyte function. LPV and RTV but not ATV increased oxidative stress leading to defective lipid storage [22]. Accordingly, in human subcutaneous and visceral adipocytes, RTV but not ATV affected differentiation [23]. In another study ATP/r (5/2 μmol/l) and LPV/r (10/2 μmol/l) altered adipocyte functions, arguing that the RTV boost has a major role in altering adipocyte function. Only one recent paper evaluated the effect of DRV on cultured adipocytes [24]. DRV and ATV did not alter lipid accumulation, in contrast to LPV and RTV, further stressing the safe profile of these studies reported the ability of ATV/r and LPV/r to induce insulin resistance in cultured adipocytes [11]. However, the RTV concentration was higher than in the present study. Accordingly, LPV and RTV but not unboosted ATV and DRV decreased insulin-activated glucose uptake into cultured adipocytes [24].

Regarding insulin sensitivity, our in vitro studies are in good agreement with data obtained in vivo with healthy volunteers [4,5,7]. Previous in vitro studies reported the ability of ATV/r and LPV/r to induce insulin resistance in cultured adipocytes [11]. However, the RTV concentration was higher than in the present study. Accordingly, LPV and RTV but not unboosted ATV and DRV decreased insulin-activated glucose uptake into cultured adipocytes [24].

We acknowledge some limitations. We have not evaluated human fat samples or adipocytes from SAT and VAT in particular from HIV-infected patients. Some PIs are strongly linked to serum proteins which can modify their effective concentration in a different way than in culture media containing 3% or 10% FCS. In vivo studies in healthy volunteers evaluated the effect of ATV/r and DRV/r on lipid and glucose parameters but not on adipose tissue markers [5]. This should be considered.

In conclusion, we report here that DRV/r exhibited a safe profile on cultured adipocytes in good accordance with its safe metabolic profile in HIV-infected patients. This could be related to the booster RTV concentration being lower in DRV/r combinations.

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MC-D and JC were involved in conception of the study. EC and MA were involved in technical realization. EC, MA, MC-D and JC were involved in interpretation of the data, EC, MC-D and JC were involved in drafting of the article.

Disclosure statement

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