

Antiretroviral drugs with adverse effects on adipocyte lipid metabolism and survival alter the expression and secretion of proinflammatory cytokines and adiponectin *in vitro*

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Objective: The lipodystrophy syndrome is a major adverse effect of highly active antiretroviral therapy (HAART), associated with altered circulating levels and adipose tissue mRNA expression of proinflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor (TNF) α , and adiponectin. Proinflammatory cytokines and adiponectin, which are secreted by adipose tissue, regulate fat metabolism, insulin sensitivity and adipose cell apoptosis. We examined the direct effects of individual antiretrovirals on lipid metabolism and cytokine and adiponectin production by cultured adipocytes.

Methods: Differentiating 3T3-F442A cells and differentiated 3T3-L1 adipocytes were treated for 12 or 4 days, respectively, with protease inhibitors (PIs) indinavir, nelfinavir, amprenavir, lopinavir and ritonavir, or nucleoside reverse transcriptase inhibitors (NRTIs) stavudine and zidovudine, at near- C_{max} concentrations. Lipid metabolism was estimated by Oil Red O staining of intracellular lipids, mRNA expression of fatty acid synthase and adipocyte lipid binding protein 2, and insulin activation of lipogenesis. Apoptosis was estimated by flow cytometry. The expression and secretion of proinflammatory cytokines (IL-6, TNF α and IL-1 β) and adiponectin were evaluated by real-time reverse transcription PCR and ELISA.

Results: Chronic treatment of 3T3-F442A differentiating adipocytes and differentiated 3T3-L1 adipocytes with PIs and NRTIs reduced lipid accumulation, mRNA expression of lipid markers and insulin-induced lipogenesis. IL-6, TNF α , IL-1 β and adiponectin expression and secretion were markedly altered in differentiating 3T3-F442A adipocytes. PIs had either no effect on differentiated 3T3-L1 adipocytes (TNF α expression and secretion) or their effect was less marked than in 3T3-F442A cells. Indinavir and amprenavir did not alter cytokine secretion and expression by mature adipocytes. The effects of stavudine and zidovudine on differentiating and mature adipocytes were similar, despite the difference in treatment procedure. The drugs with the strongest effect on TNF α expression also increased adipocyte apoptosis, in contrast to the drugs that only moderately increased TNF α expression.

Conclusions: These results suggest that increased cytokine and decreased adiponectin secretion and expression induced by some PIs and NRTIs may contribute to the adipose tissue loss (via apoptosis and lipid leakage) and insulin resistance associated with the lipodystrophy syndrome.

Introduction

Many patients taking highly active antiretroviral therapy (HAART) combining protease inhibitors (PIs) and nucleoside analogue reverse transcriptase inhibitors (NRTIs) develop a lipodystrophy syndrome characterized by peripheral lipoatrophy and/or increased central adiposity, with or without metabolic abnormalities and insulin resistance [1–4]. The underlying mechanisms are unclear, but it has been suggested

that PIs are linked to insulin resistance and dyslipidaemia, while NRTIs promote lipoatrophy [2–4]. Some PIs rapidly induce metabolic abnormalities in healthy subjects [5,6] and some NRTIs can induce mitochondrial toxicity and apoptosis in patients' fat tissue [7–11]. The lipodystrophy syndrome is more frequent and more severe in patients treated with both PIs and NRTIs [2,12].

These antiretroviral drugs have been shown to have direct adverse effects on cultured adipocytes: some PIs (indinavir, nelfinavir and ritonavir) induce insulin resistance and alter lipid metabolism [13–16] and also reduce adipocyte differentiation and survival [13,15,17–19], thus leading to adipose cell depletion, while the thymidine analogues (stavudine and zidovudine) can alter lipid metabolism [17,18] and induce apoptosis.

Studies using transgenic murine models have provided strong evidence that altered fat distribution precedes insulin resistance and metabolic complications [20]. Adipose tissue is a major determinant of insulin resistance, particularly through the secretion of proinflammatory cytokines, tumour necrosis factor (TNF) α [21], interleukin-6 (IL-6) [22] and IL-1 β [23]. Some of the mechanisms whereby these cytokines inhibit insulin signalling have been elucidated [21,22,24,25]. TNF α , IL-6 and IL-1 β can interfere with adipose cell differentiation [21,26] and TNF α can increase adipocyte apoptosis [27], and these effects may be involved in HAART-induced lipodystrophy. Indeed, elevated circulating levels and mRNA expression of TNF α and IL-6 have been found in serum and adipose tissue of lipodystrophic patients on HAART. Circulating levels of IL-1 β are also increased in these patients [19,28–31]. Johnson *et al.* [31] recently reported that the adipose tissue of lipodystrophic patients secreted increased levels of TNF α .

Adiponectin is an adipocyte-secreted protein that acts through an endocrine mechanism on its target tissues (liver and muscle) and plays a positive role in insulin sensitivity [21]. In lipodystrophic and insulin-resistant patients on HAART, adiponectin circulating levels [28,32–36] and mRNA expression are reduced in adipose tissue [28,33]. Moreover, a recent mouse study [37] showed that ritonavir reduced the plasma adiponectin concentration and increased plasma triglyceride and free fatty acid levels, pointing to a role of adiponectin in the aetiology of the effects of PIs on lipid metabolism.

Here we examined whether long-term treatment of differentiating 3T3-F442A and differentiated 3T3-L1 adipocytes with PIs (indinavir, nelfinavir, amprenavir, lopinavir and ritonavir) and NRTIs (stavudine and zidovudine) near their respective C_{\max} values affected the secretion and mRNA expression of proinflammatory cytokines and adiponectin.

Materials and methods

Materials

Powdered forms of indinavir (Merck Sharp & Dohme Laboratories, Clermont Ferrand, France), nelfinavir (Agouron Pharmaceuticals, San Diego, Calif., USA),

amprenavir (Vertex Pharmaceuticals, Cambridge, Mass., USA), lopinavir, ritonavir (Abbott France, Rungis, France), stavudine (Bristol-Myers Squibb Virology, Princeton, NJ, USA) and zidovudine (GlaxoSmithKline, Marly-le-Roi, France) were provided by the manufacturers.

Cell culture and treatment

3T3-F442A and 3T3-L1 murine preadipocytes were cultured and induced to differentiate as previously described [13,22]. The effects of PIs and NRTIs were tested in long-term experiments on differentiating 3T3-F442A cells (from day –4 to +7 of differentiation) and differentiated 3T3-L1 adipocytes (from day +8 to +12 of differentiation) at near- C_{\max} values: indinavir 10 μ M, ritonavir 10 μ M, nelfinavir 10 μ M (see [18] for references), amprenavir 10 μ M [38], lopinavir 10 μ M [39], zidovudine 1 μ M [40] and stavudine 10 μ M [41].

Adipokine measurements

Murine IL-6, IL-1 β and TNF α concentrations were determined on day 8 and day 12 of differentiation in 24-h supernatants of 3T3-F442A and 3T3-L1 cells, respectively, using Quantikine Mouse sandwich ELISA kits (R&D Systems, Inc., Minneapolis, Minn., USA) with the following detection limits: IL-6: 3.1 pg/ml, IL-1 β 3.0 pg/ml and TNF α 5.1 pg/ml. Murine adiponectin concentrations were determined on day 8 and day 12 of differentiation in 24-h supernatants of 3T3-F442A and 3T3-L1 cells using the sandwich ELISA kit from B-Bridge International, Inc. (Sunnyvale, Calif., USA), which has a detection limit of 15.6 pg/ml.

Lipid metabolism

Lipid metabolism was assessed on day 8 (3T3-F442A cells) or day 12 (3T3-L1 adipocytes) of differentiation by measuring the mRNA expression of adipogenic markers [adipocyte lipid-binding protein 2 (aP2) and fatty acid synthase (FAS)] and by lipid staining with Oil Red O. Staining was quantified at 520 nm after solubilization in 10% SDS. The results are expressed as % \pm SEM of the untreated control values.

Lipogenesis was studied on day 8 (3T3-F442A cells) or day 12 (3T3-L1 adipocytes) of differentiation in cells cultured for 24 h in serum-free medium. Insulin (100 nmol/l) was added for 30 min and, after a further 30 min of incubation with 0.5 μ Ci/well [U - 14 C] glucose (302 mCi/mmol; Amersham Biosciences, Saclay, France), the cells were washed and labelled lipids were extracted with chloroform/methanol (1/2). After 30 min on ice, the lower phase was collected, evaporated and counted in scintillation fluid. The results are expressed as % \pm SEM of untreated control values.

RNA preparation and real-time RT-PCR

Total RNA was isolated with the RNeasy kit (Qiagen, Inc., Valencia, Calif., USA). Complementary DNA was synthesized using random hexamers and AMV reverse transcriptase (Promega Biosciences, Inc., San Luis Obispo, Calif., USA). Real-time PCR was performed with the LightCycler system (Roche Diagnostics, Meylan, France). PCR reactions were performed using the LightCycler SYBR green fluorophore. The following primers were used: IL-6, 5'-CTG CAA GAG ACT TCC ATC CAG TT-3' and 5'-GAA GTA GGG AAG GCC TGG-3'; IL-1 β , 5'-ACA CTC CTT AGT CCT CGG CCA-3' and 5'-CCA TCA GAG GCA AGG AGG AA-3'; TNF α , 5'-GAG GCA CTC CCC CAA AAG AT-3' and 5'-TGA TGA GAG GGA GGC CAT TT-3'; adiponectin, 5'-AAG GAC AAG GCC GTT CTC T-3' and 5'-TAT GGG TAG TTG CAG TCA GTT G-3'; aP2, 5'-AAC ACC GAG ATT TCC TT-3' and 5'-ACA CAT TCC ACC ACC AG-3'; FAS, 5'-TGC TCC CAG CTG CAG GC-3' and 5'-GCC CGG TAG CTC TGG GTG TA-3'; and 18S ribosomal RNA, 5'-GAG CGA AAG CAT TTG CCA AG-3' and 5'-GGC ATC GTT TAT GGT CGG AA-3'. Each sample was normalized on the basis of its 18S rRNA content.

Cell survival

Apoptosis was evaluated by flow cytometry. On day 8 (3T3-F442A cells) or day 12 (3T3-L1) of differentiation, cells were collected, fixed in ice-cold 70% ethanol at -20°C and then stained with 20 $\mu\text{g/ml}$ propidium iodide in the presence of 100 $\mu\text{g/ml}$ ribonuclease A for 30 min at 37°C in the dark. DNA content was analysed by flow cytometry (Facs Calibur; Becton Dickinson, Mountain View, Calif., USA). The results are expressed as % \pm SEM of apoptotic cells among total cells.

Statistical analysis

Results are means \pm SEM of the indicated number of independent experiments. Statistical significance was determined using the non-parametric Mann-Whitney U test. The threshold of significance was set at $P=0.05$.

Results

Effects of PIs and NRTIs on adipocyte lipid metabolism

We first investigated the effects of the drugs on lipid metabolism in differentiating 3T3-F442A cells and mature 3T3-L1 adipocytes. As shown by Oil Red O staining (Figure 1A), the PIs (indinavir, nelfinavir, lopinavir and ritonavir) and NRTIs (stavudine and zidovudine) significantly reduced lipid accumulation (by 25–40%) on day 8 in 3T3-F442A adipocytes (treated throughout the differentiation program). Amprenavir-treated cells had normal lipid levels. These results were in keeping with the lower mRNA expression of FAS and

aP2 (Table 1), two markers of lipid status, and with the reduced capacity of insulin to activate lipogenesis (Figure 1B) in PI- and NRTI-treated cells.

A 4-day treatment of mature 3T3-L1 adipocytes with PIs (excluding indinavir and amprenavir) and NRTIs also reduced cellular lipid levels (Figure 1A), FAS and aP2 mRNA expression (Table 1) and insulin-induced lipogenesis (Figure 1B). These results indicate that some PIs and NRTIs can alter the lipid metabolism of cultured adipocytes, regardless of their differentiation status.

Effect of PIs and NRTIs on proinflammatory cytokine secretion and expression

The PIs (except for amprenavir) increased the secretion of IL-6 (1.8- to 2.0-fold) and IL-1 β (1.8- to 1.9-fold) (Figure 2A, B) in 3T3-F442A adipocytes. The increases in secretion were closely matched by increases in expression (2.3- to 3.1-fold for IL-6 and 2.1- to 2.6-fold for IL-1 β) (Table 2). Indinavir and nelfinavir increased TNF α secretion (2.1- and 2.1-fold, respectively) (Figure 2C) and mRNA expression (2.7- and 3-fold, respectively) (Table 2) by 3T3-F442A adipocytes, whereas ritonavir and lopinavir had lesser effects on TNF α secretion (1.4- and 1.2-fold, respectively) (Figure 2C) and mRNA expression (1.9- and 1.4-fold increases, respectively) (Table 2). Chronic treatment with amprenavir altered neither TNF α secretion nor expression by 3T3-F442A differentiating adipocytes.

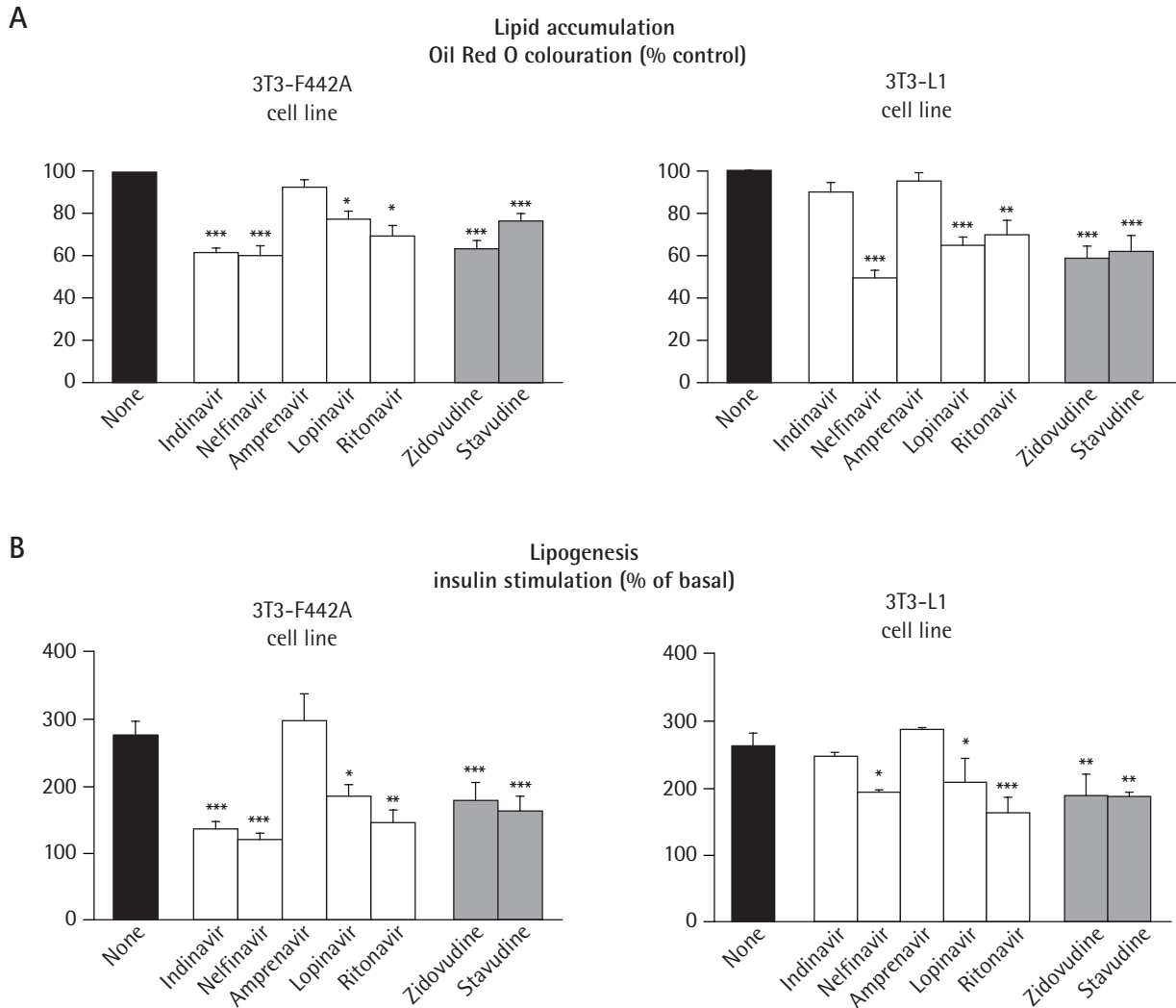
A 4-day PI treatment also affected cytokine secretion (Figure 2) and mRNA expression (Table 2) by mature 3T3-L1 adipocytes. The effects of nelfinavir, lopinavir, ritonavir and amprenavir on IL-6 and IL-1 β secretion and expression were similar to their effects on 3T3-F442A differentiating adipocytes. However, none of the PIs modified TNF α secretion or expression, and indinavir had no effect in 3T3-L1 adipocytes. Except for IL-6, the basal levels of cytokine secretion were in the same range in 3T3-F442A and 3T3-L1 adipocytes.

The thymidine analogues stavudine and zidovudine similarly altered proinflammatory cytokine secretion in differentiating 3T3-F442A and mature 3T3-L1 adipocytes, as follows: 1.8- to 2.7-fold for IL-6 (Figure 2A), 1.2- to 1.5-fold for IL-1 β (Figure 2B) and 1.5- to 2-fold for TNF α (Figure 2C). The increases in secretion were closely matched by increases in expression: 1.7- to 2.7-fold for IL-6, 1.4- to 2.8-fold for IL-1 β and 2.3- to 3.3-fold for TNF α (Table 2).

Thus, in contrast to the PIs, the NRTIs affected lipid metabolism and cytokine secretion and expression independently of adipocyte differentiation status.

Effect of PIs and NRTIs on adiponectin secretion and expression

As shown in Figure 3A, all the PIs tested (indinavir, nelfinavir, amprenavir, lopinavir and ritonavir)

Figure 1. Chronic effects of PIs and NRTIs on lipid metabolism in 3T3-F442A and 3T3-L1 adipocytes

Differentiating 3T3-F442A cells and fully differentiated 3T3-L1 adipocytes were treated with PIs (indinavir, nelfinavir, amprenavir, ritonavir and lopinavir 10 μ M) or NRTIs (zidovudine 1 μ M and stavudine 10 μ M). (A) On day 8 (3T3-F442A) or day 12 (3T3-L1) of differentiation, cells were fixed and stained with Oil Red O; staining was quantified at 520 nm and expressed as % \pm SEM of untreated controls. * P <0.05, ** P <0.01, *** P <0.001 versus control (black bars). (B) On day 7 (3T3-F442A) or day 11 (3T3-L1) of differentiation, cells were starved overnight and the effect of insulin on [14 C] glucose incorporation into lipids was evaluated as described in Materials and methods. The experiments were repeated three times on triplicate samples. The results were normalized to the protein content and expressed as % \pm SEM of the baseline value. * P <0.05, ** P <0.01, *** P <0.001 versus insulin-stimulated lipogenesis in untreated cells (black bars).

markedly reduced adiponectin secretion (by 50–95%) by differentiating 3T3-F442A adipocytes. Adiponectin mRNA expression fell by 15–70% in PI-treated cells (Figure 3B). A 4-day treatment of 3T3-L1 mature adipocytes with the PIs (except for amprenavir) also reduced adiponectin secretion and expression, albeit to a lesser extent (20–75 and 20–30%, respectively) (Figure 3, right panels).

Stavudine and zidovudine also reduced adiponectin secretion (by 75% and 70%, respectively) and expression (by 35% and 30%, respectively) by 3T3-F442A adipocytes (Figure 3, left panels). The effects were lower than those of indinavir, ritonavir and lopinavir

(90–95% reduction in secretion). NRTI treatment of mature 3T3-L1 adipocytes also inhibited adiponectin secretion (by 50–65%) and expression (by 20%) (Figure 3, right panels), indicating that the effects of NRTIs are independent of differentiation status.

Effect of PIs and NRTIs on adipocyte apoptosis

The drugs that markedly increased TNF α secretion (Figure 2C) and expression (Table 2) in 3T3-F442A cells (indinavir, nelfinavir, stavudine and zidovudine) also increased apoptosis, whereas those that moderately increased TNF α production (ritonavir, lopinavir and amprenavir) did not (Table 3). NRTIs, in contrast

Table 1. Chronic effects of PIs and NRTIs on the expression of the adipogenic markers FAS and aP2 in 3T3-F442A and 3T3-L1 adipocytes

| | 3T3-F442A cell line | |
|------------|----------------------------------|----------------------------------|
| | FAS mRNA level (% of control) | aP2 mRNA level (% of control) |
| None | 100 | 100 |
| Indinavir | 64.1 ± 5.1*** | 43.2 ± 4.1*** |
| Nelfinavir | 65.0 ± 6.7*** | 61.3 ± 3.1*** |
| Amprenavir | 96.3 ± 8.0 | 90.2 ± 8.9 |
| Lopinavir | 60.4 ± 14.3*** | 27.8 ± 2.7*** |
| Ritonavir | 85.4 ± 9.4*** | 60.2 ± 6.9*** |
| Zidovudine | 53.8 ± 7.0*** | 53.1 ± 6.9*** |
| Stavudine | 56.3 ± 5.4*** | 52.1 ± 9.4*** |

| | 3T3-L1 cell line | |
|------------|----------------------------------|----------------------------------|
| | FAS mRNA level (% of control) | aP2 mRNA level (% of control) |
| None | 100 | 100 |
| Indinavir | 94.5 ± 13.3 | 94.2 ± 11.0 |
| Nelfinavir | 59.5 ± 10.9*** | 67.3 ± 8.6*** |
| Amprenavir | 104.9 ± 12.4 | 93.4 ± 11.9 |
| Lopinavir | 72.9 ± 11.5* | 66.8 ± 9.5*** |
| Ritonavir | 76.9 ± 10.5* | 62.3 ± 11.7*** |
| Zidovudine | 61.0 ± 17.8* | 40.2 ± 7.8*** |
| Stavudine | 61.9 ± 7.4*** | 63.9 ± 11.4*** |

Total RNA was extracted and subjected to real-time PCR as described in Materials and methods. FAS and aP2 mRNA levels, normalized to 18S rRNA expression, were determined relative to untreated cells. Results are % ± SEM of control values in 6–15 experiments performed in duplicate. **P*<0.05, ****P*<0.001 versus control.

to PIs, induced apoptosis of mature 3T3-L1 adipocytes (Table 3), to an extent related to their capacity to increase TNFα production.

Discussion

We tested the individual effects of 4- to 12-day exposure to antiretroviral drugs on differentiating (3T3-F442A) and fully differentiated (3T3-L1) adipocytes in culture. With the exception of amprenavir, the PIs and the NRTIs markedly affected lipid metabolism, regardless of differentiation status, with decreased lipid accumulation and mRNA expression of the adipogenic markers FAS and aP2, and inhibition of insulin-induced lipogenesis. In contrast, the effects of indinavir were clearly dependent on differentiation status, as they were only observed with differentiating 3T3-F442A adipocytes [14].

Chronic treatment of 3T3-F442A and 3T3-L1 adipocytes with PIs or NRTIs led to increased secretion of the inflammatory cytokines IL-6, IL-1β and TNFα. Nelfinavir, ritonavir, lopinavir, stavudine and zidovudine

Table 2. Chronic effects of PIs and NRTIs on cytokine mRNA expression in 3T3-F442A and 3T3-L1 adipocytes

| | IL-6 mRNA level (% of control) | |
|------------|--------------------------------|-------------------|
| | 3T3-F442A cell lines | 3T3-L1 cell lines |
| None | 100 | 100 |
| Indinavir | 265.3 ± 14.1*** | 99.6 ± 13.6 |
| Nelfinavir | 299.8 ± 20.0*** | 224.4 ± 46.8** |
| Amprenavir | 110.6 ± 5.9 | 102.0 ± 11.3 |
| Lopinavir | 146.1 ± 23.7*** | 152.5 ± 30.4** |
| ritonavir | 194.5 ± 19.1*** | 88.3 ± 9.1 |
| Zidovudine | 274.1 ± 30.3*** | 196.9 ± 18.8*** |
| Stavudine | 169.3 ± 12.2*** | 171.0 ± 21.5*** |

| | IL-1β mRNA level (% of control) | |
|------------|---------------------------------|-------------------|
| | 3T3-F442A cell lines | 3T3-L1 cell lines |
| None | 100 | 100 |
| Indinavir | 221.5 ± 27.0*** | 95.5 ± 10.4 |
| Nelfinavir | 257.8 ± 26.4*** | 180.1 ± 48.0* |
| Amprenavir | 121.2 ± 13.8 | 106.7 ± 17.3 |
| Lopinavir | 201.7 ± 30.4*** | 168.6 ± 28.5** |
| Ritonavir | 251.2 ± 31.8*** | 182.5 ± 54.0*** |
| Zidovudine | 282.5 ± 35.7*** | 185.3 ± 30.2*** |
| Stavudine | 197.2 ± 15.2*** | 137.0 ± 14.0** |

| | TNFα mRNA level (% of control) | |
|------------|--------------------------------|-------------------|
| | 3T3-F442A cell lines | 3T3-L1 cell lines |
| None | 100 | 100 |
| Indinavir | 265.4 ± 30.5*** | 109.375 ± 14.1 |
| Nelfinavir | 299.9 ± 33.9*** | 125.4 ± 27.4 |
| Amprenavir | 110.6 ± 17.2 | 104.0 ± 13.8 |
| Lopinavir | 146.1 ± 18.3*** | 101.1 ± 44.1 |
| Ritonavir | 194.5 ± 19.3*** | 98.6 ± 15.2 |
| Zidovudine | 254.4 ± 48.1*** | 315.0 ± 36.6*** |
| Stavudine | 231.5 ± 42.7*** | 333.7 ± 58.7*** |

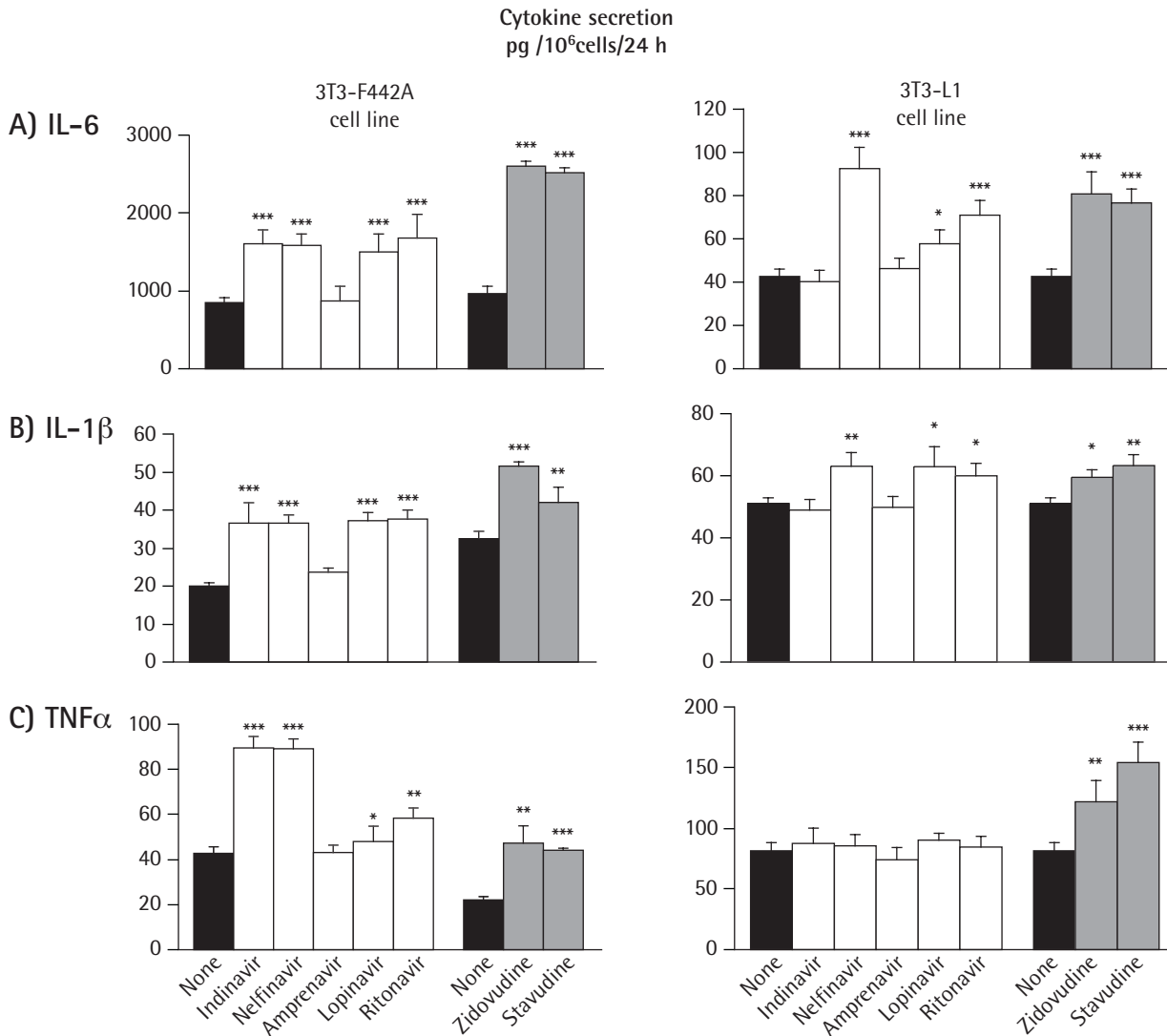
Total RNA was extracted and subjected to real-time PCR as described in Materials and methods. IL-6, IL-1β and TNFα mRNA levels, normalized to 18S rRNA expression, were determined relative to untreated cells. Results are % ± SEM of control values in 3–17 experiments performed in duplicate. **P*<0.05, ***P*<0.01, ****P*<0.001 versus control.

Table 3. Chronic effects of PIs and NRTIs on 3T3-F442A and 3T3-L1 adipocyte apoptosis

| | % of cells in apoptosis | |
|------------|-------------------------|------------------|
| | 3T3-F442A cell line | 3T3-L1 cell line |
| None | 2.8 ± 0.4 | 2.5 ± 0.4 |
| Indinavir | 12.2 ± 1.9*** | 3.1 ± 0.6 |
| Nelfinavir | 6.1 ± 1.4*** | 2.7 ± 0.4 |
| Amprenavir | 3.3 ± 3.3 | 3.2 ± 0.1 |
| Lopinavir | 3.1 ± 0.9 | 3.3 ± 0.2 |
| Ritonavir | 2.7 ± 0.2 | 3.3 ± 0.2 |
| Zidovudine | 5.7 ± 0.9*** | 8.6 ± 0.4** |
| Stavudine | 10.7 ± 1.4*** | 6.2 ± 2.2** |

Cells were collected and subjected to flow cytometry as described in Materials and methods. Results are the means ± SEM of 3–15 experiments. ***P*<0.01, ****P*<0.001 versus control.

Figure 2. Chronic effects of PIs and NRTIs on cytokine secretion in 3T3-F442A and 3T3-L1 adipocytes



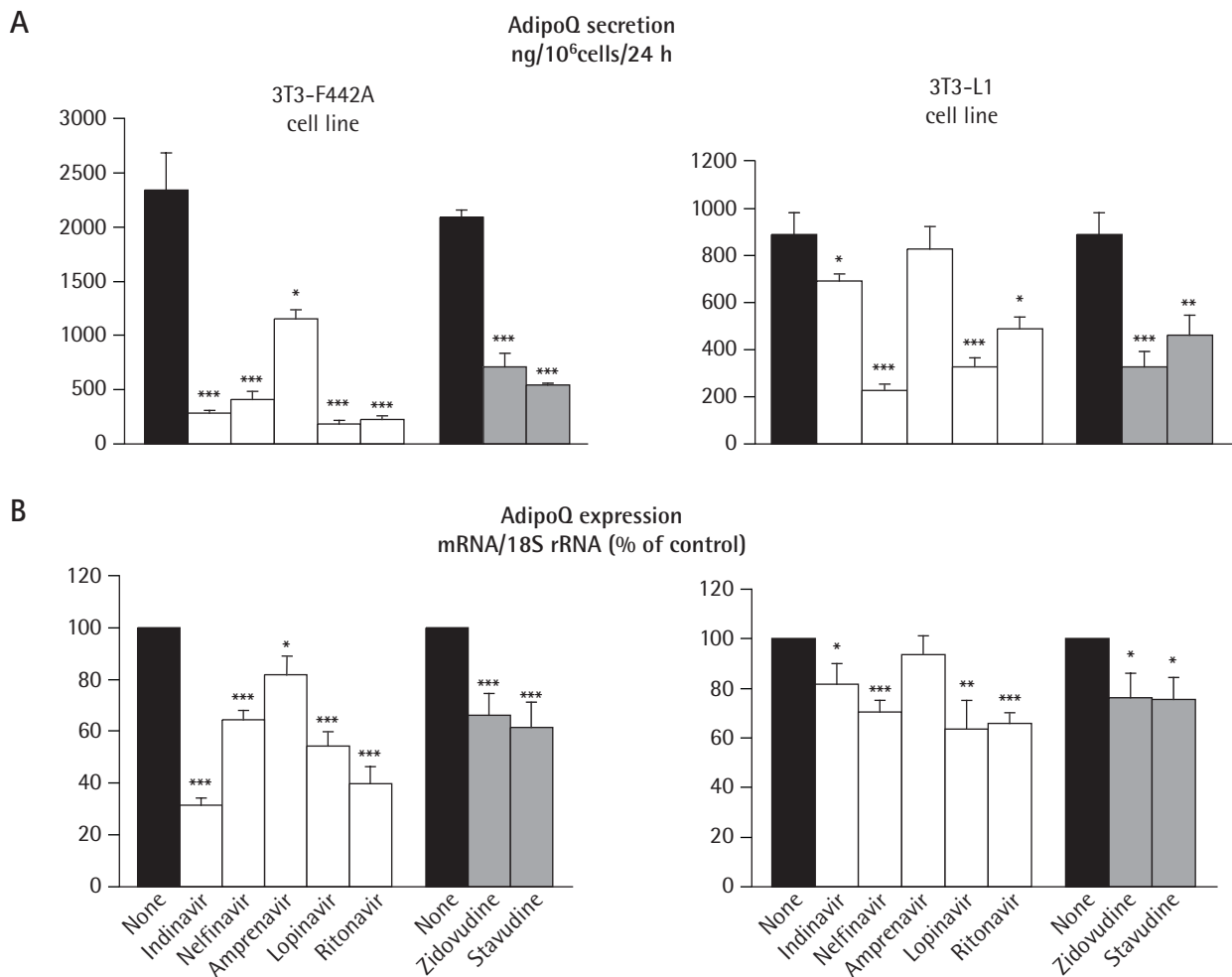
Differentiating 3T3-F442A cells and fully differentiated 3T3-L1 adipocytes were treated with PIs (indinavir, nelfinavir, amprenavir, ritonavir and lopinavir 10 μ M) or NRTIs (zidovudine 1 μ M and stavudine 10 μ M). On day 7 (3T3-F442A) or day 11 (3T3-L1) of differentiation, the medium was replaced and, 24 h later, the levels of (A) IL-6, (B) IL-1 β and (C) TNF α were determined with Quantikine Mouse ELISA tests. Results are expressed in pg/10⁶cells/24 h \pm SEM of 6–20 experiments performed in duplicate. * P <0.05, ** P <0.01, *** P <0.001 versus control (black bars).

similarly increased IL-6 and IL-1 β secretion and expression in the two cell lines. In keeping with the observed effects on lipid metabolism, indinavir failed to increase IL-6 and IL-1 β production in differentiated 3T3-L1 adipocytes, confirming the influence of adipocyte differentiation status on the effects of indinavir. Indeed, the adverse effect of indinavir [13,19] on 3T3-F442A cell differentiation is probably responsible for the cytokine hypersecretion.

IL-6 and IL-1 β appear to be targets of the PIs and NRTIs. IL-6 and IL-1 β have autocrine and paracrine effects and can alter adipocyte lipid metabolism, differ-

entiation [22,26] and survival [23], and could thus contribute to HAART-induced lipoatrophy when over-expressed. Since all cytokines function as a network [21,23], it is possible that PI and NRTI effects on IL-1 β secretion by 3T3-F442A cells resulted from increased levels of TNF α and/or IL-6, even though in 3T3-L1 mature adipocytes, PIs increased IL-1 β secretion without affecting TNF α production.

Adipocyte differentiation status also influenced the effect of PIs on TNF α production. All the PIs, except for amprenavir, increased TNF α secretion and expression by differentiating 3T3-F442A cells, whereas they had

Figure 3. Chronic effects of PIs and NRTIs on adiponectin secretion and expression in 3T3-F442A and 3T3-L1 adipocytes

Differentiating 3T3-F442A cells and fully differentiated 3T3-L1 adipocytes were treated with PIs (indinavir, nelfinavir, amprenavir, ritonavir and lopinavir 10 μ M) and NRTIs (zidovudine 1 μ M and stavudine 10 μ M). (A) On day 7 (3T3-F442A) or day 11 (3T3-L1) of differentiation, the medium was replaced and, 24 h later, the level of adiponectin was determined with the B-Bridge ELISA kit. Results are expressed in ng/10⁶cells/24h \pm SEM of 4–10 experiments performed in duplicate. * P <0.05, ** P <0.01, *** P <0.001 versus control (black bars). (B) Total RNA was extracted and subjected to real-time PCR as described in Materials and methods. Adiponectin mRNA levels normalized to 18S rRNA expression were determined relative to untreated control cells. Results are % \pm SEM of control values in 6–12 experiments performed in duplicate. * P <0.05, ** P <0.01, *** P <0.001 versus control (black bars).

no effect on mature 3T3-L1 adipocytes. In contrast, NRTIs increased TNF α production regardless of differentiation status. These results confirm that PIs and NRTIs alter adipocyte functions through different mechanisms; the mitochondrial toxicity of the thymidine analogues is probably implicated [8–10].

TNF α has both paracrine and autocrine effects on adipocytes and can alter adipose cell functions by decreasing differentiation [26] and insulin responsiveness [24]. A role of the TNF α system in the insulin resistance associated with lipodystrophy has been suggested, as circulating soluble TNF α receptor (TNF-R1) concentrations are increased and correlate negatively with insulin sensitivity [32]. We recently

reported [22] that high concentrations of IL-6 can alter adipocyte insulin response. Thus TNF α and IL-6 hypersecretion induced by PIs and NRTIs might contribute, at least in part, to the insulin resistance of adipose tissue.

TNF α also plays a key role in cell apoptosis and may be a driving force of PI- and NRTI-induced apoptosis of adipose cells. Apoptotic nuclei have been observed in adipose tissue of patients on HAART [11,34]. Here, the drugs that increased TNF α secretion by 3T3-F442A adipocytes (indinavir, nelfinavir, stavudine and zidovudine) also promoted their apoptosis. In contrast, ritonavir, lopinavir and amprenavir had little effect on either TNF α secretion or cell apoptosis. PIs

affected neither TNF α production nor apoptosis of 3T3-L1 mature adipocytes. The thymidine analogues that up-regulated TNF α secretion in both 3T3-F442A and 3T3-L1 cells also increased apoptosis in both cell lines.

These data suggest that HAART-induced fat cell loss could partly be mediated by the proapoptotic effect of TNF α . Apoptosis could also be mediated by TNF α -induced mitochondrial toxicity, as TNF α is known to impair respiratory chain activity and to increase the release of reactive oxygen species in a variety of cells [42].

Circulating adiponectin levels are an index of insulin sensitivity [21]. Adiponectin levels are subnormal in serum [28,32,33,35,36] and adipose tissue [28,32,33] of lipodystrophic patients on HAART. This is in keeping with the reduced adiponectin secretion and expression by 3T3-F442A and 3T3-L1 adipocytes after PI treatment, effects that might contribute to the insulin resistance observed *in vivo* [1,3,4]. Interestingly, indinavir and ritonavir, which have been found to cause lipid metabolic disorders and, probably, insulin resistance *in vivo* [5,6,37,43], decreased adiponectin secretion by 90–95%. Our results are in keeping with those of Xu *et al.* [37] obtained with mature 3T3-L1 adipocytes treated for 24 h with 20 μ M nelfinavir, indinavir and ritonavir. Stavudine and zidovudine also reduced adiponectin secretion (by 50–70%), regardless of adipocyte differentiation status, suggesting that they could aggravate the effect of PIs in HAART-treated patients. In 3T3-L1 mature adipocytes, our finding that NRTIs can induce both lipid leakage and apoptosis is in line with the lipoatrophic effect of NRTIs [2–4].

Thiazolidinediones have been shown to increase adiponectin expression [21] and to decrease TNF α and IL-6 expression [21,22] in experimental models, and might therefore have beneficial effects on HAART-induced lipodystrophy. However, clinical data concerning patients on HAART are controversial: thiazolidinediones improved [44–46] or not [47,48] lipoatrophy, although rosiglitazone increased adiponectin secretion [46,47,49].

IL-6, IL-1 β and TNF α can suppress adiponectin expression by adipocytes [21], supporting the concept that these adipokines might function as a network, each influencing the production of the others and interacting in the regulation of local fuel metabolism. Thus, the impact of PIs and NRTIs on adiponectin production could be explained in part by our finding that they increased IL-6, IL-1 β and TNF α secretion and expression.

In conclusion, our data show that individual PIs and NRTIs can directly affect adipocyte functions and induce hypersecretion of the cytokines involved in apoptosis, lipid depletion and insulin resistance, which

could be an important link in the pathogenesis of HAART-related lipodystrophy.

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References

1. Carr A, Samaras K, Burton S, Law M, Freund J, Chisholm DJ & Cooper DA. A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. *AIDS* 1998; **12**:51–58.
2. Carr A, Miller J, Law M & Cooper DA. A syndrome of lipoatrophy, lactic acidaemia and liver dysfunction associated with HIV nucleoside analogue therapy: contribution to protease inhibitor-related lipodystrophy syndrome. *AIDS* 2000; **14**:25–32.
3. Jain RG, Furfine ES, Pedneault L, White AJ & Lenhard JM. Metabolic complications associated with antiretroviral therapy. *Antiviral Research* 2001; **51**:151–177.
4. Miller J, Carr A, Emery S, Law M, Mallal S, Baker D, Smith D, Kaldor J & Cooper DA. HIV lipodystrophy: prevalence, severity and correlates of risk in Australia. *HIV Medicine* 2003; **4**:293–301.
5. Noor MA, Lo JC, Mulligan K, Schwarz JM, Halvorsen RA, Schambelan M & Grunfeld C. Metabolic effects of indinavir in healthy HIV-seronegative men. *AIDS* 2001; **15**:11–18.
6. Purnell JQ, Zambon A, Knopp RH, Pizzuti DJ, Achari R, Leonard JM, Locke C & Brunzell JD. Effect of ritonavir on lipids and post-heparin lipase activities in normal subjects. *AIDS* 2000; **14**:51–57.
7. Nolan D, Hammond E, Martin A, Taylor L, Herrmann S, McKinnon E, Metcalf C, Latham B & Mallal S. Mitochondrial DNA depletion and morphologic changes in adipocytes associated with nucleoside reverse transcriptase inhibitor therapy. *AIDS* 2003; **17**:1329–1338.
8. Walker UA, Bickel M, Lutke Volksbeck SI, Ketelsen UP, Schofer H, Setzer B, Venhoff N, Rickerts V & Staszewski S. Evidence of nucleoside analogue reverse transcriptase inhibitor-associated genetic and structural defects of mitochondria in adipose tissue of HIV-infected patients. *Journal of Acquired Immune Deficiency Syndromes* 2002; **29**:117–121.
9. Pace CS, Martin AM, Hammond EL, Mamotte CD, Nolan DA & Mallal SA. Mitochondrial proliferation, DNA depletion and adipocyte differentiation in subcutaneous adipose tissue of HIV-positive HAART recipients. *Antiviral Therapy* 2003; **8**:323–331.
10. Shikuma CM, Hu N, Milne C, Yost F, Waslien C, Shimizu S & Shiramizu B. Mitochondrial DNA decrease in subcutaneous adipose tissue of HIV-infected individuals with peripheral lipoatrophy. *AIDS* 2001; **15**:1801–1809.
11. Domingo P, Matias-Guiu X, Pujol RM, Francia E, Lagarda E, Sarnbeat MA & Vazquez G. Subcutaneous adipocyte apoptosis in HIV-1 protease inhibitor-associated lipodystrophy. *AIDS* 1999; **13**:2261–2267.
12. Mallal SA, John M, Moore CB, James IR & McKinnon EJ. Contribution of nucleoside analogue reverse transcriptase

- inhibitors to subcutaneous fat wasting in patients with HIV infection. *AIDS* 2000; **14**:1309–1316.
13. Caron M, Auclair M, Vigouroux C, Glorian M, Forest C & Capeau J. The HIV protease inhibitor indinavir impairs sterol regulatory element-binding protein-1 intranuclear localization, inhibits preadipocyte differentiation, and induces insulin resistance. *Diabetes* 2001; **50**:1378–1388.
 14. Lenhard JM, Furfine ES, Jain RG, Ittoop O, Orband-Miller LA, Blanchard SG, Paulik MA & Weiel JE. HIV protease inhibitors block adipogenesis and increase lipolysis *in vitro*. *Antiviral Research* 2000; **47**:121–129.
 15. Caron M, Auclair M, Sterlingot H, Kornprobst M & Capeau J. Some HIV protease inhibitors alter lamin A/C maturation and stability, SREBP-1 nuclear localization and adipocyte differentiation. *AIDS* 2003; **17**:2437–2444.
 16. Murata H, Hruz PW & Mueckler M. Indinavir inhibits the glucose transporter isoform GLUT4 at physiologic concentrations. *AIDS* 2002; **16**:859–863.
 17. Dowell P, Flexner C, Kwiterovich PO & Lane MD. Suppression of preadipocyte differentiation and promotion of adipocyte death by HIV protease inhibitors. *Journal of Biology & Chemistry* 2000; **275**:41325–41332.
 18. Roche R, Poizot-Martin I, Yazidi CM, Compe E, Gastaut JA, Torresani J & Planells R. Effects of antiretroviral drug combinations on the differentiation of adipocytes. *AIDS* 2002; **16**:13–20.
 19. Vernochet C, Azoulay S, Duval D, Guedj R, Ailhaud G & Dani C. Differential effect of HIV protease inhibitors on adipogenesis: intracellular ritonavir is not sufficient to inhibit differentiation. *AIDS* 2003; **17**:2177–2180.
 20. Gavrilova O, Marcus-Samuels B, Graham D, Kim JK, Shulman GI, Castle AL, Vinson C, Eckhaus M & Reitman ML. Surgical implantation of adipose tissue reverses diabetes in lipoatrophic mice. *Journal of Clinical Investigation* 2000; **105**:271–278.
 21. Fasshauer M & Paschke R. Regulation of adipocytokines and insulin resistance. *Diabetologia* 2003; **46**:1594–1603.
 22. Lagathu C, Bastard JP, Auclair M, Maachi M, Capeau J & Caron M. Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. *Biochemical & Biophysical Research Communications* 2003; **311**:372–379.
 23. Zhang HH, Kumar S, Barnett AH & Eggo MC. Dexamethasone inhibits tumor necrosis factor- α -induced apoptosis and interleukin-1 β release in human subcutaneous adipocytes and preadipocytes. *Journal of Clinical Endocrinology & Metabolism* 2001; **86**:2817–2825.
 24. Ruan H & Lodish HF. Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor- α . *Cytokine Growth Factor Review* 2003; **14**:447–455.
 25. Mito N, Hiyoshi T, Hosoda T, Kitada C & Sato K. Effect of obesity and insulin on immunity in non-insulin-dependent diabetes mellitus. *European Journal of Clinical Nutrition* 2002; **56**:347–351.
 26. Tanaka T, Itoh H, Doi K, Fukunaga Y, Hosoda K, Shintani M, Yamashita J, Chun TH, Inoue M, Masatsugu K, Sawada N, Saito T, Inoue G, Nishimura H, Yoshimasa Y & Nakao K. Down regulation of peroxisome proliferator-activated receptor γ expression by inflammatory cytokines and its reversal by thiazolidinediones. *Diabetologia* 1999; **42**:702–710.
 27. Prins JB, Niesler CU, Winterford CM, Bright NA, Siddle K, O'Rahilly S, Walker NI & Cameron DP. Tumor necrosis factor- α induces apoptosis of human adipose cells. *Diabetes* 1997; **46**:1939–1944.
 28. Lihn AS, Richelsen B, Pedersen SB, Haugaard SB, Rathje GS, Madsbad S & Andersen O. Increased expression of TNF- α , IL-6, and IL-8 in HALS: implications for reduced adiponectin expression and plasma levels. *American Journal of Physiology. Endocrinology & Metabolism* 2003; **285**:E1072–1080.
 29. Christeff N, Melchior JC, de Truchis P, Perronne C & Gougeon ML. Increased serum interferon α in HIV-1 associated lipodystrophy syndrome. *European Journal of Clinical Investigation* 2002; **32**:43–50.
 30. Kannisto K, Sutinen J, Korshennikova E, Fisher RM, Ehrenborg E, Gertow K, Virkamaki A, Nyman T, Vidal H, Hamsten A & Yki-Jarvinen H. Expression of adipogenic transcription factors, peroxisome proliferator-activated receptor γ co-activator 1, IL-6 and CD45 in subcutaneous adipose tissue in lipodystrophy associated with highly active antiretroviral therapy. *AIDS* 2003; **17**:1753–1762.
 31. Johnson JA, Albu JB, Engelson ES, Fried SK, Inada Y, Ionescu G & Kotler DP. Increased systemic and adipose tissue cytokines in patients with HIV-associated lipodystrophy. *American Journal of Physiology. Endocrinology & Metabolism* 2004; **286**:E261–271.
 32. Vigouroux C, Maachi M, Nguyen TH, Coussieu C, Gharakhanian S, Funahashi T, Matsuzawa Y, Shimomura I, Rozenbaum W, Capeau J & Bastard JP. Serum adipocytokines are related to lipodystrophy and metabolic disorders in HIV-infected men under antiretroviral therapy. *AIDS* 2003; **17**:1503–1511.
 33. Sutinen J, Korshennikova E, Funahashi T, Matsuzawa Y, Nyman T & Yki-Jarvinen H. Circulating concentration of adiponectin and its expression in subcutaneous adipose tissue in patients with highly active antiretroviral therapy-associated lipodystrophy. *Journal of Clinical Endocrinology & Metabolism* 2003; **88**:1907–1910.
 34. Jan V, Cervera P, Maachi M, Baudrimont M, Kim M, Vidal H, Girard PM, Levan P, Rozenbaum W, Lombes A, Capeau J & Bastard JP. Altered fat differentiation and adipocytokine expression are inter-related and linked to morphological changes and insulin resistance in HIV-1-infected lipodystrophic patients. *Antiviral Therapy* 2004; **9**:555–564.
 35. Addy CL, Gavrilova A, Tsiodras S, Brodovicz K, Karchmer AW & Mantzoros CS. Hypoadiponectinemia is associated with insulin resistance, hypertriglyceridemia, and fat redistribution in human immunodeficiency virus-infected patients treated with highly active antiretroviral therapy. *Journal of Clinical Endocrinology & Metabolism* 2003; **88**:627–636.
 36. Tong Q, Sankale JL, Hadigan CM, Tan G, Rosenberg ES, Kanki PJ, Grinspoon SK & Hotamisligil GS. Regulation of adiponectin in human immunodeficiency virus-infected patients: relationship to body composition and metabolic indices. *Journal of Clinical Endocrinology & Metabolism* 2003; **88**:1559–1564.
 37. Xu A, Yin S, Wong L, Chan KW & Lam KS. Adiponectin ameliorates dyslipidemia induced by the human immunodeficiency virus protease inhibitor ritonavir in mice. *Endocrinology* 2004; **145**:487–494.
 38. Wire MB, Ballow C, Preston SL, Hendrix CW, Piliero PJ, Lou Y & Stein DS. Pharmacokinetics and safety of GW433908 and ritonavir, with and without efavirenz, in healthy volunteers. *AIDS* 2004; **18**:897–907.
 39. Gutierrez F, Padilla S, Navarro A, Masia M, Hernandez I, Ramos J, Esteban A & Martin-Hidalgo A. Lopinavir plasma concentrations and changes in lipid levels during salvage therapy with lopinavir/ritonavir-containing regimens. *Journal of Acquired Immune Deficiency Syndromes* 2003; **33**:594–600.
 40. Cato A 3rd, Qian J, Hsu A, Levy B, Leonard J & Granneman R. Multidose pharmacokinetics of ritonavir and zidovudine in human immunodeficiency virus-infected patients. *Antimicrobial Agents & Chemotherapy* 1998; **42**:1788–1793.
 41. Dudley MN, Graham KK, Kaul S, Geletko S, Dunkle L, Browne M & Mayer K. Pharmacokinetics of stavudine in patients with AIDS or AIDS-related complex. *Journal of Infectious Diseases* 1992; **166**:480–485.
 42. Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA & Fiers W. Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of

- mitochondrial radical generation. *Journal of Biological Chemistry* 1992; **267**:5317–5323.
43. Riddle TM, Kuhel DG, Woollett LA, Fichtenbaum CJ & Hui DY. HIV protease inhibitor induces fatty acid and sterol biosynthesis in liver and adipose tissues due to the accumulation of activated sterol regulatory element-binding proteins in the nucleus. *Journal of Biological Chemistry* 2001; **276**:37514–37519.
44. Calmy A, Hirschel B, Hans D, Karsegard VL & Meier CA. Glitazones in lipodystrophy syndrome induced by highly active antiretroviral therapy. *AIDS* 2003; **17**:770–772.
45. Gelato MC, Mynarcik DC, Quick JL, Steigbigel RT, Fuhrer J, Brathwaite CE, Brebbia JS, Wax MR & McNurlan MA. Improved insulin sensitivity and body fat distribution in HIV-infected patients treated with rosiglitazone: a pilot study. *Journal of Acquired Immune Deficiency Syndromes* 2002; **31**:163–170.
46. Hadigan C, Yawetz S, Thomas A, Havers F, Sax PE & Grinspoon S. Metabolic effects of rosiglitazone in HIV lipodystrophy: a randomized, controlled trial. *Annals of Internal Medicine* 2004; **140**:786–794.
47. Carr A, Workman C, Carey D, Rogers G, Martin A, Baker D, Wand H, Law M, Samaras K, Emery S & Cooper DA. No effect of rosiglitazone for treatment of HIV-1 lipodystrophy: randomised, double-blind, placebo-controlled trial. *Lancet* 2004; **363**:429–438.
48. Sutinen J, Hakkinen AM, Westerbacka J, Seppala-Lindroos A, Vehkavaara S, Halavaara J, Jarvinen A, Ristola M & Yki-Jarvinen H. Rosiglitazone in the treatment of HAART-associated lipodystrophy – a randomized double-blind placebo-controlled study. *Antiviral Therapy* 2003; **8**:199–207.
49. Sutinen J, Kannisto K, Korshennikova E, Fisher RM, Ehrenborg E, Nyman T, Virkamaki A, Funahashi T, Matsuzawa Y, Vidal H, Hamsten A & Yki-Jarvinen H. Effects of rosiglitazone on gene expression in subcutaneous adipose tissue in highly active antiretroviral therapy-associated lipodystrophy. *American Journal of Physiology. Endocrinology & Metabolism* 2004; **286**:E941–949.

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