LONG TERM TREATMENT BY LOPINAVIR/RITONAVIR INDUCES A REDUCTION IN PERIPHERAL ADIPOSE DEPOTS IN MICE

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SUMMARY

Highly active antiretroviral therapy (HAART) of HIV infected patients is associated with adverse effects such as lipodystrophy and hyperlipidemia. The lipodystrophic syndrome is characterised by a peripheral lipoatrophy and/or fat accumulation in abdomen and neck. In order to get insights into the physiopathological mechanisms underlying this syndrome we treated mice with protease inhibitors (PI) over a long period of time. Whereas atazanavir treated mice presented the same circulating triglyceride concentration as control mice, lopinavir/ritonavir treated mice became rapidly hypertriglyceridemic, 200mg/dl versus 80mg/dl in control or atazanavir treated animals. These results in mice reproduce the human metabolic disorder. White adipose tissue (WAT) was analysed after eight weeks of treatment. Compared to control or atazanavir treated mice, lopinavir/ritonavir induced a significant 25% weight reduction in the peripheral inguinal WAT depot. By contrast, the profound epididymal WAT depot was not affected. This effect was associated with a 5.5 fold increase in SREBP-1c gene expression only in the inguinal depot. Our results demonstrated that the long-term treatment of mice with PI constitutes an interesting experimental model to study some aspects of the lipoatrophy induced by HAART.
INTRODUCTION

Highly active antiretroviral therapy (HAART) combines various protease inhibitors (PI), nucleoside analogue reverse transcriptase inhibitors (NRTI) and non-nucleoside analogue reverse transcriptase inhibitors (NNRTI). Whereas HAART efficiently suppresses HIV replication, long-term treatment of HIV-infected patients has been associated with a lipodystrophic syndrome and metabolic complications, one of the most common being hypertriglyceridemia (6). The HAART-associated lipodystrophy is characterised by peripheral fat wasting in the face and limbs and accumulation of visceral fat, breast hypertrophy and cervical fat-pads (buffalo hump) (7-9). Metabolic complications are preferentially associated to PI and appear much more rapidly than lipodystrophy (20). It is not known whether the latter is a long-term consequence of the metabolic disorder or whether it corresponds to distinct molecular mechanisms. Because of the lack of pertinent experimental model, the pathogenesis and physiopathological mechanisms by which PI/NRTI/NNRTI cause HAART-associated lipodystrophy and metabolic disorders remain to be elucidated.

PI/NRTI/NNRTI can interfere with adipocyte differentiation and adipocyte-specific gene expression (16, 18, 22, 26-29). However the reported effects are very variable, positive or negative, depending on the molecule and on the experimental model studied. Furthermore, these cellular studies do not explain why HAART leads, in one case, to peripheral lipoatrophy and, in the other case, to abdominal hypertrophy of the adipose tissue. Short-term treatment (10 days) of mice evidenced a hyperlipidemic effect of the PI ritonavir (21). This effect correlated with the activation of SREBP-1c in liver and white adipose tissues (WAT).

Because HAART is the association of several drugs, it is impossible to discriminate the precise contribution of each antiretroviral compound. In order to get insights into the physiopathological mechanisms underlying the lipodystrophy syndrome we long-term treated...
mice with PI. We found that the lopinavir/ritonavir treatment reduces specifically the peripheral inguinal WAT depots of adult treated mice.
MATERIALS AND METHODS

Animals and drug administration

C57BL/6J male mice were housed on a 12h light/dark schedule and had free access to water and food. Lopinavir/ritonavir association (ABT-378/r™ oral solution, Abbott Laboratories) or Atazanavir (Reyataz™, Bristol-Myers-Squibb laboratories) were administered by oral gavage, 200µl/animal, once daily. Control mice received an equal volume of 0.5% hydroxymethylcellulose by pipet. Body weights were measured weekly.

The experiments were conducted following the standard ethical guidelines (European Union guidelines on animal laboratory care) and approved by the Faculty de Medicine of Nice ethical committee.

Pharmacokinetic analysis

C57BL/6J male mice were treated daily with Atazanavir or Lopinavir/Ritonavir for 3 days. The fourth day, mice were anaesthetised with pentobarbital (9mg/mouse) and 500µl of blood samples were collected from the retroorbitary sinus at various times after the last gavage. PIs plasma concentrations were determined by HPLC using a reverse phase column and a DAD detector; the limit of quantification was 50 ng/ml. AUC in plasma were estimated by using a non compartmental analysis model (Winonlin 3.2 software).

Collection of tissues and serum analysis

Following blood sampling, serum was isolated by centrifugation and immediately stored at -80°C until analysis. Inguinal white adipose tissue, epididymal white adipose tissue, interscapular brown adipose tissue (BAT) and liver were excised and weighed. For RNA isolation samples of epididymal and inguinal white adipose tissues of left side were placed on ice and immediately homogenized with TRIZOL reagent (Invitrogen Life Technologies).
Quantification of triglycerides was performed using the Triglycerides 100 biochemical kit (ABX diagnostics, Montpellier, France). Glycemia was determined by Accu-Check active bands (Roche Diagnostics, Mannheim, Germany).

Adipose tissue histology.

The epididymal and inguinal white adipose tissue of right side were formalin-fixed and paraffin-embedded. The sections were stained with hematoxylin-eosin-safranin for light microscopic examination.

Analysis of mRNA levels by Real-Time Quantitative PCR

Total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) and gene expression analysis was performed with the ABI Prism 7000 (Applied Biosystem) and SYBR Green Reagents (Eurogentec, Seraing, Belgium) as already described (2). Briefly, cDNAs were synthesized from 2µg of total RNA using Superscript II reverse transcriptase (Invitrogen). Primers set were designed according to the manufacturer software. Samples contained 1xSYBR Green Master Mix, 0.5µM primers and 1/10 synthesized cDNA in a 25µl volume. The PCR conditions were as follows: 10 min at 95°C, then 40 cycles of 15 s at 94°C, 30 s at 60°C and 1min at 72°C. We used 36B4 as the reference gene.

Primer sequences are the following:

FAS F: 5’TGCTCCAGCTGCAGGC3’
FAS R: 5’GCCCGGTAGCTCTGGGTGTA3’
SREBP1c F: 5’GGAGCCATGGATTGCACATT3’
SREBP1c R: 5’GCTTCCAGAGAGGAGGCCAG3’
PPARγ F: 5’CTGTTTTTATGCTGTTATGGGTGAAA3’
PPARγ R: 5’GCACCATGCTCTGGGTCAA3’
Adiponectin F: 5’TCATGCCGAAGATGACGTTACT3’
Adiponectin R: 5’CCATCCAACCTGCACAAGTTC3’
36B4 F: 5’TCCAGGCTTTGGGCATCA3’

36B4 R: 5’CTTTATCAGCTGCACATCACTCAGA3’.
RESULTS

In order to set up an experimental model to study the HAART-associated lipodystrophy, control mice were compared to mice receiving two long-term IP treatments: the classical association of lopinavir/ritonavir (LPV/r), strongly involved in HAART, and the new compound atazanavir (ATV) which induces rare metabolic disorders in patients (12, 19).

Pharmacokinetic analysis

Pharmacokinetic analysis was performed to determine the dose regimen necessary to obtain in mice circulating drug concentrations similar to human therapeutic levels. Two lopinavir/ritonavir (LPV/r) doses, 200/50 and 400/100mg/kg (15 to 30 fold the human dose), were given daily to animals by oral gavage. The fourth treatment day, LPV and RTV concentrations were determined at 0, 2, 4, 6, 8 and 12hr after the last gavages. In humans the areas under the curve (AUC) value of lopinavir, on a 12 hours period, is comprised between 52 and 76µg.h/ml (10) (14). Our results show that lopinavir was eliminated rapidly in mice, the 200mg/kg/day dose giving impregnation similar to human dosages, with 95µg.h/ml AUC, whereas impregnation by the 400mg/kg/day dose was four fold higher, with 255µg.h/ml AUC (Figure 1). Ritonavir was also eliminated rapidly and its concentration peaked at 2µg/ml, for the 50mg/kg dose, which is similar to human dosages (data not shown). Long-term treatment at the400/100mg/kg/day dose appeared highly toxic, leading to death of most of the mice after few weeks (data not shown). In the case of atazanavir, we also chose a concentration 15 fold higher than in humans and found that the 90mg/kg/day dose gave a peak of circulating concentration after 1h of 12µg/ml, which decreased rapidly (data not shown). The calculated AUC is 32.7µg.h/ml, giving impregnation similar to human dosages (whose AUC is 28µg.h/ml). Our results show that half-life and steady-state levels of these molecules are shorter in mice as in humans. An important pharmaceutical parameter is the fraction of drugs
that binds to circulating proteins. Differences in this proportion between humans and mice could introduce a bias in the results. Association of LPV/r and ATV to plasma binding proteins in different species (including human and mice) has been analysed by others and are available on the European Medicine Agency web site: http://www.emea.eu.int. Published results show no significant differences between human and mice for these molecules. Therefore we postulate that the impregnation and the proportion of active molecules we obtained in mice are comparable to that reached in human therapy.

**Lopinavir/ritonavir (LPV/r) induces hypertriglyceridemia in mice**

Retro-orbital blood sampling was performed on control C57Bl6J male mice and mice treated daily with three different doses of LPV/r (100/25, 200/50 and 300/75mg/kg/day) for two weeks. Samples were analysed for glucose and triglyceride contents. The three LPV/r treatments induced hypertriglyceridemia in mice (2.2, 5.1 and 3.8 fold the triglyceride concentration of control mice, respectively) after two weeks (Figure 2A). This effect was stable over the time and persisted in eight weeks-treated animals (Figure 2B). Glycemia was measured in these animals and no change was induced by the various LPV/r treatments (Figure 2C for the 200/50mg/kg/day dose and data not shown). Our results reproduce the dyslipidemia syndrome associated with HAART in humans and the observation that these effects are much more frequent and substantial than changes in glucose levels (25). The 200/50mg/kg/day dose was used in the following experiments, a dose which did not appeared toxic and induced reproducible hypertriglyceridemia.

We then analysed the metabolic parameters of mice treated for 8 weeks either with LPV/r (200/50mg/day) or atazanavir (ATV) (90mg/kg/day) compared with the control treated mice. Whereas no change was observed in glycemia (data not shown), only the LPV/r treatment induced hypertriglyceridemia (2.5 fold the triglyceride concentration of control or ATV-treated mice), confirming the absence of metabolic effect of ATV (Figure 2D).
Lopinavir/ritonavir (LPV/r) reduces the peripheral white adipose depot in long-term treated mice

In preliminary experiments, 6 or 8 weeks old mice were treated for height weeks with LPV/r at 200/50mg/kg/day. At those ages mice are still growing and, over a period of 8 weeks, the weight of control mice went up from 20 to 27g and from 25 to more than 30g, respectively. In those cases, LPV/r treatment had a marked inhibitory effect on growth; the weight of treated mice did not increase or increased much more slowly than in control mice (Figure 3A). This effect of the 200/50mg/kg LPV/r dose on young mice is probably related to the relatively toxic effect of LPV/r, already observed at higher doses. We then, performed the experiments by starting with 10 weeks old mice and ATV-treated mice were included in those experiments. No effect on weight was noticed between the three groups of mice, their starting weight was around 30g +/-2 for all the mice, and this value did not change significantly during the treatments (Figures 3B and 4). After 8 weeks, mice were sacrificed, and various tissues were carefully dissected and weighed. Two different groups of mice were treated in the same conditions, whose results are presented in Figure 4A. Compared to controls, the weight of the peripheral inguinal adipose depot was significantly reduced by 25% by the LPV/r treatment. A representative lipoatrophic effect of the LPV/r treatment on peripheral inguinal adipose depot is presented in figure 4B. No significant change was observed in the size of adipocytes of LPV/r treated animals, suggesting that the lipoatrophic effect was due to a remodelling of the tissue rather than a cellular effect. By contrast, the epididymal fat depot and the brown adipose tissues were not significantly modified (Figure 4A). Compared to control mice, ATV treatment did not induce significant changes in any of the tissues we investigated (Figure 4A).

Lopinavir/ritonavir (LPV/r) induces SREBP-1c gene expression in the peripheral white adipose depot
In order to study the molecular mechanisms involved in this tissue specific effect, RNAs from both inguinal and epididymal fat depots were extracted and analysed by real-time RT-PCR for gene expression of various adipocyte markers. Compared to controls, results show that SREBP-1c and FAS expressions were strongly induced, more than 5 fold, in the inguinal WAT of LPV/r treated mice (Figure 5). By contrast, in the epididymal fat pad, which weight is not affected by LPV/r, the treatment did not affect significantly the expression of these genes. In both WAT depots, gene expression of adiponectin and PPARγ adipogenic markers was not modified by the LPV/r treatment.
DISCUSSION

In order to get insights into the physiopathological mechanisms underlying the lipodystrophy syndrome we treated mice with protease inhibitors for long periods. We found that the lopinavir/ritonavir association induces hypertriglyceridemia and reduces specifically peripheral inguinal WAT depots of 10 weeks-old mice treated for 8 weeks. The metabolic disorder observed in our animal model reproduces the dyslipidemia observed in the human disease. The HAART-associated lipodystrophic syndrome in humans is also reproduced in mice, but only partially. Particularly, the peripheral lipoatrophy is effectively detected in mice at the level of inguinal WAT depots, whereas other tissues are not affected. However, this effect on mice does not match completely the observations reported in humans, for example we did not notice any change at the level of the other WAT depots.

In a recently published study (11) the authors analysed the effects of a ritonavir long-term treatment on 5 weeks-old mice. They found that the drug induces a general lipoatrophy and exerts a potent blocking effect on the general growth of the animals. Similarly to their data, we also noticed that drug treatment of young animals, while they are still growing, led to potent inhibition of their growth. Furthermore, we observed that higher doses led to a rapid death of the animals. Therefore, it is possible that, in young animals, the observed effects may be related to a toxic unspecific effect rather than a lipodystrophic potential. By contrast, starting the treatment with 10 weeks-old adult animals did not affect their growth. Therefore, it is likely that treating animals after puberty, when growth has slowed down or stopped, is more relevant to HAART-associated lipodystrophy described in adult humans.

Importantly, lopinavir/ritonavir (LPV/r) reduces the peripheral white adipose depot in long-term treated mice without affecting the other fat depots. Compared to visceral adipose tissue, peripheral/subcutaneous adipose tissue display important differences in their biological
characteristics and properties (see for review (15)). These differences vary from metabolic pathways to adipocyte differentiation capacities. Whether the specific LPV/r effect on peripheral tissue is due to one or several of these characteristics remain to be determined. Interestingly, potent activators of peroxisome proliferator-activated receptor γ (PPARγ), such as thiazolidinedione (TZD), increase adipocyte differentiation of subcutaneous preadipocytes but have no effect on adipocyte differentiation of visceral precursors (1). We found no effect of LPV/r on PPARγ expression, suggesting that the reduction of peripheral WAT by LPV/r is not due to a PPARγ downregulation. However, one possibility to be tested is that TZD could counteract the LPV/r-induced reduction of peripheral WAT by increasing adipogenesis in this tissue.

We found that the reduction in peripheral WAT depot induced by the LPV/r treatment is associated to an increase in gene expression of the transcription factor SREBP-1c and of fatty acid synthase (FAS), an SREBP-1c-target gene. Gene expression of the adipocyte markers PPARγ and adiponectin was not affected. Interestingly, in the same mice, these gene regulations were not found in the epididymal WAT depot, whose weight was not modified by the drug treatment. SREBP-1c activation has been largely involved in control of adipocyte differentiation, in development of adipose tissue and more recently in HAART-associated lipodystrophy. Although SREBP-1c has been first shown to promote adipocyte differentiation in vitro (24), in vivo transgenic experiments demonstrated that its overexpression in WAT is associated with impaired adipogenesis and lipoatrophic phenotype (13, 23). Furthermore, several studies have shown that PIs treatment results in regulation of SREBP-1c activity and, concomitantly, inhibition of adipogenesis, both in vitro (4, 5, 18) and in vivo, in mice and humans (3, 21). The effects of ritonavir and indinavir PIs on SREBP1-c are complex; ritonavir increases mature nuclear active form of the protein both in vitro (18) and in mice (21), whereas indinavir decreases total SREBP-1c protein expression and alters its subcellular
localisation (5). Because of its known function in the regulation of fatty acid synthesis, SREBP-1c activation could directly participate in the hypertriglyceridemia associated to PI treatment. Accordingly, our results showed that LPV/r induced both hypertriglyceridemia and increased gene expression of SREBP-1c and FAS. Although the precise implication of SREBP-1c in lipodystrophy is unclear (see review by Nerurkar et al. (17)), our results strongly suggest that SREBP-1c gene expression is linked to lopinavir-induced lipoatrophy of peripheral WAT in mice. One can hypothesize that the high variability of the lipodystrophy syndrome observed in humans reflects the various effects of PIs on expression and activity of genes involved in adipogenesis such as SREBP-1c. This hypothesis is strengthened by our results with atazanavir. By contrast to LPV/r, atazanavir induces neither lipoatrophy of peripheral WAT depot nor change in SREBP-1c gene expression. This absence of drug effect on mice is in agreement with preliminary clinical observations. Since our experiments were performed with the association of lopivanir and ritonavir, further experiments, testing each drug individually, are required to know whether the effects are only due to lopinavir or if there is also a contribution of ritonavir. Likewise, since atazanavir is now also administered in association with ritonavir, the effects of this association in mice should be evaluated.

In conclusion, our results validated the use of mice as an experimental model to explore some aspects of the physiopathology of HAART-associated lipodystrophy in humans. In our experimental animal model, starting the treatment with adult mice seems a critical parameter to obtain reliable effects. In order to get a model closer to the human disease and to evidence additional clinical aspects relevant to HAART-associated lipodystrophy, it should be interesting to modify the treatment by increasing its duration and/or by adjusting the dose regimen to further approach human AUC. Alternatively, testing other PIs, alone or in association, could also reveal interesting clinical aspects.
ACKNOWLEDGEMENTS

This work was supported by grants from ANRS (Agence Nationale de Recherches sur le SIDA) and "Ensemble contre le SIDA". We thank F. Bost for its support.

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LEGEND TO FIGURES

FIGURE 1: Pharmacokinetic analysis of lopinavir

Mice were daily treated for four days with the LPV/r at either 200/50mg (lopi 200)/ or 400/100mg (lopi 400) /kg/day. At indicated time after the last gavage, blood samples were analysed by HPLC to determine drug concentration. Values represent the means of three animals, +/- SEM.

FIGURE 2: Effect of treatments on triglyceridemia and glycemia

(A) Mice were treated daily for 2 weeks with increasing doses of the LPV/r: 100/25, 200/50 and 300/75mg/kg/day, or control treated, as indicated. Blood samples were analysed for triglyceride concentration. Histogram shows the mean values for 4 animals per condition. (B) Mice were treated daily for 2 to 8 weeks, as indicated, with the LPV/r at 200/50mg/kg/day (LPV/r), or excipient alone (Con) and analysed for triglyceride concentration. Values are means of 4 to 10 animals per group. (C) Mice were treated daily for 2 weeks with the LPV/r at 200/50mg/kg/day (LPV/r), or excipient alone (Con) and Glycemia was measured. Histogram shows the mean values for 4 animals per condition. (D) Mice were treated daily for 8 weeks with the LPV/r at 200/50mg/kg/day (LPV/r), or atazanavir at 90mg/kg/day (ATV) or excipient alone (Con), and triglyceride concentration was measured. Histogram shows the mean values from 23 (Con), 17 (LPV/r) or 12 (ATV) animals. Values are given +/- SEM, N.S.: non significant.

FIGURE 3: Effects of LPV/r treatment on body weights of young and adult mice
6 weeks (6w), 8 weeks (8w) (A) or 10 weeks (10w)-old mice (B) were treated daily for 8 weeks with the LPV/r at 200/50mg/kg/day (LPV/r), or control treated (C) and weighed every week. Values are means of 6 to 10 animals per group.

**FIGURE 4: Effects of LPV/r or ATV on body and tissue weights**

10 weeks-old mice were treated daily for 8 weeks with the LPV/r at 200/50mg/kg/day (LPV/r), or atazanavir at 90mg/kg/day (ATV) or control treated (Con). (A) Mice were analysed for their body weights and inguinal WAT (WAT ing), epididymal WAT (WAT epi) and BAT (BAT) depots were dissected and weighed. Values are normalised to 1 for control animals +/- SEM. Inguinal WAT was significantly affected by the LPV/r treatment: with p=0.0004. (B) Representative histological analysis of inguinal WAT depots of either control or LPV/r-treated mice. D: Dermis, WAT: white adipose tissue, F: Fibrous trabecula. The magnification is x10.

**FIGURE 5: Expression of adiponectin, PPARγ, SREBP-1c and FAS genes in WAT**

mRNAs from epididymal (epi) and inguinal (ing) WAT depots were extracted from control, ATV and LPV/r treated mice and analysed by Real-Time RT-PCR with specific primers for adiponectin (adipoN), PPARγ (PPARγ), SREBP-1c (srebp) and FAS (FAS) genes. Values were corrected for equal 36B4 expression and normalised to 1 for control animals +/- SEM. *= p<0.05.
Fig. 2

A

![Bar chart showing TG (mg/dl) levels for different LPV/r doses (100/25, 200/50, 300/75)].

B

![Line graph showing TG (mg/dl) levels over time (weeks)].

Con. vs. LPV/r, N.S.
Fig. 2

![Graph showing glycemia and TG levels](image-url)
Figure 3
Figure 4A

Relative ratio / control

P=4.10^{-4}

- Con, n=23
- LPVr, n=17
- ATV, n=12
Figure 5

Relative ratio / control

Figure 5
ERRATUM

Long-Term Treatment with Lopinavir-Ritonavir Induces a Reduction in Peripheral Adipose Depots in Mice

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Volume 50, no. 12, p. 3998–4004, 2006. Page 3999, column 2, line 3: “mice receiving two long-term intraperitoneal treatments” should read “mice receiving two long-term treatments.”