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Inhibition of Human Preadipocyte Proteasomal Activity by HIV Protease Inhibitors or Specific Inhibitor Lactacystin Leads to a Defect in Adipogenesis, Which Involves Matrix Metalloproteinase-9

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ABSTRACT

In a previous publication, we reported that human immunodeficiency virus (HIV) protease inhibitors (PIs) inhibited the differentiation of human preadipocytes in primary culture, reducing the expression and secretion of matrix metalloproteinase 9 (MMP-9). The present work was performed to clarify this mechanism. Interestingly, HIV-PIs have been reported to be inhibitors of the proteasome complex, which is known to regulate nuclear factor (NF)-κB activation and transcription of its target genes, among them MMP-9. We thus investigated the potential involvement of the proteasome in the antiadipogenic effects of HIV-PIs. The effect of four HIV-PIs was tested on preadipocyte proteasomal activity, and chronic treatment with the specific proteasome inhibitor lactacystin was performed to evaluate alterations of adipogenesis and MMP-9 expression/secretion. Finally, modifications of the NF-κB pathway induced by either HIV-PIs or lactacystin were studied. We demonstrated that preadipocyte proteasomal activity was decreased by several HIV-PIs and that chronic treatment with lactacystin mimicked the effects of HIV-PIs by reducing adipogenesis and MMP-9 expression/secretion. Furthermore, we observed an intracellular accumulation of the NF-κB inhibitor, IκBβ, with chronic treatment with HIV-PIs or lactacystin as well as a decrease in MMP-9 expression induced by acute tumor necrosis factor-α stimulation. These results indicate that inhibition of the proteasome by specific (lactacystin) or nonspecific (HIV-PIs) inhibitors leads to a reduction of human adipogenesis, and they therefore implicate deregulation of the NF-κB pathway and the related decrease of the key adipogenic factor, MMP-9. This study adds significantly to recent reports that have linked HIV-PI-related lipodystrophic syndrome with altered proteasome function, endoplasmic reticulum stress, and metabolic disorders.

Proteasomes are highly conserved multimeric peptidases present in all eukaryotic cells. Whereas various forms of proteasomes exist, the 20S proteasome (multicatalytic core of all proteasomes) and the 26S proteasome (20S core capped with two 19S regulatory units) are major proteasomes. Proteasomes were initially thought to be just recyclers of damaged or misfolded proteins, but over the last decade the activity of this enzymatic complex has been found to be of critical importance for many cellular functions. Cell-cycle progression, oncogenesis, apoptosis, regulation of gene expression, and inflammation or immune surveillance are all physiological functions regulated by the proteasome pathway (Kisselev and Goldberg, 2001). Among the substrates of the proteasome, cyclins (e.g., cyclin B1), cyclin-dependent kinase inhibitors (e.g., p21 and p27), tumor suppressors (e.g., p53), and inhibitors (IκB) or precursors (e.g., p105) of the transcription factor NF-κB are well established (Kisselev and Goldberg, 2001; Adams, 2004). Accordingly, the proteasome complex has emerged as an attractive target for cancer therapy, and numerous proteasome inhibitors have been developed (Adams, 2004; Voorhees and Orlowski, 2006).

ABBREVIATIONS: IκB, inhibitor of nuclear factor-κB; NF-κB, nuclear factor-κB; HIV, human immunodeficiency virus; PI, protease inhibitor; AT, adipose tissue; MMP, matrix metalloproteinase; SQV, saquinavir; NFV, nelfinavir; TNF, tumor necrosis factor; Bay 11-7802, (E)-3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile; IDV, indinavir; RTV, ritonavir; RFU, relative fluorescence units; TBST, Tris-buffered saline/Tween 20; RT, reverse transcriptase; PCR, polymerase chain reaction; aP2, fatty acid binding protein; HSL, hormone-sensitive lipase; ANOVA, analysis of variance. MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; TG, triglyceride.

Recently, human immunodeficiency virus (HIV) protease inhibitors (PIs), central components of highly active antiretroviral therapy, have been reported by some authors to directly inhibit the activities of murine and human 20S and/or 26S proteasomes in purified fractions or cellular extracts (André et al., 1998; Gaedicke et al., 2002; Pajonk et al., 2002; Piccinini et al., 2002). Indeed, several cleavage sites used by the HIV protease, which were once thought to be unique and distinct from those of mammalian proteases (predicting inhibitor selectivity), were found to be similar to those recognized by the 20S proteasome (Schmidtke et al., 1999). Although HIV-PIs have been successfully used in clinical therapy of HIV infection, they were also rapidly associated with the emergence of a lipodystrophic syndrome observed in patients treated with highly active antiretroviral therapy, which includes dyslipidemia, insulin resistance, central adiposity, and peripheral lipoatrophy. It is now clear that the mechanism associated with PI-induced metabolic abnormalities is multifactorial (for reviews, see Hui, 2003; Rudich et al., 2005) but whether the action of HIV-PIs on proteasomal activity is related to their side effects has been poorly investigated to date.

We previously reported, in accordance with in vivo studies performed on adipose tissue biopsies from HIV-infected lipodystrophic patients (Bastard et al., 2002; Lloreta et al., 2002), that PIs reduced the differentiation of human preadipocytes (i.e., adipocyte precursors) in primary culture isolated from subcutaneous abdominal adipose tissue (AT) (Bourlier et al., 2005). This effect was attributed to an indirect action of HIV-PIs on the expression and secretion of the matrix metalloproteinase-9 (MMP-9), an enzyme implicated in extracellular matrix remodeling and significantly involved in the human adipogenic process in vitro (Bourlier et al., 2005). Because the MMP-9 promoter contains an NF-κB site (Sato and Seiki, 1993; St-Pierre et al., 2004) and because proteasomal activity is involved in the activation of NF-κB pathway, we investigated the potential role of the proteasome in the antiadipogenic effect of HIV-PIs. We demonstrate here that HIV-PIs, particularly saquinavir (SQV) and nelfinavir (NFV), were direct inhibitors of the human preadipocyte 20S and 26S proteasomes. We found that chronic lactacystin (a specific proteasome inhibitor) treatment mimicked the effects of HIV-PIs by decreasing the differentiation process and the expression and secretion of MMP-9. Chronic treatment with either HIV-PIs (SQV or NFV) or lactacystin led to the accumulation of the IκBβ protein, the inhibitor of the transcription factor NF-κB and a substrate of the proteasome. Finally, acute treatment with TNF-α (activator of NF-κB) induced a potent increase in MMP-9 expression, which was significantly reduced by SQV, lactacystin, or (*E*)-3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile (Bay 11-7082) (inhibitor of NF-κB activation) cotreatments. Overall, our data indicate that HIV-PIs, by affecting proteasomal activity, alter the NF-κB pathway and implicate the reduction of MMP-9 activity in their antiadipogenic effect.

Materials and Methods

Cell Culture and Treatment. Chemicals were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Cell culture reagents were either from Life Technologies (Cergy Pontoise, France), Roche Diagnostics (Meylan, France), or Cambrex Biosciences (Verviers, Belgium).

Human subcutaneous abdominal white AT was obtained from moderately overweight women undergoing plastic surgery (mean age 46 ± 2 years and mean body mass index 27.5 ± 0.7 kg/m^2). The isolation of human AT-derived stromal cells and the culture of stromal preadipocytes differentiated into adipocytes were performed as described previously (Bouloumié et al., 2001). In brief, sterile AT was cut into small pieces and digested under agitation with collagenase (2 mg/ml) (Serva; Coger, Paris, France) for ~1 h at 37°C. After centrifugation, washing, and filtration steps, the stromal cells were suspended in Dulbecco's modified Eagle's medium F-12 supplemented with 10% fetal bovine serum and plated at 60,000 cells/cm^2. After 24 h, the medium was replaced by a medium consisting of Dulbecco's modified Eagle's medium F-12 supplemented with 33 μM biotin, 17 μM pantothenate, and 50 μg/ml gentamicin (basal medium) in the presence of 66 nM insulin, 1 nM triiodothyronine, 100 nM cortisol, and 10 μg/ml human transferrin (adipogenic medium) and 1 μg/ml ciglitazone for the first 3 days. After the 3-day priming period, the cells were used for experiments or cultured in the adipogenic medium supplemented with 1) the proteasome inhibitor lactacystin or 2) HIV-PIs IDV (kindly provided by Merck Laboratories, Rahway, NJ) or SQV (kindly provided by Roche, Welwyn Garden City, UK), RTV (kindly provided by Abbott Laboratories, Chicago, IL), or NFV (kindly provided by Roche Diagnostics, Mannheim, France) for 10 days. Lactacystin and HIV-PIs were dissolved in pure dimethyl sulfoxide. Control cells were treated using an appropriate concentration of vehicle (<0.25%) after verification that dimethyl sulfoxide was without effect on the various parameters analyzed (i.e., lipogenic index, adipogenic gene, or MMP secretion). Medium was replaced every 2 days. Before some experiments, cells were placed overnight in basal medium supplemented with the various treatments.

The medium was then collected and used in zymography analysis. Cellular triglyceride and DNA contents were determined using kits from Sigma-Aldrich and Molecular Probes (PicoGreen; Interchim, Montluçon, France), respectively, according to the manufacturer's instructions. DNA content was used to normalize data (triglyceride content and MMP secretion) and to evaluate any cytotoxic effect of treatments.

Gelatin Zymography. Proteins with gelatinolytic activity (including the gelatinase A "MMP-2" and the gelatinase B "MMP-9") were identified by electrophoresis in the presence of SDS in 8% polyacrylamide gels containing 1 mg/ml gelatin. In brief, culture medium aliquots (20 μl) were directly loaded onto gels, and, after electrophoresis, proteins were renatured by exchanging SDS with 2.5% Triton X-100 (20-min incubation repeated twice). The gels were then incubated for 16 h at 37°C in 50 mM Tris-HCl (pH 8.8), 5 mM CaCl$_2$, and 0.02% NaN$_3$ and stained with Coomassie Blue. The presence of gelatinolytic activity in the culture medium aliquots was visualized as clear areas on an otherwise blue gel. Migration of proteins was compared with that of prestained molecular weight markers. The gels were scanned by an imaging densitometer and quantified using the NIH Image program (developed at the National Institutes of Health, Bethesda, MD).

Fluorimetric Proteasome Activity Assay. Chymotrypsin-like proteasome activity was performed on 96-well plates using the specific fluorogenic substrate N-succinyl-Leu-Leu-Val-Tyr$_7$-amido-4-methylcoumarin (Sigma). In brief, cells contained in one well were scraped in 35 μl of lysis buffer containing 25 mM Tris-HCl (pH 7.5), 0.1% Nonidet P-40, 10% glycerol, 5 mM MgCl$_2$, 1 mM ATP, and 10 mM KCl. Protein concentration was determined using a Bio-Rad kit (Bio-Rad, Marnes-la Coquette, France), and 10 μg were incubated alone or with increasing concentrations of lactacystin (0.01–10 μM) or HIV-PIs (1, 10, and 50 μM) for 30 min at 37°C in 20 mM Tris-HCl (pH 8), 0.5 mM EDTA, and 0.035% SDS to assess 20S proteasome activity or in 20 mM Tris-HCl (pH 8), 1 mM ATP, and 2 mM MgCl$_2$ to assess 26S proteasome activity, according to the method of Rock et al. (1994). Then, fluorogenic substrate was added to a final concentration of 50 μM, and fluorescence was monitored at $\lambda_{excitation}$ 360

nm/$\lambda_{emission}$ 460 nm every 5 min over a 2-h period using a Fluoroscan Ascent FL (Labsystems, Cergy Pontoise, France). Chymotrypsin-like proteasome activities of 20S and 26S were evaluated by the maximal rate (relative fluorescence units per minute) on seven separate measurements in each well.

Western Blot. For Western blot analysis of IκBα and IκBβ, cells contained in two wells were scraped in 70 μl of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM Na$_3$VO$_4$, 1% Nonidet P-40, 10% glycerol, and a mix of protease inhibitors (Complete; Roche Diagnostics). The protein concentration was determined, and 35 to 50 μg of protein were loaded and separated by electrophoresis on a 12% SDS-polyacrylamide gel under denaturing conditions. After transfer to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA) and Ponceau staining to verify equal loading of the lanes, membranes were blocked for 1 h with TBST [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20] containing 5% nonfat milk, following by incubation overnight at 4°C with primary antibody against IκBα (ab7545) or IκBβ (ab7547) (Abcam, Cambridge, UK) at a dilution of 1:1000 in TBST containing 1% nonfat milk. Then, membranes were washed with TBST and incubated for 1 h with a peroxidase-conjugated secondary antibody (Pierce Chemical, Brebières, France) at a dilution of 1:50,000 in TBST containing 1% nonfat milk and washed again. The immunocomplexes were detected using a chemiluminescence reagent kit (SuperSignal West Dura Extended Duration Substrate; Pierce Chemical, Rockford, IL). Human Daudi cell lysate (Imgenex, San Diego, CA) was used as positive control.

For Western blot analysis of NF-κB p65, cells contained in 12 wells were washed twice with cold phosphate-buffered saline and prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce) according to the manufacturer's instructions. Protein concentrations in nuclear and cytoplasmic extracts were determined as described above: 20 to 45 μg of proteins were loaded and separated by electrophoresis on a 12% SDS-polyacrylamide gel electrophoresis under denaturing conditions. After transfer to polyvinylidene difluoride membranes and Ponceau staining to verify equal loading of the lanes, a WesternBreeze Chemiluminescent Western Blot Immunodetection Kit (Invitrogen, Cergy Pontoise, France) was used for signal detection. Briefly and according to the manufacturer's instructions, membranes were blocked, washed, and then incubated with a primary antibody against NF-κB p65 N-terminal (Santa Cruz Biotechnology, Le Perray-en-Yvelines, France) at a dilution of 1:1000. After washing, membranes were incubated with an alkaline phosphatase-conjugated secondary antibody provided with the kit, and immunocomplexes were detected after the addition of chemiluminescent substrate. Human nuclear extract from A-431 cells (Santa Cruz Biotechnology) was used as positive control.

The autoradiographs were scanned by an imaging densitometer and quantified using the NIH Image program. To normalize results, the densitometric value of each band of interest (i.e., IκBα, IκBβ, and NF-κB p65) was reported to the densitometric value of total loaded proteins in the corresponding lane, obtained after scanning and quantification of the Ponceau staining (NIH Image program).

Real-Time RT-PCR. Changes in mRNA levels from specific genes were quantified by real-time RT-PCR. Total RNAs were extracted using a QIAGEN RNeasy Mini Kit according to the manufacturer's instructions, and RNA concentrations were determined using a Molecular Probes fluorimetric assay (RiboGreen; Interchim). RNA (0.5 μg) was reverse-transcribed using the Superscript II system (Invitrogen) according to the manufacturer's instructions. Reverse transcription was also performed without Thermoscript enzyme on RNA samples to check for any genomic DNA contamination. PCR primers were designed using Primer Express software according to the recommendations of Applied Biosystems (Courtaboeuf, France). The forward and reverse primer sequences for fatty acid binding protein (aP2), hormone-sensitive lipase (HSL), MMP-9, MMP-2, and IκBβ were as follows (5′–3′): aP2, GCATGGCCAAAC-CTAACATGA (forward) and CCTGGCCCAGTATGAAGGAAA (re-

verse); HSL, GTGCAAAGACGGAGGACCACTCCA (forward) and GACGTCTCGGAGTTTCCCCTCAG; MMP-9, CCCTGGAGACCTG-AGAACCA (forward) and CCACCCGAGTGTAACCATAGC (reverse); MMP-2 CACCCATTTACACCTACACCAAGA (forward) and AGAGCTCCTGAATGCCCTTGA (reverse), and IκBβ TACGACGAC-ATTGTGGTTCACA (forward) and GGGTCGTCAGGAAGAGGTTTT (reverse).

Each amplification reaction was performed with 15 ng of cDNA sample in duplicate in 96-well optical reaction plates with a Gene-Amp 7500 sequence detection system. The PCR mixture contained forward and reverse primer mix (final concentration: 900 nM for HSL, MMP-9, or MMP-2 and 300 nM for aP2 or IκBβ) and SYBR Green PCR Master Mix. For ribosomal RNA control (18S rRNA), a mixture containing primers and fluorogenic probe mix, TaqMan Universal PCR Master Mix (Applied Biosystems) was used. All reactions were performed under the same conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were analyzed with the GeneAmp 7500 software, and all values were normalized to the levels of 18S rRNA. An interindividual variability of 10.46% within the control values was observed for all PCR data (aP2, HSL, MMP-2, MMP-9, and IκBβ).

Statistical Analysis. Values are expressed as means $\pm$ S.E.M. from n independent experiments. Statistical analysis was performed using Student's t test for paired data or one-way analysis of variance (ANOVA) coupled with post hoc Dunnett's multiple-comparison test (GraphPad Prism 4; GraphPad Software Inc., San Diego, CA). Values of $p < 0.05$ were considered statistically significant.

Results

HIV-PIs or Lactacystin Reduced Proteasome Activity from Human Preadipocyte Homogenates. To validate our hypothesis, we first investigated the effect of HIV-PIs on proteasome activity of human preadipocytes. After the 3-day priming period, undifferentiated preadipocytes were scraped into specific lysis buffer, and cellular homogenates were obtained. Then, homogenates were pretreated (30 min, 37°C) with increasing concentrations of the proteasome inhibitor lactacystin (from 0.01 to 10 μM) or HIV-PIs (1, 10, and 50 μM), and 20S and 26S chymotrypsin-like proteasome activities from these lysates were determined by in vitro fluorescence assays with the specific fluorogenic substrate N-succinyl-Leu-Leu-Val-Tyr$_7$-amido-4-methylcoumarin.

As expected, lactacystin reduced, in a concentration-dependent manner, the fluorescence emission induced by the cleavage of the substrate by the 20S and 26S proteasome (control maximal rate: 1.62 $\pm$ 0.84 and 1.13 $\pm$ 0.21 RFU/min, respectively) (Fig. 1, top and bottom). Similar results were obtained with another known proteasome inhibitor, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) (data not shown). As shown on the top of Fig. 1, HIV-PIs, except IDV, decreased chymotrypsin-like activity of the 20S proteasome in a concentration-dependent manner with the following rank of efficiency: SQV $\geq$ NFV > RTV. Higher concentrations of HIV-PIs were necessary to reduce the 26S chymotrypsin-like proteasome activity in the cellular extracts (Fig. 1, bottom).

Chronic Lactacystin Treatment Decreased the Differentiation of Human Preadipocytes. To determine whether the inhibition of the proteasome per se could affect the human adipocyte differentiation process, primary cultures of human preadipocytes were placed in an adipogenic medium in the presence of 0.1 or 0.5 μM lactacystin for 10 days. Cellular triglyceride was quantified, and the expression of two adipocyte differentiation markers, aP2 and HSL, was analyzed by real-time RT-PCR.
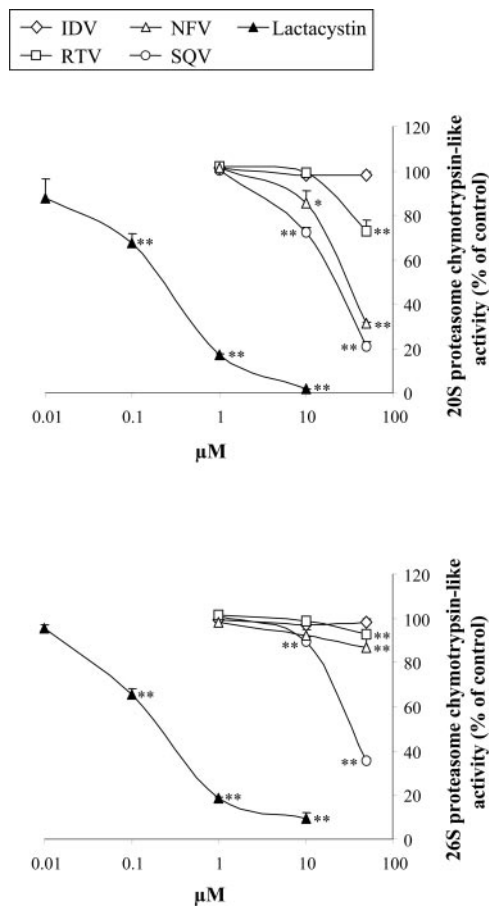
Fig. 1. Effect of HIV-PIs on chymotrypsin-like proteasome activity in human preadipocyte homogenates. Stromal preadipocytes were isolated from human subcutaneous AT. After the 3-day priming period, cells were washed and maintained overnight in basal medium before being scrapped in specific lysis buffer. Ten micrograms of protein obtained from cellular homogenates were then preincubated (30 min, 37°C) with or without increasing concentrations of lactacystin (0.01 and 10 μM) or HIV-PIs (1, 10, and 50 μM). The proteasome fluorogenic substrate N-succinyl-Leu-Leu-Val-Tyr$_7$-amido-4-methylcoumarin was added and fluorescence ($\lambda_{excitation}$ 360 nm/$\lambda_{emission}$ 460 nm) was recorded every 5 min over a 2-h period. Data are means ± S.E.M. of maximal rate (relative fluorescence units per minute) for seven separate measurements, expressed as a percentage of the control for four to five independent experiments. ∗, $p <$ 0.05 versus control; ∗∗, $p <$ 0.01 versus control.

As clearly shown in Fig. 2A, 0.5 μM lactacystin led to a strong inhibition of the adipocyte differentiation process because treated cells exhibited less cytoplasmic accumulation of lipid droplets. Quantification of cellular triglyceride (TG) content, used as a lipogenic index, demonstrated that lactacystin treatment significantly decreased adipogenesis (36% decrease in triglyceride concentration, i.e., 26.58 ± 8.28 mg of TGs/mg of DNA for controls to 13.68 ± 3.12 mg of TGs/mg of DNA for lactacystin) (Fig. 2B). Moreover, the analysis of aP2 and HSL mRNA levels showed that lactacystin led to a reduction of the expression of both adipocyte differentiation markers (41 and 60% decreases in aP2 and HSL mRNA expression, respectively) (Fig. 2B). It has to be noticed that lactacystin at 0.1 μM had no effect on the differentiation process and that both 0.1 and 0.5 μM lactacystin were free of any cytotoxic effects (data not shown). However, the use of concentrations higher than 1 μM was associated with cell death within several days (data not shown).

Chronic Lactacystin Treatment of Human Preadipocytes Reduced MMP-9 Expression and MMP-9 Gelatinase Activity Released into the Medium. MMP-9 activity has been shown 1) to be involved in the human adipocyte differentiation process (Bouloumié et al., 2001; Bourlier et al., 2005) and 2) to be reduced in human preadipocytes treated by HIV-PIs (Bourlier et al., 2005). To evaluate whether the inhibition of the proteasome per se could affect MMP-9 secretion and expression, overnight-conditioned medium from preadipocytes treated with 0.5 μM lactacystin for 10 days was collected to be analyzed by gelatin zymography, and real-time RT-PCR analysis was performed on mRNAs.
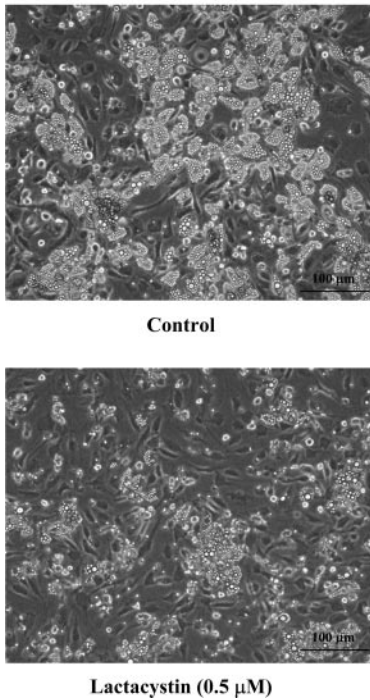
As observed in Fig. 3A, MMP-9 (92 kDa) and MMP-2 (72 kDa) proforms (artificially activated by the gelatin zymography technique) were the major forms detectable in preadipocyte-conditioned media. The active form was visible for MMP-2 (62 kDa) but not for MMP-9 (82 kDa). Densitometric analysis of the lytic areas showed that lactacystin treatment reduced the MMP-9 gelatinase activity (proform) released by treated preadipocytes (53.2 ± 2.7% of the control value, $n =$ 4, $p <$ 0.001). Interestingly, this decrease of MMP-9 gelatinase activity in the medium, corresponding to the proform, was not accompanied by an increase of the active-MMP-9 form (still not detectable), suggesting that this reduction was due to a decrease in the secretion of the enzyme rather than to an increase in the maturation. In contrast to MMP-9, the MMP-2 gelatinase activity (proform + active forms) was not affected by lactacystin treatment (112.2 ± 11.8% of the control value, $n = 4$).

The analysis of MMP mRNA expression (Fig. 3B) was consistent with the data obtained by gelatin zymography because lactacystin treatment led to a statistically significant reduction of MMP-9 expression (45% decrease), whereas no change was observed for MMP-2 expression. Thus, the observed decrease of MMP-9 gelatinase activity released into the medium under lactacystin treatment was probably due to a reduction of MMP-9 expression.

Chronic Treatment with HIV-PIs or Lactacystin Had No Effect on IκBα but Increased IκBβ Protein Level in Human Preadipocytes. To identify the link between the inhibition of the proteasome activity and the decrease in MMP-9 expression, we studied the effect of chronic lactacystin or HIV-PI treatment on IκBα and IκBβ proteins, known substrates of the proteasome. The degradation of these two well-known IκB family members leads to NF-κB translocation to the nucleus (Whiteside and Israël, 1997) and transcription of NF-κB target genes, among them MMP-9 (St-Pierre et al., 2004). Human preadipocytes were placed in an adipogenic medium and treated for 10 days with lactacystin or various HIV-PIs at concentrations for which no cytotoxic effect was detected and the inhibition of the differentiation was maximal according to our previous publication (Bourlier et al., 2005). Cellular extracts were obtained and analyzed by the Western blot technique.

As shown in Fig. 4A, none of the treatments led to the accumulation of IκBα in human preadipocytes. In contrast, the quantification of the IκBβ band clearly indicated that SQV, NFV, and lactacystin treatment increased the total cellular level of IκBβ protein (83, 36, and 75% increases, respectively) in human preadipocytes (Fig. 4B). This effect was not observed with the two other HIV-PIs, IDV and RTV.
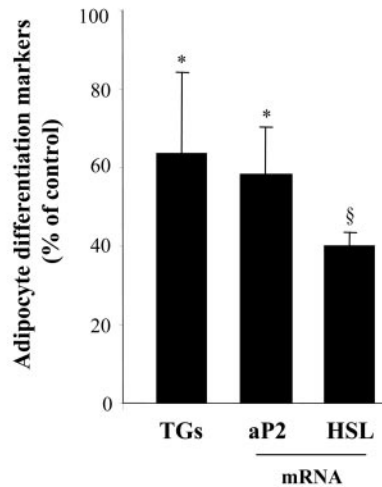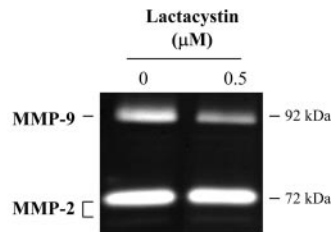
A

Control

Lactacystin (0.5 μM)

B

Fig. 2. Effect of chronic lactacystin treatment on the differentiation of human preadipocytes. Stromal preadipocytes from human subcutaneous AT were cultured in an adipogenic medium in the presence of 0.5 μM lactacystin or its vehicle (control). A, light microscopy analysis was performed after 10 days of treatment. Photomicrographs (100×) are representative of five independent experiments. B, cellular TGs, as well as aP2 and HSL mRNA expression, were determined in cells maintained overnight in basal medium with treatment. Cellular triglycerides were normalized to DNA content. For aP2 and HSL expression, total RNAs were extracted from cells and reverse-transcribed, and real-time RT-PCR analysis was performed using specific cDNA primers. The results obtained were normalized to the levels of 18S rRNA. The presented data are means ± S.E.M. expressed as a percentage of the control for five independent experiments. ∗, $p < 0.05$ versus control; §, $p < 0.001$ versus control.
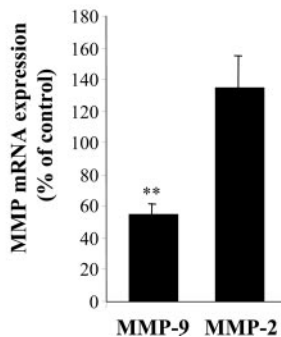
A

B

Fig. 3. Effect of chronic lactacystin treatment on MMP-9 and -2 gelatinase activities released into the medium and on their expression by human preadipocytes. Stromal preadipocytes from human subcutaneous AT were cultured in an adipogenic medium with 0.5 μM lactacystin or its vehicle (control). After 10 days of culture, the cells were washed and maintained overnight in basal medium with or without treatment. A, culture media were then collected and analyzed by gelatin zymography. The presented gel is representative of four independent experiments. Quantification of the lytic areas was performed with the NIH Image program. B, total RNAs were extracted from cells and real-time RT-PCR analysis was performed using specific primers for MMP-9 and MMP-2 cDNAs. The results were normalized to the levels of 18S rRNA. The presented data are means ± S.E.M. expressed as a percentage of the control for five independent experiments. ∗∗, $p < 0.01$ versus control.

It has to be noticed that homogeneity of the controls (i.e., IDV, RTV, NFV, SQV, and lactacystin controls) was checked using one-way ANOVA (not significant, $p = 0.83$). The control raw value obtained for the densitometric analysis of IκBβ band normalized to total loaded proteins per lane was 0.095 ± 0.019 (arbitrary units).

To verify that the increase in IκBβ protein level was not due to an effect of treatments on IκBβ expression, RT-PCR analysis was performed on mRNAs of preadipocytes treated for 10 days with NFV (5 μM), SQV (10 μM), or lactacystin (0.5 μM), when accumulation of the protein was observed. As shown in Fig. 4C, none of the treatments significantly affected IκBβ expression, suggesting that the accumulation of the protein in the cytosol was due mainly to a decrease of its degradation, i.e., a decrease in proteasomal activity. However, a contribution of IκBβ mRNA expression with NFV can not be excluded, whereas no statistical significance was found compared with control values ($p = 0.07$, $n = 6$), according to the particular variability of the data in this condition. As for the Western blot experiment, homogeneity of the controls (i.e., NFV, SQV, and lactacystin controls) was checked using one-way ANOVA (not significant, $p = 0.90$). The control raw value obtained for the relative amount of IκBβ mRNA normalized to 18S rRNA (i.e., $2^{\Delta Ct} \times 10^4$) was 1.42 ± 0.25 (arbitrary units).

Chronic Treatment with SQV or Lactacystin Did Not Lead to NF-κB p65 Accumulation in the Cytoplasm of Human Preadipocytes. According to the very similar profile in IκBβ protein increase between SQV and lactacystin chronic treatment, we measured the NF-κB p65 activity in 10-day-treated preadipocytes because this subunit contains the transactivation domain necessary for gene transcription (Hayden and Ghosh, 2004) and has been recently implicated
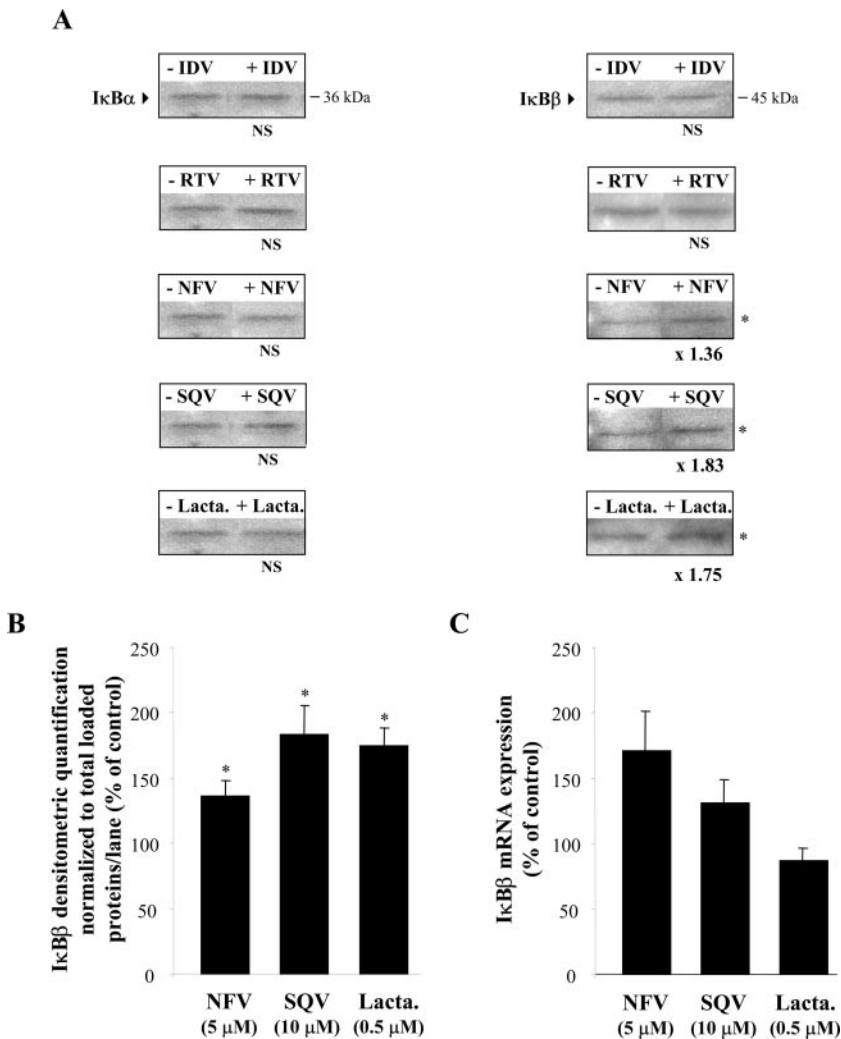
A

B

C

Fig. 4. Effect of chronic HIV-PIs or lactacystin treatment on IκBα and IκBβ protein level in human preadipocytes. Stromal preadipocytes from human subcutaneous AT were cultured in an adipogenic medium with vehicle (control) or in the presence of lactacystin (Lacta.; 0.5 μM) or HIV-PIs: IDV (50 μM), RTV (10 μM), SQV (10 μM), or NFV (5 μM). After 10 days of culture, the cells were washed and maintained overnight in basal medium with treatment before being scrapped in a specific lysis buffer. Thirty-five to 50 μg of total proteins obtained after lysis were then analyzed by Western blot using a specific antibody. A, representative autoradiographs of the IκBα and the IκBβ blots from four to seven independent experiments are shown. Statistically significant results are expressed as ratio of the control values. NS, nonsignificant. ∗, $p < 0.05$ versus control. B, quantification of the corresponding bands was performed with the NIH Image program and normalized to the density of the total loaded proteins per lane, obtained from the analysis of the Ponceau staining. The control value of the densitometric ratio was 0.095 ± 0.019. The presented data are means ± S.E.M. expressed as a percentage of the control. ∗, $p < 0.05$ versus control. C, total RNAs were extracted from cells and reverse-transcribed, and real-time RT-PCR analysis was performed using specific primers for IκBβ cDNAs. The results were normalized to the levels of 18S rRNA. Control value of the $2^{\Delta Ct} \times 10^4$ was 1.42 ± 0.25. The presented data are means ± S.E.M. expressed as a percentage of the control for five to six independent experiments.

in adipocyte differentiation of the 3T3-L1 cell line (Berg et al., 2004). Unfortunately, we were unable to detect NF-κB p65 activity in any of our conditions (control or treated preadipocytes) using the TransAM NF-κB chemiluminescent transcription factor assay kit (Active Motif Europe, Rixensart, Belgium), following the manufacturer's instructions. Assays were performed with either cellular or nuclear preadipocyte extracts, but signal corresponding to free active NF-κB p65 was always at the blank level (even with the highest protein concentration required), whereas signal from the positive control (Jurkat nuclear extract) of the kit was detected. One possible explanation was a very low presence of active NF-κB p65 under basal conditions.

We decided to also perform NF-κB p65 Western blot analysis of cytoplasmic and nuclear extracts from 10-day-treated preadipocytes to investigate whether NF-κB p65 translocation was affected by IκBβ protein accumulation induced by SQV or lactacystin. As observed in Fig. 5A (left panel), NF-κB p65 subunit was undetectable ($n = 3$) or not analyzable ($n = 2$) in nuclear extracts from control or treated human preadipocytes. This observation was in agreement with the negative results obtained with the TransAM assay kit. However, NF-κB p65 was clearly present in the cytoplasmic extracts, but quantification of the corresponding bands did not reveal any significant difference in protein level between control

versus treated preadipocytes (Fig. 5B). To validate the cytoplasmic to nuclear translocation of the NF-κB p65 assay, control experiments were performed using human preadipocytes stimulated with TNF-α (25 ng/ml) for 1 h. Indeed, TNF-α is a standard activator of the NF-κB pathway in a variety of cells (Viatour et al., 2005). As observed in Fig. 5A (right panel), cytoplasmic NF-κB p65 was decreased with TNF-α compared with controls (55% decrease, $n = 3$, $p < 0.05$). Furthermore and although no NF-κB p65 was detectable in the nucleus, a new band of ~35 kDa was clearly visible under TNF-α stimulation. This band has already been identified as a product of nuclear proteolysis of NF-κB p65 (N-terminal fragment), termed NF-κB p35, and observed in the physiological turnover of the transcription factor (Cressman and Taub, 1994). This result indicates that NF-κB p65 has been translocated to the nucleus, validating our technique.

Acute Treatment with SQV, Lactacystin, or BAY 11-7082 Reduced TNF-α-Induced MMP-9 Expression in Human Preadipocytes. Because one of the possible limitations of our experiments to clearly identify NF-κB was that cells were under basal conditions, we decided to investigate the acute effect of SQV and lactacystin on MMP-9 expression induced by TNF-α. After the 3-day priming period, human preadipocytes were placed in an adipogenic medium and
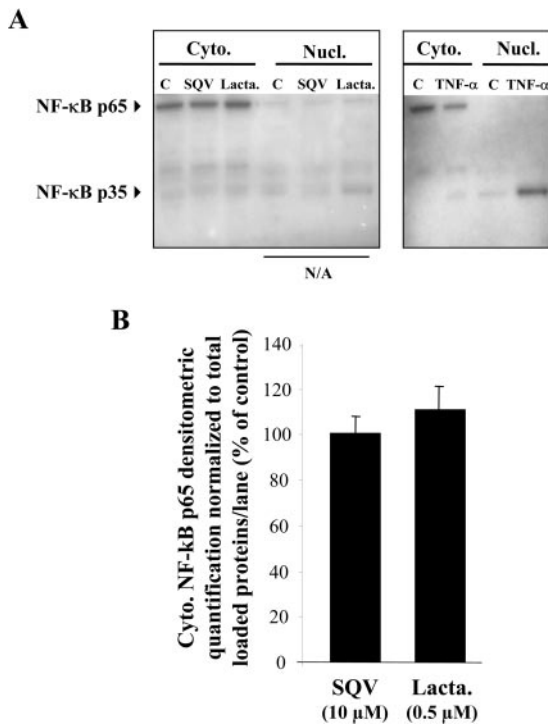
Fig. 5. Effect of chronic SQV or lactacystin (Lacta.) treatment on cytoplasmic and nuclear NF-κB p65 protein level in human preadipocytes. Stromal preadipocytes from human subcutaneous AT were cultured in an adipogenic medium with vehicle (control) or in the presence of lactacystin (0.5 μM) or SQV (10 μM). After 10 days of culture, the cells were washed and maintained overnight in basal medium with treatment before being scrapped and prepared using NE-PER nuclear and cytoplasmic extraction reagents kit. Twenty to 45 μg of proteins from cytoplasmic (Cyto.) or nuclear (Nucl.) extracts were then analyzed by Western blot using a specific anti-NF-κB p65 subunit antibody. A representative autoradiograph of five independent experiments is shown (A, left panel). Quantification of the corresponding bands in the cytoplasmic extracts was performed with the NIH Image program and normalized to the density of the total loaded proteins per lane, obtained from analysis of Ponceau staining (B).The data presented are means ± S.E.M. expressed as a percentage of the control. N/A, not analyzable. To validate the technique, human preadipocytes were acutely stimulated with TNF-α (25 ng/ml, 1 h), and Western blot analysis was performed. A representative autoradiograph of 3 independent experiments is shown (A, right).

treated for 24 h with TNF-α (25 ng/ml) alone or in cotreatment with SQV (10 μM), lactacystin (0.5 μM), or BAY 11-7082 (1 μM), an inhibitor of IκB phosphorylation, which is involved in the NF-κB activation (Pierce et al., 1997). MMP-9 expression was analyzed by real-time RT-PCR.

As shown in Fig. 6, TNF-α treatment strongly enhanced MMP-9 expression (10 times) in human preadipocytes. As expected, cotreatment with BAY 11-7082, the inhibitor of the NF-κB pathway, reduced the MMP-9 expression induced by TNF-α (27% decrease). Similar results were obtained with SQV or lactacystin cotreatment, because MMP-9 expression was decreased by 48 and 22%, respectively, implicating NF-κB as the link between proteasome activity and MMP-9 expression in our experiments. It should be noted that similar data were obtained when SQV was replaced by NFV (5 μM) or when stimulation was performed with interleukin-1β (20 ng/ml) in place of TNF-α (data not shown). Compared with TNF-α, which has been shown to involve preferentially IκBα, interleukin-1β is known to induce both IκBα and IκBβ degradation when activating the NF-κB pathway (Thompson et al., 1995).
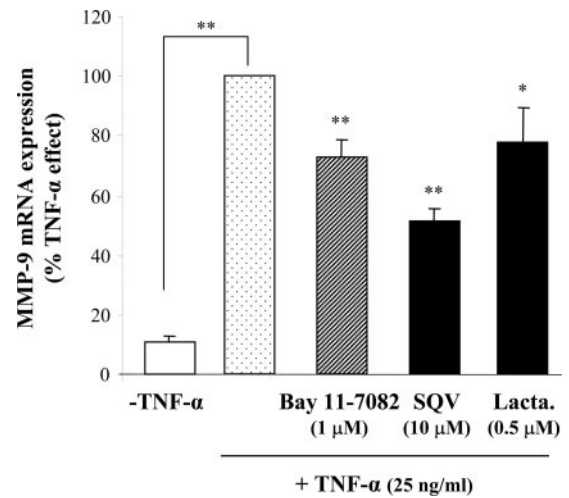

Fig. 6. Effect of acute SQV or lactacystin treatment on TNF-α-induced MMP-9 expression in human preadipocytes. Stromal preadipocytes were isolated from human subcutaneous AT. After the 3-day priming period, cells were placed in an adipogenic medium for 24 h, with or without TNF-α (25 ng/ml) alone or in cotreatment with lactacystin (0.5 μM), SQV (10 μM), or BAY 11-7082 (1 μM), an inhibitor of NF-κB activation. Total RNAs were then extracted from cells and reverse-transcribed, and real-time RT-PCR analysis was performed using specific primers for MMP-9 cDNAs. The results were normalized to the levels of 18S rRNA. The presented data are means ± S.E.M. expressed as a percentage of the TNF-α effect alone for three to four independent experiments. *, p < 0.05 versus TNF-α alone; **, p < 0.01 versus TNF-α alone.

Discussion

We previously reported that HIV-PIs reduced the differentiation of human preadipocytes in primary culture. This effect was attributed to the HIV-PI-induced decrease of MMP-9 expression and secretion since we showed that blocking of MMP-9 activity by pharmacological agents led to the inhibition of human adipogenesis (Bourlier et al., 2005). In the present work, we report that HIV-PIs decreased the activity of the two major proteasome complexes, the 20S and the 26S proteasomes, in human preadipocytes. Interestingly, we showed that lactacystin, a proteasome inhibitor, mimicked the effects of HIV-PIs in reducing both human adipogenesis and MMP-9 expression and secretion. Finally, some of our results suggested that perturbations of the NF-κB pathway are the link between the inhibition of proteasomal activity and the decrease in MMP-9 expression. Indeed, even if we were unable to detect NF-κB p65 signal changes under basal conditions, we did observe that chronic treatments (10 days) with HIV-PIs (SQV and NFV) or lactacystin induced cellular IκBβ accumulation. Furthermore, SQV or lactacystin reduced the expression of MMP-9 induced by acute treatment (24 h) with TNF-α, a classic activator of NF-κB.

The inhibition of proteasomal activity and, more precisely, proteasome chymotrypsin-like activity by HIV-PIs was initially reported few years ago by André et al., (1998) and linked to a decrease of antigen presentation and T-cell response in infected mice. Here we show, for the first time, that proteasomes from human adipocyte precursors, which are major cell targets of HIV-PIs involved in the lipodystrophic syndrome, are also directly inhibited by several HIV-PIs at therapeutic concentrations. These data agree with recent observations made with the murine the 3T3-L1 cell line model of preadipocytes (Parker et al., 2005). As have other investigators, we found that the 20S proteasome was more

sensitive to HIV-PIs than the 26S proteasome (Gaedicke et al., 2002; Pajonk et al., 2002). One reason may be the different tertiary structure between the 20S and 26S proteasomes, leading to better accessibility of the inhibitors to the proteolytic sites of the 20S proteasome (Piccinini et al., 2005, Voorhees and Orlowski, 2006). We found that all HIV-PIs did not have the same potency in inhibiting the chymotrypsin-like proteasome activity in our model. Indeed SQV and NFV were the most effective, followed by RTV, whereas IDV was without effect. Similar observations were made in the rare publications comparing the effect of HIV-PIs on human proteasomal activity measured on purified proteasome from erythrocytes (Piccinini et al., 2002, 2005; Parker et al., 2005). It has to be noted that even if all HIV-PIs were weaker inhibitors of proteasomal activity than the specific lactacystin inhibitors in our in vitro assay, SQV and NFV (not RTV nor IDV), can accumulate greatly into cells (20 to >80 times) (Jones et al., 2001; Janneh et al., 2003). Thus, it can be expected that intracellular concentrations of these HIV-PIs are much higher that the ones tested here.

Interestingly, SQV and NFV were the HIV-PIs with the major effect on the differentiation of human preadipocytes in our previous work (Bourlier et al., 2005). We thus investigated whether a blockade of proteasomal activity can affect human adipogenesis. We showed that chronic lactacystin treatment of preadipocytes in an adipogenic medium led to a decrease of the various differentiation markers studied (triglyceride content and aP2 and HSL expression). Although some authors have already implicated the proteasome in adipogenesis, their observations were on a different model, the murine 3T3-L1 cell line, using more or less specific proteasome inhibitors (calpain/proteasome), and contradictory results were obtained (Patel and Lane, 1999; Nguyen et al., 2000; Prince et al., 2002). As expected and in association with the reduction of adipogenesis, we found that lactacystin treatment, as that with HIV-PIs, selectively decreased MMP-9 expression and activity released into the culture medium by preadipocytes. The reduction of MMP-9 activity per se may be responsible for the antiadipogenic effect of lactacystin since we have previously demonstrated that treatment of human preadipocytes with pharmacological inhibitors of MMP-9 activity decreased their differentiation (Bourlier et al., 2005). However we cannot exclude any other additional phenomenon implicated in adipogenesis that might be disturbed by proteasome inhibitor treatment.

Reduction of MMP-9 secretion with proteasome inhibitors has previously been reported for distinct cellular types in vitro involving NF-κB pathway perturbations (Ikebe et al., 1998; Kolev et al., 2003; Lu and Wahl, 2005). We were not able to find any modification in the activity and repartition of NF-κB p65 within the cytosol and the nucleus in association with chronic SQV or lactacystin treatment in our model, using two different techniques (enzyme-linked immunosorbent assay-based TransAM NF-κB assay kit and Western blot). This may be partly explained by the fact that in the resting conditions that we studied, the part of active NF-κB p65 was too weak to be detected. This hypothesis is in agreement with one of the rare published reports on the subject showing that NF-κB p65 is mostly cytosolic in unstimulated human preadipocytes (Chung et al., 2005). Nevertheless, we showed that the same chronic treatment with NFV, SQV, or lactacystin (but not with IDV nor RTV) led to intracellular

accumulation of IκBβ protein. In contrast, none of the HIV-PIs or lactacystin was effective in modifying the IκBα protein level. These results agree with the presumed roles of these two members of the same family because 1) IκBα has been correlated to transient activation of NF-κB and IκBβ to persistent activation that yields a more permanent change (Thompson et al., 1995) and 2) IκBβ, but not IκBα, has been shown to function as a classic cytoplasmic inhibitor of NF-κB dimers in resting cells (Malek et al., 2001). Because both NF-κB inhibitors have been shown to be substrates of the proteasome (Weil et al., 1997) and because no parallel increase in IκBβ mRNA expression was observed under our conditions, the increase in IκBβ protein most probably reflects the reduction of its degradation resulting from proteasomal activity inhibition induced by the treatments. Furthermore, a different set of experiments conducted with the classic NF-κB pathway activator TNF-α (Viatour et al., 2005) showed that MMP-9 expression was up-regulated within 24 h of treatment in our model, but also that this up-regulation could be reduced either by SQV, lactacystin, or the NF-κB inhibitor, Bay 11-7082. Overall, according to these data on resting and TNF-α-stimulated human preadipocytes, we can easily imagine that the decrease in MMP-9 expression observed with lactacystin and some HIV-PIs (SQV and NFV) arose in part from perturbations in the NF-κB pathway.

To our knowledge, this is the first investigation showing that HIV-PIs are potent inhibitors of human preadipocyte proteasomal activity and that chronic low-level proteasome inhibition reduces human adipogenesis. Disorders of the NF-κB pathway, resulting from proteasome inhibition may partly explain the decrease in MMP-9 expression and secretion potentially responsible for adipogenesis reduction (Bourlier et al., 2005) and observed with both lactacystin and HIV-PI treatment. Our in vitro results suggest that HIV-PI-induced perturbation of preadipocyte to adipocyte balance noted in clinical studies (Bastard et al., 2002; Lloreta et al., 2002) may also target preadipocyte proteasome function. Interestingly, a role for the proteasome in more metabolic aspects of the HIV-related lipodystrophic syndrome has previously been reported. Indeed, apolipoprotein B and sterol regulatory element binding protein degradation have been shown to be altered by HIV-PI-related proteasome inhibition and involved in lipid metabolism disorders (Liang et al., 2001; Hui, 2003). More recently, Parker et al. (2005) has linked the reduction of proteasomal activity induced by HIV-PIs and the dyslipidemia to endoplasmic reticulum stress. Finally, attention is now being focused on HIV-PI-induced proteasomal alterations and disturbances of glucose metabolism because proteasome dysfunction and/or endoplasmic reticulum stress has been closely related to insulin resistance (Ozcan et al., 2004; Rome et al., 2004). All of this information will help to better understand the etiology of the lipodystrophic syndrome but may also be of great interest for the recent clinical use of proteasome inhibitors in cancer therapy (i.e., bortezomib treatment for multiple myeloma) and possible effects on adipose tissue development.

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