Regulation of adipocyte lipolysis by degradation of the perilipin protein: Nelfinavir enhances lysosome-mediated perilipin proteolysis

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Running title: lipolysis through proteolysis of perilipin

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Abstract

A decrease in the lipid droplet-associated protein perilipin may constitute a mechanism for enhanced adipocyte lipolysis under non-stimulated (basal) conditions, and increased basal lipolysis has been linked to whole-body metabolic dysregulation. Here we investigated whether the lipolytic actions of the HIV protease inhibitor, nelfinavir, are mediated by decreased perilipin protein content, and studied the mechanisms by which it occurs. Time-course analysis revealed that the decrease in perilipin protein content preceded the increase in lipolysis. A causative relationship was suggested by demonstrating that nelfinavir potently increased lipolysis in adipocytes derived from mouse embryonal fibroblasts (MEF) expressing perilipin, but not in MEF adipocytes devoid of perilipin, and that adenoviral mediated over-expression of perilipin in 3T3-L1 adipocytes blocked nelfinavir’s lipolytic actions. Nelfinavir did not alter mRNA content of perilipin, but rather decreased perilipin proteins t1/2 from >70h to 12h. Protein degradation of perilipin in both control and nelfinavir-treated adipocytes could be prevented by inhibiting lysosomal proteolysis using leupeptin or NH₄Cl, but not by the proteasome inhibitor MG-132. We propose that proteolysis of perilipin involving the lysosomal protein degradation machinery may constitute a novel mechanism for enhancing adipocyte lipolysis.

Introduction

The lipid droplet-associated protein perilipin is thought to function as a critical regulator of basal (non-stimulated) adipocyte lipolysis (1,2), a process central to maintain normal energy balance and utilization (3,4). In response to lipolytic stimulation by beta-adrenergic agents, PKA-mediated phosphorylation of perilipin renders it a facilitator of lipolysis, at least partly by altering the subcellular localization of cellular lipases (5,6). Conversely, in the absence of a lipolytic stimulus (basal or constitutive lipolysis), perilipin inhibits lipase(s) activity, an action commonly described as the "barrier function" between cellular lipases and their substrate (triglycerides comprising the lipid droplets)(7,8). The regulatory role of this function of perilipin was suggested by demonstrating that TNFα treatment of murine adipocytes reduced perilipin expression while increasing lipolysis, whereas overexpression of perilipin A blocked the lipolytic effect of TNF (9). The physiological role of perilipin as an inhibitor of basal lipolytic rate was confirmed in perilipin knockout mice, which exhibit a lean phenotype attributed to elevated basal lipolysis (10,11). In humans, recent evidence suggests that polymorphisms in the perilipin gene may predict vulnerability to obesity (12-15). In studies where perilipin protein expression was examined in isolated adipocytes, perilipin was found to be decreased in obese individuals, suggesting a mechanism for the elevated levels of circulating fatty acids typically observed in obesity (16,17). Intriguingly, protein expression of perilipin does not always correlate with the mRNA level (17-19), suggesting that post-transcriptional mechanisms participate in regulating perilipin...
protein content, and thereby, adipocyte lipolytic rate.

Enhanced rate of lipolysis is likely a common metabolic side-effect of Highly Active Anti-Retroviral Therapy (HAART). Patients receiving HAART develop a dyslipidemia characterized by enhanced lipid mobilization and elevated levels of circulating free fatty acids (FFA)(20). In addition, the lipolysis rate was increased when assessed directly in-vivo (21). Furthermore, dyslipidemia in HAART patients, particularly in patients receiving certain HIV protease inhibitors, is frequently associated with fat tissue redistribution (loss of subcutaneous "peripheral" fat and accumulation of intra-abdominal fat). Thus, enhanced lipolysis may contribute to both the frequently observed loss of subcutaneous fat mass, as well as to the dyslipidemia (22).

Investigating the cellular mechanisms for enhanced adipocyte lipolysis in response to HIV protease inhibitors (HPI) revealed that nelfinavir-induced lipolysis rate in 3T3-L1 adipocytes was associated with decreased protein levels of perilipin (23). Furthermore, the HIV protease inhibitor ritonavir had no effect on cAMP levels nor on PKA activity in 3T3-L1 adipocytes (24). This suggests that the increase in lipolysis rate in response to HIV protease inhibitors is mediated by alterations downstream of PKA (24). In the present study we investigated whether the nelfinavir-associated decrease in perilipin plays a causative role in the increased rates of basal lipolysis. Furthermore, we investigate the underlying cellular mechanisms by which HPI reduces adipocyte perilipin protein content.

**Experimental Procedures**

**Materials:** Tissue culture medium, serum, and antibiotic solutions were obtained from Biological Industries (Beit-Haemek, Israel). Recombinant human insulin was from Novo Nordisk (Bagsvaerd, Denmark). Anti-Perilipin, anti-adipocyte differentiation related protein (ADRP) and anti-hormone-sensitive lipase (HSL) antibodies and recombinant adenoviruses expressing perilipin A or green fluorescent protein (GFP) were generated as previously described (8,9,25). Anti-glucose transporter 4 (GLUT4) antibodies were from Chemicon International Inc. (Temecula, Calif., USA). Peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, protein G-Sepharose, protein A-Sepharose and [35S]-methionine were from Amersham Life Sciences (Buckingham, United Kingdom). Alexa488-conjugated anti-rabbit IgG antibody was from Molecular Probes (Eugene, Oregon, USA). FFA measurement kit was obtained from Roche (Mannheim, Germany). Two sources of Nelfinavir were tested: Roche Pharmaceuticals (through Tel Aviv branch, Israel), and Shanghai 21CEC Pharma Ltd. (London, UK). All other chemicals (including glycerol measurement kit) were obtained from Sigma Chemical Co (St. Louis, Mo., USA).

**Cell Culture:** 3T3-L1 pre-adipocytes (American Type Culture Collection) were grown in DMEM and differentiated exactly as previously described (23,26,27). Cells were used 9–11 days following differentiation induction, when exhibiting >90% adipocyte phenotype.

**Adipocyte differentiated from Mouse Embryonic Fibroblasts (MEF):** Stable lines of MEF pre-adipocytes overexpressing PPARγ were generated from immortalized MEFs of perilipin-null mice (MEF-/−) or wild-type controls (MEF+/+) as described (6,28). PPARγ was introduced by retroviral-mediated transduction followed by puromycin selection. MEF were cultured, as described (6), in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 1 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µg/ml amphotericin B. At confluence cells were exposed to a pro-differentiation regimen consisting of dexamethasone (1 µM), insulin (5 µg/ml), isobutylmethylxanthine (IBMX, 0.5 mmol/l), and rosiglitazone (5 µM). After 2 days, the medium was changed and the cells were maintained in medium containing insulin for 4 days, then in medium without insulin for an additional 4–5 days before use. Cells used for all experiments exhibited >90% adipocyte morphology by light microscopy.

For nelfinavir treatment, differentiated adipose cells were incubated in serum-free DMEM supplemented with 0.5% RIA-grade bovine serum albumin (BSA), with or without nelfinavir, and in the presence or absence of indicated inhibitors. The final nelfinavir medium concentrations were achieved by the appropriate dilutions of a 100 mM stock solution prepared in 100% ethanol. Final ethanol concentrations of up to 0.04% were without measurable effects on the parameters measured in this study. As in
previous publications (23,26,29), cell viability was monitored by protein recovery and MTT assays, excluding compromised cell viability under the conditions used.

**Adenovirus-mediated perilipin over-expression:** Cells were infected as previously described (8). Briefly, 3T3-L1 fibroblasts placed in 12-well plates were cultured in DMEM containing 10% bovine calf serum and differentiated as described above. Adipocytes were infected on day 3 of differentiation with a multiplicity of infection of ~100 plaque-forming units/cell, by incubation with Lipofectamine Plus reagent (Invitrogen) for 3 h at 37 °C. Eight days later, infected adipocytes were used for the experiments, with at least 70-80% of cells exhibiting increased perilipin content. As a control, recombinant adenovirus expressing green fluorescent protein (GFP) was used.

**Cell Lysates and Western Blots:** After treatments, cells were rinsed three times with PBS and scraped into ice-cold lysis buffer containing 50 mmol/l Tris-HCl, pH 7.5, 0.1% (wt/vol) Triton X-100, 1 mmol/l EDTA, 1 mmol/l EGTA, 50 mmol/l NaF, 10 mmol/l Na β-glycerophosphate, 5 mmol/l sodium pyrophosphate, 1 mmol/l sodium vanadate, and 0.1% (vol/vol) 2-mercaptoethanol, and inhibitors (a 1:1,000 dilution of protease inhibitor cocktail [Sigma]). Lysates were gently shaken for 15 min at 4 °C, centrifuged (12,000 g, 15 min at 4 °C), and the fraction between the pellet and adipose cake was collected. Protein concentration was determined using the Bio-Rad Bradford method procedure (Munich, Germany). Protein samples were resolved on 10% SDS-polyacrylamide gel electrophoresis and subjected to Western blot, followed by quantitation using video densitometry analysis, as described previously (27,30). In each experiment the intensity of the band derived from control cells was assigned a value of 1 arbitrary unit, and the intensity of all treatment groups was expressed as the fold value of control.

**Lipolysis:** For basal lipolysis measurements, cells were rinsed three times with PBS and incubated for 1 h with Krebs-Ringer phosphate buffer (KRPB) (HEPES 50 mmol/l, pH 7.4, NaCl 128 mmol/l, KCl 4.7 mmol/l, CaCl$_2$ 1.25 mmol/l, MgSO$_4$ 1.25 mmol/l, and sodium phosphate 10 mmol/l) supplemented with 1% RIA-grade BSA, as previously described (31). FFA concentrations in KRPB buffer were determined using a commercial kit (Roche, Mannheim, Germany), following the instructions of the manufacturer, and calculated according to a palmitic acid standard curve. Glycerol was measured using a glycerol 3-phosphate oxidase trinder kit (Sigma), as described (31).

**Quantitative real-time polymerase chain reaction:** cDNA was prepared using Reverse-iT™ 1st Strand Synthesis Kit (ABgene, UK) and quantitative real-time PCR assays were carried out for perilipin A and for 18S rRNA exactly as previously described (32). The following primers were used:

- **murine perilipin A:**
  - sense: GGCCTGGACGACAAAAACC;
  - anti-sense: CAGGATGGGCTCCATGAC;

- **murine 18S rRNA:**
  - sense: CGCCGCTAGAGGTTGAATTCT;
  - anti-sense: CATTCTTGCAAAATGCTTCT;

**Pulse-Chase Experiments:** Prior to $[^{35}S]$methionine incorporation the cells were preincubated for 1 h with methionine free media in order to deplete the cells from intracellular methionine. Next, cells were pulse-labeled for 5 h with 100 µCi/ml $[^{35}S]$methionine added to methionine free media. Pulse-labeling reaction was terminated by three washes with PBS supplemented with 1,000-fold unlabeled methionine. Cells were subsequently chased for the indicated time periods in serum-free DMEM, then washed and lysed in ice-cold RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA) supplemented with proteases inhibitors (a 1:100 dilution of protease inhibitor cocktail [Sigma]). The cell lysates were prepared as described above. The protein concentration was determined by BCA assay (Pierce) and 0.5 mg of cell lysate protein was used for immunoprecipitation. Anti-perilipin A antibody was linked to pre-washed sepharose A+G beads mix (1:1). Ninety µl of beads mixture was incubated (by gentle shaking) with anti-perilipin A antibody at 4°C overnight, after which the samples were centrifuged for 1 min at 12,000 rpm and the supernatant was discarded. Prior to immunoprecipitation, the lysates were pre-cleared by incubation (shaking) with 90 µl of sepharose-protein A+G mixture for 20 min at 4°C. Then, the samples were centrifuged for 1
min at 12,000 rpm, and the supernatants were added to the sepharose bead-bound perilipin antibodies. The immunoprecipitation reaction was performed by gentle shaking at 4°C for 3 h. The immunoprecipitated complex was pelleted by centrifugation at 5000 rpm at 4°C for 2 min, washed three times with cold RIPA buffer, and mixed with a 10% SDS Laemmli sample buffer (final SDS concentration 2%). The samples were boiled at 100°C for 5 min, centrifuged and the liquid fraction was resolved by SDS-PAGE electrophoresis. The gel was dried using a vacuum gel drier and [35S]methionine-labeled perilipin A in immunoprecipitates was detected directly by autoradiography using phosphoImager (Fujifilm FLA-5100).

Immunofluorescence: Cells were grown on glass coverslips. After treatment, cells were fixed in 3% paraformaldehyde (PFA) for 30 min, washed, and incubated with 100 mM glycine for 10 min. Following another wash with PBS, cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 15 min followed by 1 h incubation in 5% goat serum-supplemented PBS. Next, the cells were incubated with primary anti-perilipin A antibody (dilution of 1:100 in the above blocking solution) for 1 h at room temperature. Cells were washed 3 times with PBS and incubated with Alexa488-conjugated anti-rabbit IgG antibody (1:750 dilution in blocking solution) for 1 hour, at room temperature, washed, and mounted on glass slides. The images were taken at mid z-axis height of the cells, using a Zeiss laser confocal IF microscope. Acquisition parameters were kept constant within the experiment to allow comparison between resulting signal intensities.

Statistical analysis: Data are expressed as means ± SEM. Each treatment was compared with control, and statistical significance of differences between two groups was evaluated using the Student’s t test. The criterion for significance was set at $P < 0.05$.

Results

Decreased perilipin content in 3T3-L1 adipocytes precedes the increase in basal lipolysis rate induced by nelfinavir

We have previously demonstrated that 3T3-L1 adipocytes exposed to nelfinavir exhibit increased constitutive (basal) lipolysis along with decreased expression of the lipid droplet-associated protein, perilipin (23). As a first step in elucidating the role of perilipin in nelfinavir-mediated increase in lipolysis, we compared the chronology of the reduction in perilipin protein with measurements of lipolysis. Time-course analysis was performed by assessing perilipin content and glycerol release to the medium following incubation of 3T3-L1 adipocytes with 30 μM nelfinavir (Figure 1). Two hours of nelfinavir treatment were sufficient to induce a statistically significant 15.5±3.1% decrease in perilipin level compared to time 0 (Figure 1B). The progressive decrease in perilipin content over time (reaching 76.9±11.8% at 24 h), was a specific response to nelfinavir, as HSL and β-actin protein content remained stable (Figure 1A). In contrast to the early reduction in perilipin content to nelfinavir, a significant increase in basal lipolysis rate appeared only after eight hours of nelfinavir treatment (Figure 1B), corresponding to a 50% decrease in perilipin. We utilized indirect immunofluorescence and laser confocal microscopy to determine whether gross alterations in cellular morphology and subcellular localization of perilipin occurred in response to nelfinavir. Nelfinavir treatment resulted in a decrease in perilipin signal intensity, without apparent alterations in its distribution (Figure 1C), or in overall cellular morphology (not shown). Collectively, these findings indicate that a decrease in perilipin cell content temporally precedes nelfinavir-induced lipolysis, and that nelfinavir treatment results in a progressive reduction of perilipin protein expression.

A causative role for decreased perilipin expression in nelfinavir-induced lipolysis

To establish a causative relationship between a decrease in perilipin content and an increase in lipolysis induced by nelfinavir, we undertook two complementary approaches: First, we investigated whether nelfinavir increases basal lipolysis in an adipocyte cellular model devoid of perilipin. Second, we determined whether over-expression of perilipin, using an adenovirus expression system, inhibits nelfinavir-induced lipolysis.

For the first set of experiments, we used adipocytes differentiated from mouse embryonic fibroblasts (MEFs) of perilipin knockout (MEF-/-) or wild-type (MEF+/+) mice. As reported recently (6), MEF-/- adipocytes exhibit similar
adipogenesis potential to that of MEF+++. Furthermore, consistent with the phenotype of perilipin knockout mice, differentiated MEF-- were observed to have an increased basal lipolysis rate compared to MEF+++(Figure 2A). In response to 18 h of nelfinavir treatment, MEF++ adipocytes exhibited a decrease in perilipin content, whereas the expression of HSL was not significantly altered (Figure 2B), consistent with the effects observed in 3T3-L1 adipocytes [(23), Figure 1]. Thus, MEF derived adipocytes represent a reliable adipocyte model system to examine the relationship between perilipin and lipolysis in response to nelfinavir. Subsequently, we investigated whether the absence of perilipin altered the ability of nelfinavir to increase basal lipolysis. MEF-- and MEF++ adipocytes were treated with nelfinavir for 18 h, after which glycerol release to the medium was assessed. MEF++ cells exhibited a robust increase in basal lipolysis after nelfinavir treatment (nelfinavir concentrations of 30 µM and 45µM increased lipolysis by 13.2±3.1, and 24.5±5.3 -fold, respectively), while only a minor increase was observed in MEFs -/- (Figure 2C). Similar results were observed when measuring FFA release (data not shown). This finding is consistent with the notion that the effect of nelfinavir on basal lipolysis requires the presence of perilipin.

If the decrease in perilipin levels is a required step in the induction of lipolysis by nelfinavir (Figure 1), over-expression of perilipin should prevent the lipolytic response to the drug. To over-express perilipin in 3T3-L1 adipocytes we utilized adenoviruses expressing perilipin A under the control of the CMV promoter (Ad-Peri), or a control adenovirus encoding the green fluorescent protein (GFP) under the regulation of the same promoter (Ad-GFP) (9). Cells infected by Ad-Peri exhibited increased perilipin content compared to cells infected with Ad-GFP (Figure 3A and 3B). In cells where perilipin was overexpressed, as in those infected with the control virus, nelfinavir decreased perilipin protein content in a dose-dependent manner (Figure 3A, 3B). Importantly, perilipin over-expression, as compared to adipocytes transduced with Ad-GFP, significantly blocked the action of nelfinavir to increase adipocyte lipolysis (Figure 3C).

Enhanced lysosomal degradation of perilipin is involved in decreasing its protein content by nelfinavir

Given the causative role of decreased perilipin expression in nelfinavir-mediated increase in basal lipolysis, we assessed the mechanism by which this reduction occurs. The amount of perilipin A mRNA (controlled for 18S rRNA) was unaltered by nelfinavir treatment, as assessed by quantitative real-time PCR using primers specific for this isoform of perilipin (Figure 4A). There was also no significant change in perilipin B mRNA content (data not shown). This observation suggested that nelfinavir regulated perilipin through post-transcriptional mechanisms. To assess perilipin protein half-life, we performed cycloheximide (CHX) chase studies in which cells were incubated with 5 µg/ml CHX. This concentration of the protein synthesis inhibitor resulted in over 95% inhibition of 35S-methionine incorporation to total protein (data not shown). Under such conditions the amount of perilipin over time reflects its degradation rate. In control cells, the estimated perilipin protein half-life was over 70 h (Figure 4B), suggesting that it is a highly stable protein. In contrast, nelfinavir-treated 3T3-L1 adipocytes, in which protein synthesis was inhibited by CHX, revealed a 50% decrease in perilipin protein content by 12 h (Figure 4B). To further verify that nelfinavir enhanced the protein degradation of perilipin, we used a pulse-chase approach. Cells were pulsed for 5 h with 35S-methionine in methionine-free media, after which radioactive methionine was thoroughly washed and the cells were incubated in regular medium supplemented with unlabelled methionine in the presence or absence of nelfinavir for 12 and 24 h. To determine radioactive methionine content in perilipin, cell lysates were prepared and subjected to immunoprecipitation using anti-perilipin antibodies. The immunoprecipitate was subjected to SDS-PAGE followed by autoradiography to determine the amount of radio-labeled perilipin. Consistent with the CHX chase approach, after 24 h chase the loss of radioactivity in perilipin was undetectable in control cells (Figure 4C).
incubated with nelfinavir exhibited greatly enhanced loss in 35S-methionine-containing perilipin. The calculated $t_{1/2}$ of perilipin using this approach was 12h, remarkably similar to that determined by CHX chase.

In order to assess which protein degradation system is involved in the enhanced perilipin proteolysis induced by nelfinavir, we used inhibitors of proteasomal or of lysosomal proteolysis. MG-132 is considered a specific 26S-proteasome inhibitor, whereas leupeptin is a reversible and competitive intra-lysosomal proteolysis inhibitor that specifically inhibits serine and some cysteine proteases such as cathepsin B, trypsin, endoproteinase Lys-C, Kallikrein, papain, and thrombin. Consistent with a previous report in adipocytes, MG-132 had no effect on perilipin content in control adipocytes (33), and did not affect its levels in nelfinavir-treated cells (Figure 5A and 5B). In contrast, inhibiting the proteasome resulted in marked increase in ADRP (Figure 5A and 5C), a protein known to be proteasomally degraded (34). Leupeptin treatment of control cells resulted in 40% increase in perilipin content ($p=0.015$), and when present during incubation of 3T3-L1 adipocytes with nelfinavir, almost completely prevented the decrease in perilipin content (Figure 5A and 5B). To further corroborate these findings, cells were treated with NH4Cl, which blocks lysosomal proteolysis by inhibiting lysosomal acidification. Like leupeptin, preventing lysosomal acidity tended to increase perilipin content in control cells, and significantly prevented the decrease in perilipin protein content in nelfinavir-treated cells (Figure 5A and 5B). Finally, consistent with the causative role of decreased perilipin in nelfinavir-induced lipolysis, both leupeptin and NH4Cl resulted in 35.3±10.7% ($p=0.007$) and 71.9±17.8% ($p=0.001$) inhibition of glycerol release induced by the drug, respectively.

**Discussion**

Two novel observations emerge from the present study: 1. the decrease in adipocyte perilipin content plays a causative role in the enhanced basal lipolytic rate induced by nelfinavir. 2. lysosomal protein degradation processes are likely involved in the regulation of perilipin content, and hence, in the regulation of lipolysis.

**Regulation of adipocyte lipolysis by proteolytic degradation of perilipin**

Enhanced adipocyte basal lipolytic flux is a common feature in conditions associated with insulin resistance, including obesity, type 2 diabetes and the lipodystrophy syndrome associated with HAART (3,4,21). At least two studies suggested that obesity was indeed associated with decreased perilipin protein content (17,18). Since perilipin has been shown to inhibit basal lipolysis rate, reduced expression of this protein likely contributes to enhanced basal lipolytic flux associated with obesity. This is supported by the increased basal lipolytic rate observed in perilipin knockout mice (10,11) and in adipocytes derived from this mouse model (Figure 2A), as well as by the demonstration that a decrease in perilipin induced by either nelfinavir or by TNF-$\alpha$ plays a causative role in the induction of lipolysis [Figures 1-3 and (9), respectively]. It is interesting to note that the reduction in perilipin protein content caused by TNF-$\alpha$ treatment is associated with reduced perilipin mRNA levels, while nelfinavir does not decrease perilipin mRNA. Mechanisms controlling the protein stability of perilipin in adipocytes are largely unknown, but may be functionally important, as changes in the protein content of perilipin are not always associated with alterations in mRNA levels (17-19). In the present study, direct measurements of the half-life of perilipin protein in adipocytes revealed that it is a highly stable protein under control conditions, with a $t_{1/2}$ exceeding 70h (Figure 4B and 4C), and that enhanced protein degradation, not a change in its transcription rate, accounts for the decreased expression of perilipin in response to nelfinavir. Thus, an important area for future research will be to determine whether enhanced proteolytic degradation of perilipin constitutes a major process for altering perilipin expression in response to endogenous cues, thereby regulating basal adipocyte lipolysis rate.

**Involvement of lysosomal proteolysis of perilipin in regulating lipolysis**

If regulating perilipin content is indeed a functional means of controlling lipolysis in adipocytes, it is important to determine how perilipin expression is regulated. A small but statistically significant increase in perilipin protein content was observed when adipocytes were treated with the lysosomal proteases inhibitor leupeptin for 18h (Figure 5A and 5B). In contrast, the proteasomal inhibitor MG-132...
did not alter perilipin content, while markedly preventing the proteolytic degradation of ADRP, another lipid droplet associated protein known to be degraded by the proteasome (34). This observation seemingly contradicts a recently published finding (35). Yet, this study, which assessed perilipin protein stability and degradation, utilized non-adipocyte cells (Chinese Hamster Ovary cells) exogenously expressing perilipin. Therefore, we hypothesize that in adipocytes, perilipin protein degradation occurs in lysosomes, and that this process is enhanced by nelfinavir. Furthermore, perilipin's degradation through the lysosome versus ADRP's degradation by the proteasome provides a novel mechanism to explain the reciprocal regulation of these two lipid-droplet associated proteins.

Lysosomal degradation of intracellular proteins can occur as part of cellular microautophagy or macroautophagy (36,37). Intriguingly, autophagy can be triggered by oxidative stress (38,39), which has been shown to be induced in nelfinavir-treated adipocytes (32,40). This could be mediated by oxidative stress-induced impairment in Akt/PKB activation (27), as this kinase was shown to inhibit autophagy (41). Nevertheless, micro- and macro-autophagy are non-specific mechanisms, whereas some degree of selectivity was observed in the enhanced lysosomal degradation of perilipin induced by nelfinavir (Figures 1 and 5). While chaperone-mediated autophagy is a selective proteolytic mechanism (42,43), its described substrates are all soluble cytosolic proteins, whereas perilipin is lipid anchored. Thus, whether perilipin is targeted to the lysosomes through chaperone-mediated autophagy, and if so, whether it becomes detached from the lipid droplet, is worth investigating in future studies. Finally, a recent study suggests that perilipin may undergo regulated translocation to the plasma membrane (44), raising the theoretical possibility that perilipin traffics between the phospholipids monolayer of the lipid droplet, the plasma membrane and the lysosome.

In conclusion, studying the regulation of lipolysis by the HIV protease inhibitor nelfinavir has provided new insights into the protein degradation of perilipin as a cellular mechanism that likely involves the lysosomal degradation machinery. Future studies will determine whether lysosomal degradation of perilipin is important in physiological states such as obesity and in response to inflammatory cytokines.

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References:
1. Greenberg, A. S., Egan, J. J., Wek, S. A., Garty, N. B., Blanchette-Mackie, E. J., and Londos, C. (1991) J Biol Chem 266(17), 11341-11346
2. Greenberg, A. S., Egan, J. J., Wek, S. A., Moos, M. C., Jr., Londos, C., and Kimmel, A. R. (1993) Proc Natl Acad Sci U S A 90(24), 12035-12039
3. Holm, C. (2003) Biochem Soc Trans 31(Pt 6), 1120-1124
4. Londos, C., Gruia-Gray, J., Brasaemle, D. L., Rondinone, C. M., Takeda, T., Dwyer, N. K., Barber, T., Kimmel, A. R., and Blanchette-Mackie, E. J. (1996) Int J Obes Relat Metab Disord 20 Suppl 3, S97-101
5. Tansey, J. T., Sztalryd, C., Hlavin, E. M., Kimmel, A. R., and Londos, C. (2004) IUBMB Life 56(7), 379-385
6. Miyoshi, H., Souza, S. C., Zhang, H. H., Strissel, K. J., Christoffolete, M. A., Kovsan, J., Rudich, A., Kraemer, F. B., Bianco, A. C., Obin, M. S., and Greenberg, A. S. (2006) J Biol Chem 281(23), 15837-15844
7. Brasaemle, D. L., Rubin, B., Harten, I. A., Gruia-Gray, J., Kimmel, A. R., and Londos, C. (2000) J Biol Chem 275(49), 38486-38493
8.
Souza, S. C., Muliro, K. V., Liscum, L., Lien, P., Yamamoto, M. T., Schaffer, J. E., Dallal, G. E., Wang, X., Kraemer, F. B., Obin, M., and Greenberg, A. S. (2002) *J Biol Chem* 277(10), 8267-8272.

9.
Souza, S. C., de Vargas, L. M., Yamamoto, M. T., Lien, P., Franciosa, M. D., Moss, L. G., and Greenberg, A. S. (1998) *J Biol Chem* 273(38), 24665-24669.

10.
Martinez-Botas, J., Anderson, J. B., Tessier, D., Lapillonne, A., Chang, B. H., Quast, M. J., Goreinstein, D., Chen, K. H., and Chan, L. (2000) *Nat Genet* 26(4), 474-479.

11.
Tansey, J. T., Szalay, C., Gruia-Gray, J., Reitman, M. L., Deng, C. X., Li, C., Kimmel, A. R., and Londos, C. (2001) *Proc Natl Acad Sci U S A* 98(11), 6494-6499.

12.
Corella, D., Qi, L., Sorli, J. V., Godoy, D., Portoles, O., Coltell, O., Greenberg, A. S., and Ordovas, J. M. (2005) *J Clin Endocrinol Metab* 90(9), 5121-5126.

13.
Corella, D., Qi, L., Tai, E. S., Deurenberg-Yap, M., Tan, C. E., Chew, S. K., and Ordovas, J. M. (2006) *J Clin Endocrinol Metab* 91(9), 3251-3261.

14.
Ballesta, J., Anderson, J. B., Tessier, D., Lapillonne, A., Chang, B. H., Quast, M. J., Goreinstein, D., Chen, K. H., and Chan, L. (2000) *Nat Genet* 26(4), 474-479.

15.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2004) *Obes Res* 12(11), 1758-1765.

16.
Corella, D., Qi, L., Tai, E. S., Tan, C. E., Shen, H., Chew, S. K., and Ordovas, J. M. (2005) *Diabetes Care* 29(6), 1313-1319.

17.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

18.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Diabetes Care* 29(6), 1313-1319.

19.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

20.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

21.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Diabetes Care* 29(6), 1313-1319.

22.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

23.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

24.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

25.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

26.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

27.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

28.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

29.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

30.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

31.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

32.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

33.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

34.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

35.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.

36.
Qi, L., Shen, H., Larson, I., Schaefer, E. J., Greenberg, A. S., Tregouet, D. A., Corella, D., and Ordovas, J. M. (2005) *Obes Res* 13(6), 448-456.
37. Marino, G., and Lopez-Otin, C. (2004) *Cell Mol Life Sci* **61**(12), 1439-1454
38. Kiffin, R., Bandyopadhyay, U., and Cuervo, A. M. (2006) *Antioxid Redox Signal* **8**(1-2), 152-162
39. Moore, M. N., Allen, J. I., and Somerfield, P. J. (2006) *Mar Environ Res* **62 Suppl**, S420-425
40. Vincent, S., Tourniaire, F., El Yazidi, C. M., Compe, E., Manches, O., Plannels, R., and Roche, R. (2004) *J Acquir Immune Defic Syndr* **37**(5), 1556-1562
41. Ellington, A. A., Berhow, M. A., and Singletary, K. W. (2006) *Carcinogenesis* **27**(2), 298-306
42. Dice, J. F., Terlecky, S. R., Chiang, H. L., Olson, T. S., Isenman, L. D., Short-Russell, S. R., Freundlieb, S., and Terlecky, L. J. (1990) *Semin Cell Biol* **1**(6), 449-455
43. Majeski, A. E., and Dice, J. F. (2004) *Int J Biochem Cell Biol* **36**(12), 2435-2444
44. Aboulaich, N., Vener, A. V., and Stralfors, P. (2006) *J Biol Chem* **281**(17), 11446-11449
Figure legends:

**Figure 1:** Nelfinavir-induced decrease in perilipin level precedes the increase in basal lipolysis rate

Differentiated 3T3-L1 adipocytes were incubated with 30 μM nelfinavir for the indicated time periods. At each time point lipolysis rate was determined by measuring glycerol release, and cells were lysed and analyzed by Western blot using anti-perilipin, anti-HSL or anti-β-actin antibodies (A). Representative blots of 5-9 independent experiments are shown. (B) Perilipin content (□) and lipolysis (glycerol release, ◆) were followed for up to 24 h incubation with nelfinavir, and expressed as relative to time 0 (which was assigned a value of 100%). Results are expressed as the mean ± SE. *p<0.05 vs. time 0. (C) Following incubation with or without nelfinavir (30 μM, 18 h), 3T3-L1 adipocytes were fixed and indirect immunofluorescence detection of perilipin was performed, using anti-perilipin A Ab, followed by laser confocal microscopy, as described in Experimental Procedures. Shown are panels from two independent experiments. Scale bar 10 μm.

**Figure 2:** Nelfinavir increases basal lipolysis in adipocytes differentiated from mouse embryonal fibroblasts from wild-type, but not from perilipin knockout mice

Differentiated 3T3-L1 adipocytes, or adipocytes derived from mouse embryonal fibroblasts of wild-type or perilipin knockout mice (MEF+/+ and MEF-/-, respectively), were treated with or without the indicated concentrations of nelfinavir for 18 h. Then, cells were incubated for an additional hour in KRPB buffer, and glycerol or FFA release was assessed (A). (B) Cells were lysed and analyzed by Western blot using specific antibodies against perilipin and HSL. Representative blots from 5 independent experiments are shown. (C) Lipolysis rates of MEF+/+ and MEF-/- following nelfinavir treatment, measured by glycerol release and expressed as fold of untreated cells. Results are expressed as the mean ± SE of 5 independent experiments. * p<0.05.

**Figure 3:** Adenovirus-mediated perilipin over-expression prevents nelfinavir-induced lipolysis.

3T3-L1 adipocytes were infected with either an adenovirus encoding perilipin A (Ad-Peri A) or the Green Fluorescent Protein (Ad-GFP), as described in the Experimental Procedures section. Eight days after infection cells were treated with the indicated concentrations of nelfinavir for 18 hours, after which glycerol release was assessed, and Western blot was used to assess the protein content of perilipin A, HSL and β-actin (A). (B) Results of densitometry analysis for perilipin A content in Ad-GFP and Ad-Peri A infected cells, expressed as fold of Ad-GFP infected, nelfinavir untreated cells. (C) Lipolysis rate (expressed as µmol of glycerol release per mg protein per hour). Values are means ± SE of three independent experiments. *p<0.05.

**Figure 4:** Nelfinavir decreases perilipin content through enhanced protein degradation.

(A) Total RNA was isolated from control or nelfinavir treated cells (30 μM, 18 h). After RT-PCR, perilipin cDNA amount was quantified by real-time PCR using primers specific for perilipin A, as described in Experimental Procedures section. The results were normalized to the amount of 18S rRNA, and are means ± SE of three independent experiments. (B) 3T3-L1 adipocytes were incubated with 5 μg/ml cycloheximide (CHX) alone or in the presence of 30 μM nelfinavir. At each time point cells were lysed and the amount of perilipin protein in control and nelfinavir treated cells was quantified by Western blot. Shown is a representative blot and densitometry analysis of five independent experiments. (C) 3T3-L1 adipocytes were pulsed for 5 h in the presence of 100 μCi/ml [³⁵S]methionine, as described in the Experimental Procedures section. Then, cells were chased for 12 and 24 h with or without 30 μM nelfinavir. Perilipin A was immunoprecipitated and the amount of associated [³⁵S]methionine was detected by autoradiography. A representative autoradiogram and the means ± SE of three independent experiments are shown. *p<0.05 versus respective cells untreated with nelfinavir.
Figure 5: Lysosomal, but not proteasomal protein degradation inhibitors, prevent nelfinavir-induced perilipin degradation.

3T3-L1 adipocytes were incubated with or without nelfinavir (30 μM) in the absence or presence of MG-132 (10 μM) or leupeptin (10 μg/ml) or NH₄Cl (10 mM) for 18 h. Afterwards, cells were lysed and analyzed by Western blot using anti-perilipin, anti-ADRP or anti-β-actin antibodies. (A) Representative blots of 5-9 independent experiments are shown. Results of densitometry analysis for perilipin A (B) and ADRP (C) are shown as fold of untreated control cells. MG – MG-132, Leu – leupeptin. *p<0.05 vs. cells untreated with either nelfinavir or protein degradation inhibitors.
Figure 1

A. Time (h): 0 2 4 6 8 24
Perilipin A
HSL
β-actin

B. Perilipin content (relative to control)
Glycerol release (relative to control)

C. -Nelfinavir +Nelfinavir

* * *
Figure 2

A.

![Bar graph showing Glycerol or FFA release in MEF +/+, MEF -/- mice](image)

B.

![Western blot images of Perilipin A and B, HSL in 3T3-L1 cells, and Nelfinavir concentration](image)

C.

![Bar graph showing Glycerol release fold of control in Nelfinavir treatment](image)
Figure 3

A.

| Nelfinavir (μM) | Ad-GFP | Ad-Peri A |
|----------------|--------|-----------|
| 0              | 0      | 0         |
| 30             | 0      | 0         |
| 45             | 0      | 0         |

Perilipin A →

HSL →

β-actin →

B.

![Bar chart showing Perilipin A content with Nelfinavir (μM) levels of 0, 30, and 45, comparing Ad-GFP and Ad-Peri A.]

C.

![Bar chart showing Glycerol release with Nelfinavir (μM) levels of 0, 30, and 45, comparing Ad-GFP and Ad-Peri A.]

Downloaded from http://www.jbc.org/ on March 24, 2020.
Figure 4

A.

![Bar chart showing Perilipin A / 18S rRNA levels with and without Nelfinavir]

B.

![Graph showing time course of Perilipin A protein with CHX and CHX + Nelfinavir]

C.

![Graph showing Perilipin A protein levels during a 5h pulse chase with and without Nelfinavir]

End of 5h pulse

- Nelfinavir  + Nelfinavir

Time (h)

- Nelfinavir  + Nelfinavir

Chase

Perilipin A

Perilipin A protein percent of time 0

t₁/₂ = 12h

Time (h)
Figure 5

A. - Nelfinavir + Nelfinavir
- MG Leu NH₄Cl - MG Leu NH₄Cl

Perilipin A

ADRP

β-actin

B. Perilipin A content (fold of control)

- Nelfinavir +Nelfinavir

C. ADRP content (fold of control)

- Nelfinavir +Nelfinavir
Regulation of adipocyte lipolysis by degradation of the perilipin protein: Nelfinavir enhances lysosome-mediated perilipin proteolysis
Julia Kovsan, Ronit Ben-Romano, Sandra C. Souza, Andrew S. Greenberg and Assaf Rudich

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