The HIV Protease Inhibitor Nelfinavir Induces Insulin Resistance and Increases Basal Lipolysis in 3T3-L1 Adipocytes

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HIV protease inhibitors (HPIs) are potent antiretroviral agents clinically used in the management of HIV infection. Recently, HPI therapy has been linked to the development of a metabolic syndrome in which adipocyte insulin resistance appears to play a major role. In this study, we assessed the effect of nelfinavir on glucose uptake and lipolysis in differentiated 3T3-L1 adipocytes. An 18-h exposure to nelfinavir resulted in an impaired insulin-stimulated glucose uptake and activation of basal lipolysis. Impaired insulin stimulation of glucose uptake took place at nelfinavir concentrations >10 μmol/l (EC50 = 20 μmol/l) and could be attributed to impaired GLUT4 translocation. Basal glycerol and free fatty acid (FFA) release were significantly enhanced with as low as 5 μmol/l nelfinavir, displaying fivefold stimulation of FFA release at 10 μmol/l. Yet, the antilipolytic action of insulin was preserved at this concentration. Potential underlying mechanisms for these metabolic effects included both impaired insulin stimulation of protein kinase B Ser 473 phosphorylation with preserved insulin receptor substrate tyrosine phosphorylation and decreased expression of the lipolysis regulator perilipin. Troglitazone pre- and cotreatment with nelfinavir partly protected the cells from the increase in basal lipolysis, but it had no effect on the impairment in insulin-stimulated glucose uptake induced by this HPI. This study demonstrates that nelfinavir induces insulin resistance and activates basal lipolysis in differentiated 3T3-L1 adipocytes, providing potential cellular mechanisms that may contribute to altered adipocyte metabolism in treated HIV patients. Diabetes 50:1425–1431, 2001

Curren antiretroviral treatment (highly active antiretroviral therapy [HAART]) has improved the prognosis of patients with HIV by dramatically suppressing HIV viral load, increasing CD4 counts, and reducing opportunistic infections associated with AIDS (1,2). However, it is increasingly recognized that as much as 83% of the patients receiving this treatment develop metabolic abnormalities, which include dyslipidemia (elevated triglycerides and cholesterol), central adiposity, and peripheral lipodystrophy (3,4). Interestingly, treated HIV patients who developed this clustering of metabolic abnormalities were also found to have elevated fasting insulin or C-peptide levels (5–7), strongly suggesting that these individuals develop systemic insulin resistance. Moreover, elevated C-peptide levels appear to be a reliable predictor for the development of lipodystrophy. Importantly, it is becoming increasingly recognized that these patients may be at high risk for developing premature cardiovascular morbidity as well as type 2 diabetes, emphasizing the medical significance of the metabolic dysregulation associated with HAART (4,7,8).

Which component of the antiretroviral regimen, or whether the HIV infection itself, is responsible for this seemingly serious abnormality is not fully clear. AIDS is frequently associated with hypertriglyceridemia, but with reduced total, LDL, and HDL cholesterol (9,10). Nucleoside reverse transcriptase inhibitors may also be associated with hepatic steatosis and lactic acidemia, which are thought to result from mitochondrial damage caused by these agents (11). Yet, an increasing amount of clinical and epidemiological data attributes a central role for the HIV protease inhibitors (HPIs) in the induction of insulin resistance in HAART-treated patients (3,5,6,12,13). HPIs are a central component of the HAART regimen, as they are potent inhibitors of HIV aspartyl protease, an enzyme required for normal processing of HIV proteins (14–16). It appears that all HPI compounds induce insulin resistance and lipid abnormalities to various degrees (3,17), but the mechanisms for these serious side effects are largely unknown.

Only a few studies have been published recently regarding alterations in insulin signaling and insulin-regulated metabolism caused by HPIs at the cellular level. Using different agents and incubation periods, protease inhibitors have been shown to impair insulin signaling events in...
Nelfinavir alters adipocyte metabolism.

HepG2 cells (18), to inhibit adipocyte differentiation (19, 20), and to decrease adipocyte insulin-stimulated glucose transport without affecting the insulin signaling cascade (21). Yet, despite the fact that impaired adipocyte tissue metabolism is at the center of the HPI-induced metabolic syndrome, a systematic evaluation of the effects of HPIs on fully differentiated adipocyte metabolism has not been reported.

In this study, we investigated the effect of nelfinavir, a clinically used HPI, on glucose transport and lipolysis in differentiated 3T3-L1 adipocytes, with emphasis on insulin responsiveness. We demonstrate that 18 h of exposure to nelfinavir results in increased basal lipolysis, an effect associated with reduced expression of perilipin, a protein thought to be important in the regulation of lipolysis (22, 23). In addition, with higher concentrations, nelfinavir impairs insulin stimulation of glucose transport, protein kinase B (PKB) phosphorylation, and GLUT4 translocation.

RESEARCH DESIGN AND METHODS

Cell culture and treatments. 3T3-L1 adipocytes (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, Beit-Haemek, Israel) and differentiated exactly as previously described (24–26). Fully differentiated cells (12–14 days after induction of differentiation) were incubated in serum-free DMEM supplemented with 0.5% radioimmunoassay (RIA)-grade bovine serum albumin (BSA) (Sigma, St. Louis, MO), with or without various concentrations of nelfinavir, for 18 h. For stock solution, nelfinavir (Roche Pharmaceuticals, Tel Aviv, Israel) was prepared at 100 mmol/l concentration in 100% ethanol, resulting in maximal final ethanol concentration of 0.04%. Cell viability, as assessed by protein recovery and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide test, was unaffected with up to 40 μmol/l nelfinavir. For pre- and co-treatment with troglitazone (10 μmol/l, provided by Drs. Alan Saltiel and Heidi Camp, Parke-Davis, Ann Arbor, MI), cells were incubated in full DMEM for 6 h before and then in serum-free medium during nelfinavir treatment.

Hexose transport measurements. 2-Deoxyglucose (2DG) uptake was performed after rinsing the cells with phosphate-buffered saline (PBS) and incubating them without or with 100 μmol/l insulin for 20 min, exactly as previously described (24, 26), using 50 μmol/l 2-deoxy-[3H]glucose (Nuclear Research Center, Dimona, Israel) (3.7 × 10^6 Bq/ml) for 10 min.

RESULTS

Effect of nelfinavir on glucose transport and glucose transporters. Fully differentiated 3T3-L1 adipocytes were exposed for 18 h to the indicated nelfinavir concentrations as described in RESEARCH DESIGN AND METHODS, after which 2DG uptake was measured (Fig. 1A). Although 10 μmol/l nelfinavir treatment was not associated with any significant change, higher concentrations were associated with elevated basal levels and gradually reduced insulin-stimulated 2DG uptake. With 30 μmol/l nelfinavir, net insulin effect was reduced from 310 ± 19 in control cells to 54 ± 11 pmol 2DG/mg protein per min (P < 0.001), with an EC_{50} of ~20 μmol/l. To investigate whether decreased abundance of the insulin-responsive glucose transporter GLUT4 could explain the reduction in insulin-stimulated glucose transport, total membranes were prepared and analyzed by Western blot. Figure 1B demonstrates that total membrane GLUT4 content was not significantly changed by nelfinavir at concentrations of 10–40 μmol/l. We next assessed insulin-stimulated GLUT4 translocation by immunofluorescent detection of GLUT4 in plasma membrane lawns. Figure 1C demonstrates that 30 μmol/l nelfinavir was not associated with a significant change in plasma membrane GLUT4 abundance in the non-insulin-stimulated state. Yet, after insulin stimulation, an impairment in insulin-stimulated GLUT4 translocation to the plasma membrane could be observed.

Nelfinavir alters adipocyte lipolysis. Since HPI treatment is associated with both peripheral insulin resistance as well as peripheral lipodystrophy, which suggests alterations in adipocyte lipid metabolism, we next assessed the effects of nelfinavir on adipocyte lipolysis. An 18-h exposure to nelfinavir resulted in a marked stimulation of basal lipolysis, as assessed by the increase of both FFAs and glycerol levels in the culture medium (Fig. 2A). Interestingly, this effect of nelfinavir could be observed at concentra-
trations as low as 5 μmol/l, whereas 10 μmol/l, which did not alter glucose transport activity (Fig. 1A), was already associated with a 5.74 ± 0.70-fold increase in FFA release.

To assess whether the insulin resistance observed (Fig. 1A) could be similarly demonstrated on the acute antilipolytic action of insulin, cells were submaximally stimulated with 5 nmol/l isoproteranol, without or with 100 nmol/l insulin, for 1 h after nelfinavir treatment. As depicted in Fig. 2B, FFA release in control cells was stimulated ~18-fold by 5 nmol/l isoproteranol and was decreased by 58% in the presence of insulin. In cells treated for 18 h with 5 and 10 μmol/l nelfinavir, insulin demonstrated a preserved antilipolytic effect, resulting in 63 and 54% inhibition of isoproteranol-stimulated FFA release, respectively. At 30 μmol/l nelfinavir, no significant stimulation of FFA release could be demonstrated with 5 nmol/l isoproteranol, and only a nonsignificant 9% decrease could be induced by insulin. Interestingly, in both nelfinavir-treated and untreated cells, exposure to 1 μmol/l isoproteranol resulted in a further increase in lipolysis rate compared with 5 nmol/l isoproteranol, reaching similar rates of FFA release the medium (data not shown).

Potential cellular mechanisms for nelfinavir-stimulated lipolysis and insulin resistance. Long-term regulation of lipolysis may involve alterations both in the expression level of HSL and in perilipins. The latter are believed to regulate lipolytic activity by limiting the access of HSL to the lipid droplets (28–30). Figure 3A demonstrates the effect of nelfinavir on the content of these two proteins. As shown in the upper blot, HSL protein content was not elevated in nelfinavir-treated cells compared with control cells, and it even tended to decrease with concentrations of 30 μmol/l. This finding suggests that the increased basal lipolysis shown above (Fig. 2A) cannot be attributed to increased HSL expression. The lower blot demonstrates the protein content of the two isoforms of perilipin, A and B (molecular weight 57 and 46 kDa, respectively). A gradual decrease in adipocyte perilipin content with increasing nelfinavir concentrations could be seen, beginning with as low as 10 μmol/l nelfinavir, and involved both isoforms.

To investigate the potential mechanisms by which nelfinavir treatment impairs the insulin signaling cascade, cells were incubated with nelfinavir for 18 h and then stimulated with 100 nmol/l insulin for 7 min. Tyrosine phosphorylation of a band of ~185 kDa, corresponding to IRS-1 and ~2, is shown in Fig. 3B along with an IRS-1 immunoblot. As shown, nelfinavir treatment had no effect on either IRS-1 content nor on its insulin-stimulated tyrosine phosphorylation. Similarly, insulin-stimulated tyrosine phosphorylation of the β-subunit of the insulin receptor (~95 kDa band) was unaffected by nelfinavir treatment (not shown).
suggesting that nelfinavir did not impair early steps in the insulin signaling cascade. In contrast, insulin-stimulated Ser 473 phosphorylation of PKB was significantly inhibited by nelfinavir concentrations of at least 20 μmol/l, without alterations in its total protein content (Fig. 3C). Densitometric analyses show a dose-dependent effect, which parallels the effect observed on 2DG uptake activity (Fig. 1A). These data suggest a potential role for reduced perilipin content in nelfinavir-stimulated lipolysis and an impairment in insulin-stimulated PKB phosphorylation in the induction of insulin resistance.

Insulin and thiazolidinediones significantly inhibit nelfinavir-stimulated lipolysis. We next assessed whether the induction of basal adipocyte lipolysis by nelfinavir treatment could be blocked by acute insulin treatment or by pre- and coincubation with the thiazolidinedione (TZD) insulin-sensitizer troglitazone. Figure 4A demonstrates that both agents display a significant capacity to inhibit nelfinavir-stimulated FFA release, particularly at lower concentrations of this HPI. At a nelfinavir concentration of
only troglitazone provided a mild but significant inhibition in FFA release. To determine whether troglitazone could also protect against the impairment in insulin action induced by nelfinavir, insulin-stimulated 2DG uptake was measured. As shown in Fig. 4B, insulin-stimulated 2DG uptake activity was increased by nearly 20% in troglitazone-treated control cells. This effect of troglitazone was only marginally significant when cells were cotreated with 10 μmol/l nelfinavir and was completely absent at 30 μmol/l. These results indicate that although troglitazone showed a certain capacity to protect against the metabolic effects of nelfinavir, troglitazone became less efficient with increasing doses of this agent.

**DISCUSSION**

HPI treatment, which dramatically improved the prognosis of HIV-infected patients, appears to cause or aggravate a metabolic syndrome with potentially severe consequences (7). The cellular mechanisms underlying the appearance of this syndrome are largely unknown. The clinical evidence of systemic peripheral insulin resistance, as well as the high prevalence of peripheral lipodystrophy and central adiposity, suggests that altered adipose tissue metabolism may have a central role in the development of this syndrome (4,8). Here, we demonstrate that the HPI nelfinavir, when incubated with 3T3-L1 adipocytes for an 18-h period, results in both increased basal lipolysis as well as resistance to the acute metabolic actions of insulin. These two effects display distinct sensitivities to nelfinavir, with the activation of lipolysis occurring at lower concentrations of nelfinavir than those at which the induction of insulin resistance occurs.

A recent study on the effect of HPI on glucose transport activity in differentiated 3T3-L1 adipocytes also reached the conclusion that HPIs impair insulin-stimulated glucose uptake activity (21). This impairment was associated with normal GLUT4 expression as well as with preserved insulin-stimulated tyrosine phosphorylation events. However, although impaired insulin-stimulated GLUT4 translocation associated with decreased PKB Ser 473 phosphorylation was observed in our study (Figs. 1C and 3C), these were reported as normal in the study by Murata et al. (21). This discrepancy may be attributed to the different HPI agents used in the two studies and possibly also to the concentrations and duration of incubation used.

Significant activation of basal lipolysis occurred at nelfinavir concentrations as low as 5 μmol/l, whereas the HPI effect on insulin-stimulated glucose uptake was only observed at higher concentrations (Fig. 2A). These findings suggest that these two metabolic changes are the result of independent mechanisms. In addition, although it is tempting to speculate that the insulin resistance is secondary to the activated lipolysis, the studies with troglitazone do not necessarily support this notion. Although troglitazone at least partly inhibited nelfinavir-induced lipolysis, it did not protect against the decrease in insulin-stimulated glucose uptake. The decreased efficiency of troglitazone with increasing nelfinavir concentrations may be consistent with a recent report demonstrating decreased peroxisome proliferator-activated receptor-γ protein content in nelfinavir-treated 3T3-L1 adipocytes (20). This finding may explain the reduced expression of various adipocyte-specific proteins in response to nelfinavir treatment, as well as the inhibition of the preadipocyte to the adipocyte differentiation program (20). In the present study, adipocyte differentiation was not directly assessed. Yet, GLUT4 protein

**FIG. 4.** The effect of troglitazone and insulin on basal FFA release and on insulin-stimulated 2DG uptake. A: Cells were either treated for 18 h with nelfinavir or treated for 5 h before and during nelfinavir treatment with 10 μmol/l troglitazone. Cells were then washed and incubated for an additional 1 h in KRPB, either with no additions or with 100 nmol/l insulin. FFA release was then assessed. Results are derived from three independent experiments performed at least in duplicate. *P < 0.05 and †P < 0.01 vs. cells treated with the same nelfinavir concentration but without either troglitazone or insulin. B: Cells were treated with nelfinavir without or with cotreatment with troglitazone, as described in A, rinsed, and stimulated for 20 min with 100 nmol/l insulin, after which 2DG uptake was measured. Results are derived from two independent experiments yielding identical results.

30 μmol/l, only troglitazone provided a mild but significant inhibition in FFA release. To determine whether troglitazone could also protect against the impairment in insulin action induced by nelfinavir, insulin-stimulated 2DG uptake was measured. As shown in Fig. 4B, insulin-stimulated 2DG uptake activity was increased by nearly 20% in troglitazone-treated control cells. This effect of troglitazone was only marginally significant when cells were cotreated with 10 μmol/l nelfinavir and was completely absent at 30 μmol/l. These results indicate that although troglitazone showed a certain capacity to protect against the metabolic effects of nelfinavir, troglitazone became less efficient with increasing doses of this agent.

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content was unaffected by nelfinavir treatment (Fig. 1B), whereas HSL content at the higher range of nelfinavir used, and perilipin also at lower concentrations, were found to be reduced (Fig. 3A).

Perilipins, located under basal conditions at the phospholipid interphase of the triglyceride droplet, are believed to limit HSL access to its substrate (28–30). Consequently, a reduction in their abundance results in higher HSL action on triglyceride storage pools. A recent perilipin-knockout model also largely supports an inhibitory role of perilipin on HSL action (23). Interestingly, in 3T3-L1 adipocytes, reduced perilipin content was previously suggested as a mechanism for the activation of lipolysis in response to tumor necrosis factor (TNF)-α (22). An intriguing possibility may thus be raised: that nelfinavir stimulates TNF secretion from 3T3-L1 adipocytes, which in turn acts in an autocrine loop to stimulate lipolysis. Yet, preliminary experiments could not demonstrate increased TNF secretion after nelfinavir treatment of 3T3-L1 adipocytes (unpublished observation).

The exact mechanism by which nelfinavir impaired insulin signaling is unknown. Assuming that nelfinavir inhibited a cellular protease, acting in a non-HIV protease-specific manner, our results imply that normal function of the insulin signaling network requires the action of such a protease. Interestingly, recent studies of genetic linkage analysis suggest that alterations in the protease calpain 10 gene may contribute to the development of insulin resistance and type 2 diabetes (31,32). Moreover, since a non-specific protease inhibitory action may affect multiple cellular processes, it is not surprising that diverse functions may be affected, acting in concert to lead to the development of lipodystrophy. Consistently, HPIs have been shown to interfere in the differentiation program from preadipocytes to adipocytes (19,20), possibly resulting in compromised balance between these two cell populations in adipose tissue. Induction of adipocyte apoptosis has also been documented (20,33), suggesting an additional mechanism for adipose tissue loss by HPIs. How these different mechanisms for the loss of adipose tissue and/or its triglyceride stores interrelate—for example, whether they form a continuity of events in the development of peripheral lipodystrophy—remains to be sorted out.

In the present study, we demonstrate that insulin and troglitazone may protect against certain aspects of the abnormalities induced by nelfinavir. This finding correlates with clinical data suggestive of beneficial metabolic effects of TZD treatment of HAART-treated HIV patients (34,35). The fact that a beneficial effect could be observed both clinically as well as in the 3T3-L1 adipocyte cell line model suggests the potential validity of this in vitro system for studying potential therapeutic interventions for HPI-induced adipocyte abnormalities.

ACKNOWLEDGMENTS

We acknowledge the help of Dr. Michal Hershfinkel (Department of Physiology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel) in performing the confocal microscopy studies. The technical assistance of Dr. Moti Rosenstock is highly appreciated.

REFERENCES


