The Mechanism of Insulin Resistance Caused by HIV Protease Inhibitor Therapy*  

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Retroviral protease inhibitors used as therapy for HIV-1 infection have been causally associated with serious metabolic side effects, including peripheral lipodystrophy, hyperlipidemia, insulin resistance, and in some cases, overt type 2 diabetes. The etiology of this characteristic clinical syndrome remains unknown. We demonstrate that the HIV protease inhibitor, indinavir, dramatically inhibits insulin-stimulated glucose uptake in 3T3-L1 adipocytes in a dose-dependent manner (65% inhibition observed with 100 μM indinavir). Indinavir treatment did not affect early insulin signaling events or the translocation of intracellular Glut1 or Glut4 glucose transporters to the cell surface. To determine whether indinavir may be directly affecting the intrinsic transport activity of glucose transporters, the Glut1 and Glut4 isoforms were heterologously expressed and analyzed in Xenopus oocytes. Indinavir at 100 μM had no effect on Glut1 transport activity in Xenopus oocytes, whereas Glut4 activity was significantly inhibited (45% inhibition). Similar effects on glucose transport were observed for other HIV protease inhibitors. We conclude that HIV protease inhibitors as a class are capable of selectively inhibiting the transport function of Glut4 and that this effect may be responsible for a major iatrogenic complication frequently observed in HIV patients.

The human immunodeficiency virus (HIV) encodes within its genome an aspartyl protease that is required to process its viral precursor polyproteins. This protease activity is essential for the proper formation of infectious HIV virions (1). The recent development of specific agents that target the HIV protease represents an extraordinary advance in the treatment of HIV infection. As part of a combination therapy, HIV protease inhibitors play a critical role in suppressing viral titers and increasing CD4+ lymphocyte counts, which translate to significantly reduced morbidity and mortality among HIV patients (2). Unfortunately, it now appears clear that protease inhibitor use is associated with a potentially serious syndrome of metabolic abnormalities characterized by peripheral fat wasting, central adiposity, hypertriglyceridemia, hypercholesterolemia, and insulin resistance (3–5). Hyperlipidemia and insulin resistance appear to occur at high prevalence among patients using protease inhibitors such that increased risk of premature cardiovascular disease and diabetes becomes a relevant issue (6). The prevalence of lipodystrophy has been reported to be as high as 83% according to one study (3, 5). The etiology of this metabolic syndrome associated with protease inhibitor use currently remains unknown, but its features are similar to those present in the insulin-resistant state commonly referred to as Syndrome X (7).

Recent studies have demonstrated that glucose transport into muscle is a rate-limiting step in whole body glucose disposal (reviewed in Ref. 8). Insulin acutely stimulates glucose uptake in muscle (9–12) and fat (13–15). These tissues express both the Glut1 (16, 17) and Glut4 (18–21) glucose transporter isoforms, although the latter is the predominant species. Upon insulin binding, the intrinsic tyrosine kinase activity of the insulin receptor is activated, which in turn initiates a complex signaling cascade (22). The downstream activation of a wortmannin-sensitive PI 3-kinase appears to be essential for the metabolic effects of insulin (22, 23). Ultimately, insulin signaling impinges on intracellular Glut4 vesicles, causing their rapid exocytosis and fusion with the plasma membrane (9, 13, 14). This phenomenon, known as Glut4 translocation, can account for most of the increase in cellular glucose uptake capacity stimulated by insulin in fat (24, 25) and muscle (26). Glut1 appears to contribute primarily to basal glucose uptake in both tissues (11, 24, 27).

These observations suggest a possible mechanism by which HIV protease inhibitors might induce insulin resistance. The purpose of this study was to address whether or not HIV protease inhibitors can directly affect the facilitated transport of glucose into insulin-responsive cells. We demonstrate here that HIV protease inhibitors selectively and potently decrease the intrinsic transport activity of the insulin-regulated glucose transporter isoform Glut4 without substantially affecting early insulin signaling events or Glut4 translocation. The clinical ramifications of this finding are discussed.

EXPERIMENTAL PROCEDURES

Materials—Indinavir, ritonavir, and amprenavir were obtained from Merck, Abbott, and Glaxo Wellcome, respectively. Xenopus laevis imported African frogs were purchased from Xenopus Express (Homsassa, FL). All other reagents unless otherwise specified were obtained from Sigma.

Cell Culture of 3T3-L1 Adipocytes—3T3-L1 fibroblasts obtained from the American Type Culture Collection were grown to confluence and 48 h later subjected to the differentiation protocol described previously (28). Mature 3T3-L1 adipocytes were maintained in DMEM supplemented with 10% fetal bovine serum and used 10 to 15 days post-differentiation.

2-Deoxyglucose Uptake Measurements in 3T3-L1 Adipocytes—3T3-L1 adipocytes grown in 3.5-cm dishes were serum-starved for at least 3 h and then washed three times with Krebs-Ringer phosphate buffer.
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[3H]2-Deoxyglucose uptake (50 μM cold 2-deoxyglucose) was measured in Krebs-Ringer phosphate buffer as described previously (28) for 6 min at 37 °C under basal and insulin-stimulated conditions (1 μM insulin for 20 min). When indicated, HIV protease inhibitors (indinavir, ritonavir, or amprenavir) were added to the cells at various concentrations 6 min prior to the assay. Stock solutions of indinavir, ritonavir, and amprenavir were made in water. Ritonavir was dissolved in ethanol. When adding ritonavir to cells, the final concentration of ethanol was less than 0.5%. Nonspecific uptake was measured in the presence of 20 μM cytochalasin B and subtracted from the experimental values.

Subcellular Fractionation of 3T3-L1 Adipocytes—3T3-L1 adipocytes were grown in 10-cm dishes and incubated at 37 °C for 4 h in serum-free DMEM in the absence or presence of 100 μM indinavir. After treatment with or without insulin (1 μM for 20 min), the cells were scraped in ice-cold HES buffer (20 mM HEPES, pH 7.4, 255 mM sucrose, and 1 mM EDTA) supplemented with 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and general protease inhibitors (1 μg/ml leupeptin, 1 μg/ml antipain, 5 μg/ml trypsin inhibitor, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride). After homogenization through 11 passes in a Yamato LSC homogenizer (1200 rpm) at 4 °C, subcellular fractionation by differential centrifugation was performed as described previously (29).

Immunoblot Analysis—3T3-L1 adipocyte fractions were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Glut1 and Glut4 transporters were detected using polyclonal antibodies raised against peptides corresponding to the carboxyl-terminal 16 residues of the respective transporter isoform. The autoradiographic signals were quantitated by using a PhosphorImager (Molecular Dynamics). Phosphotyrosine-containing proteins were detected using the monoclonal PY-20 antibody (Transduction Laboratories). Phospho-Akt specific antibodies (New England Biolabs) were used to detect Akt phosphorylated at threonine 308 and serine 473.

Confocal Immunofluorescence Microscopy—3T3-L1 adipocytes were grown on no. 1 glass coverslips. Cells were incubated in the absence or presence of 100 μM indinavir as described above for subcellular fractionation. After treatment with or without insulin (1 μM for 20 min), whole cells were fixed immediately in 4% paraformaldehyde and permeabilized using methanol. PM sheets adherent to the coverslip were prepared by gentle sonication as described previously (30) and subsequently fixed using 4% paraformaldehyde. Glut1 and Glut4 subcellular distributions in the prepared coverslips were visualized by indirect immunofluorescence microscopy using isoform-specific polyclonal antibodies essentially as described previously (30). Images were taken using a Bio-Rad MRC-1024 laser scanning confocal microscope.

2-Deoxyglucose Uptake Measurements in Xenopus Oocytes—X. laevis oocytes were prepared and injected as described previously (31) with 50 ng of either Glut1 or Glut4 mRNA synthesized in vitro (Megascript RNA synthesis kit, Ambion). After a 3-day incubation in Barth’s saline containing albumin at 18 °C, groups of 15–20 oocytes were washed, and [3H]2-deoxyglucose (50 μM containing albumin at 18 °C, groups of 15–20 oocytes were washed, and RNA synthesis kit, Ambion). After a 3-day incubation in Barth’s saline with or without insulin (1 μM for 20 min), the cells were scraped in ice-cold HES buffer (20 mM HEPES, pH 7.4, 255 mM sucrose, and 1 mM EDTA) supplemented with 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and general protease inhibitors (1 μg/ml leupeptin, 1 μg/ml antipain, 5 μg/ml trypsin inhibitor, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride). After homogenization through 11 passes in a Yamato LSC homogenizer (1200 rpm) at 4 °C, subcellular fractionation by differential centrifugation was performed as described previously (29).

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RESULTS AND DISCUSSION

We initially examined the effect of the HIV-1 protease inhibitor indinavir on glucose uptake in 3T3-L1 adipocytes, a system that responds robustly to insulin. When 3T3-L1 adipocytes were treated with indinavir, a statistically significant dose-dependent decrease in insulin-stimulated glucose uptake was observed with an inhibition of 63% at the maximal concentration of indinavir tested (100 μM; Fig. 1A). At 10 μM, which is within the physiologic range of plasma concentrations achieved in vivo in HIV patients (2), indinavir inhibited insulin-stimulated glucose uptake by 28% (p < 0.0001, ANOVA with Fisher PLSD posthoc analysis). Basal glucose uptake was largely unaffected by indinavir, although at 20 μM a modest increase was reproducibly observed. The inhibitory effect of indinavir on insulin-stimulated glucose uptake was very rapid as the drug was added to the cells only 6 min prior to the uptake assay. Furthermore, removal of indinavir rapidly restored normal insulin-responsive glucose uptake within 30 min (data not shown). Inhibition of insulin-stimulated glucose uptake appears to be a general property of HIV-1 protease inhibitors, as two other compounds within this class, amprenavir and ritonavir, also exhibited an effect comparable with that of indinavir (Fig. 1B).

Immunoblot analysis of 3T3-L1 adipocyte subcellular fractions with anti-phosphotyrosine antibodies revealed that insulin receptor autophosphorylation and subsequent tyrosine phosphorylation of insulin receptor substrate-1 occurred normally in cells exposed to indinavir (Fig. 2A). As the metabolic effects of insulin require PI 3-kinase activation (22, 23), the in vitro phosphorylation status of the downstream Akt kinase was assessed using phospho-Akt-specific antibodies. Indinavir was found to have no effect on the insulin-stimulated phosphorylation of Akt on threonine 308 or serine 473 (Fig. 2B), thus demonstrating that the PI 3-kinase signaling pathway remained intact. Insulin acutely stimulates glucose uptake in muscle and fat cells by triggering the translocation of intracellularly sequestered glucose transporters, predominantly the Glut4 transporter isoform, to the plasma membrane (32). 3T3-L1 adipocytes express Glut1 and Glut4 (25, 28), and both of these transporter isoforms appeared to translocate properly to the cell surface in response to insulin despite the presence of 100 μM indinavir. The glucose transporter content in the PM fractions detected by isoform-specific antibodies increased with insulin by 81%, and 63% for Glut1, and by 36% and 38% for Glut4 in control and indinavir-treated cells, respectively. Conversely, the transporter content in the low density microsome (LDM) fractions decreased by 37% and 48% for Glut1, and by 21% and 19% for Glut4 in control and indinavir-treated cells, respectively (Fig. 2C). Confocal immunofluorescence microscopy of whole cells and plasma membrane “sheets” also showed that the subcellular distribution of glucose transporters was un-
changed in indinavir-treated samples relative to control cells. Both control and indinavir-treated cells exhibited increased Glut1 and Glut4 staining at the plasma membrane upon stimulation with insulin (Fig. 2D). The rapid onset of inhibition observed in the glucose uptake assay (Fig. 1), in which indinavir was added to the cells after sufficient time had elapsed for the majority of the transporters to reach the plasma membrane following insulin stimulation (33–35), is consistent with indinavir acting at a site subsequent to the translocation of transporters to the plasma membrane. Additionally, the extent of inhibition of transport activity did not change if indinavir was added either before or after 20 min of insulin stimulation (data not shown).

Glut1 and Glut4 were heterologously expressed in X. laevis oocytes by microinjection of their respective mRNA to test the possibility that indinavir might be directly inhibiting the intrinsic transport activity of glucose transporters. Indinavir at 100 μM had no effect on Glut1 activity in Xenopus oocytes. Remarkably, however, the activity of Glut4 expressed in oocytes was inhibited by 45% at the maximal dose of indinavir tested (100 μM), an effect of comparable magnitude to that observed in insulin-stimulated 3T3-L1 adipocytes (Fig. 3 A).

Ritonavir and amprenavir also selectively inhibited Glut4 by 54 and 42%, respectively (Fig. 3 B). The data obtained in Xenopus oocytes are consistent with what is observed in 3T3-L1 adipocytes, in which basal (indinavir-resistant) and insulin-stimulated (indinavir-inhibitable) glucose uptake are largely mediated by Glut1 and Glut4, respectively (25).

From the above data, we conclude that HIV protease inhibitors unexpectedly act as potent, isoform-specific inhibitors of the transport function of the Glut4 glucose transporter. This is
the first demonstration that pharmacologic manipulation of glucose transport is feasible in a selective manner. An agent that can reversibly induce an insulin-resistant state would be a very useful tool in developing model systems that mimic the pathogenesis of type 2 diabetes.

Glut4 is predominantly expressed in tissues responsible for the bulk of whole body glucose disposal (skeletal/cardiac muscle and fat) (18–21) and is believed to be the principal transporter isofrom mediating insulin-stimulated glucose uptake at these sites. As glucose transport is the rate-limiting step for whole body glucose disposal in rodents (36–38) and in humans (39), the inhibitory effect of retroviral protease inhibitors on Glut4 is therefore likely to be the direct cause of insulin resistance observed in HIV patients receiving this class of drugs. In predisposed individuals, diabetes can result after pancreatic β cells fail to compensate for the insulin resistance. A recent clinical study employing a longitudinal design comparing fasting glucose and insulin levels may be very useful tool in developing model systems that mimic the chronic consequences are likely to be more prevalent in the future. Further drug development may be necessary to design new compounds that maintain the efficacy in the management of HIV infection, but that also minimize the detrimental effect on the glucose transport system observed in this study.

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