Long-term effects of HIV-1 protease inhibitors on insulin secretion and insulin signaling in INS-1 beta cells

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Introduction

The clinical introduction of HIV (human immunodeficiency virus)-1 protease inhibitors led to a dramatic decline in the morbidity and mortality rates of HIV-infected patients (Kaplan et al. 2000). It is, however, also increasingly well established that PIs cause insulin resistance that is associated with the long-term development of a metabolic syndrome-like disease including impaired glucose tolerance/diabetes and cardiovascular complications (Walli et al. 1998, Carr et al. 1999, Noor et al. 2001, 2002, Holmberg et al. 2002). The mechanism by which PIs induce insulin resistance is not clear. Studies with various cell lines, including 3T3-L1 adipocytes and L6-myotubes, and in rats suggest, however, that PIs acutely inhibit the cellular glucose-transport system (Murata et al. 2000, 2002, Hruz et al. 2002, Ben-Romano et al. 2003). In addition, there is evidence that, after longer incubation periods, PIs also affect insulin signaling at the level of insulin-receptor substrate (IRS)-1 phosphorylation (Schütt et al. 2000, Cammalleri & Germinario 2003), association of the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase; Schütt et al. 2000) and/or Thr308/Akt phosphorylation (Schütt et al. 2000, Rudich et al. 2001, Ben-Romano et al. 2003) in HepG2 hepatoma cells (Schütt et al. 2000) or 3T3-L1 cells (Rudich et al. 2001, Ben-Romano et al. 2003, 2004) respectively. A potential role of impaired insulin signaling in the insulin-resistance syndrome caused by PIs is also supported by a recent study on a novel activator of the insulin receptor tyrosine kinase, that was able to reverse PI-induced insulin resistance in 3T3-L1 cells and to prevent PI-induced insulin resistance in rats (Cheng et al. 2004).

PI-induced alterations in insulin signaling may not only be associated with peripheral insulin resistance. If present in beta cells, they could also lead to an impairment of insulin secretion. Here we investigated the long-term effects of HIV-1 protease inhibitors on glucose-stimulated insulin secretion from beta cells and explored whether altered insulin secretion might be related to altered insulin signaling. INS-1 cells were incubated for 48 h with different concentrations of amprenavir, indinavir, nelfinavir, ritonavir or saquinavir, stimulated with 20 mM D-glucose, and insulin determined in the supernatant. To evaluate insulin signaling, cells were stimulated with 100 nM insulin for 2 min, and insulin-receptor substrate (IRS)-1, -2 and Akt phosphorylation determined. Incubation for 48 h with ritonavir, nelfinavir and saquinavir resulted in impaired glucose-induced insulin secretion at 2.5, 5 and 5 µM respectively, whereas amprenavir or indinavir had no effects even at 20 and 100 µM respectively. The impaired insulin secretion by ritonavir, nelfinavir and saquinavir was associated with decreased insulin-stimulated IRS-2 phosphorylation, and, for nelfinavir and saquinavir, with decreased insulin-stimulated IRS-1 and Thr308-Akt phosphorylation. No such effects on signaling were observed with amprenavir or indinavir. In conclusion, certain HIV-1 protease inhibitors, such as ritonavir, nelfinavir and saquinavir, not only induce peripheral insulin resistance, but also impair glucose-stimulated insulin secretion from beta cells. With respect to the long-term effect on beta-cell function there appear to be differences between the protease inhibitors that may be clinically relevant. Finally, these effects on insulin secretion after a 48 h incubation with protease inhibitor were associated with a reduction of the insulin-stimulated phosphorylation of insulin signaling parameters, particularly IRS-2, suggesting that protease inhibitor-induced alterations in the insulin signaling pathway may contribute to the impaired beta-cell function.

Abstract

The mechanism by which chronic treatment with HIV (human immunodeficiency virus)-1 protease inhibitors leads to a deterioration of glucose metabolism appears to involve insulin resistance, and may also involve impaired insulin secretion. Here we investigated the long-term effects of HIV-1 protease inhibitors on glucose-stimulated insulin secretion from beta cells and explored whether altered insulin secretion might be related to altered insulin signaling. INS-1 cells were incubated for 48 h with various cell lines, including 3T3-L1 adipocytes and L6-myotubes, and in rats suggest, however, that PIs acutely inhibit the cellular glucose-transport system (Murata et al. 2000, 2002, Hruz et al. 2002, Ben-Romano et al. 2003). In addition, there is evidence that, after longer
insulin secretion. Thus insulin-secretion defects have been observed in mice and beta cells from mice with a beta-cell-specific insulin receptor (Kido et al. 2000) or IRS-1 (Kulkarni et al. 1999a, Kido et al. 2000) knockout. Furthermore, in IRS-2-knockout mice, insulin-secretory defects were associated with a reduction of beta-cell mass and insulin content, suggesting a role of insulin and/or insulin-like growth factor-I signaling through IRS-2 for beta-cell growth and function (Kido et al. 2000, Kubota et al. 2000, White 2002). The notion that PIs might influence insulin secretion as well is consistent with a recent study where homeostasis model assessment and hyperglycemic glucose clamps was found to be impaired in patients treated with PIs, predominantly nelfinavir (Woerle et al. 2003).

The concept that PIs directly affect the beta-cell function is also strongly supported by a recent study where acute effects (1 h) on glucose-stimulated insulin release in association with reduced glucose uptake in mouse islets and MIN6 beta cells were found (Koster et al. 2003). Here we investigate in INS-1 beta cells whether a long incubation period (48 h) with the PIs amprenavir, indinavir, nelfinavir, ritonavir or saquinavir alters glucose-stimulated insulin secretion, and whether an altered insulin secretion is associated with alterations in the insulin signaling cascade.

### Materials and Methods

#### Materials

RPMMI 1640 buffer was purchased from Gibco (Karlsruhe, Germany), insulin from Novo-Nordisk (Baegvard, Denmark) and a rat insulin ELISA kit from Mercodia (Uppsala, Sweden). Antibodies against IRS-1, IRS-2 and the PI3-kinase 85 kDa regulatory subunit were from Upstate Biotechnology (Lake Placid, NY, USA), anti-phosphotyrosine antibody was from Transduction Laboratories (Lexington, KY, USA) and Akt and Thr308-phosphotyrosine antibody was from Transduction Laboratories (Uppsala, Sweden). Antibodies against IRS-1, IRS-2 and diabetes in vivo and in vitro by monitoring the glucose-stimulated insulin secretion. The medium was replaced for 5 min and then again 30 min with Krebs-Ringer bicarbonate buffer (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 1.5 mM CaCl2, 10 mM Hepes, 2 mM NaHCO3 and 0.1% BSA; pH 7.4 at 37 °C) to which a PI and/or ethanol had been added at a similar concentration as before. A part of these cell-culture dishes was then used for the subsequent measurement of insulin secretion, another part for the measurement of insulin signaling, and another part for the measurement of cell number, viability and DNA content.

#### Insulin secretion

The medium was replaced by fresh Krebs-Ringer bicarbonate buffer that contained the respective PI and/or ethanol concentration as before and, in addition, 0 or 20 mM D-glucose. After 30 min at 37 °C, the insulin concentration in the medium was measured by a commercial rat insulin ELISA kit.

#### Insulin signaling

The medium was replaced by fresh Krebs-Ringer bicarbonate buffer that contained the respective PI and/or ethanol concentration as before and, in addition, 0 or 100 mM insulin. After 2 min at 37 °C, incubations were stopped, cells washed briefly with ice-cold PBS (137 mM NaCl, 0.27 mM KCl, 0.5 mM Na2HPO4 and 0.15 mM KH2PO4, pH 7.4) and frozen immediately in liquid nitrogen. The thin ice layer on the cells was scraped off from the dishes at −20 °C and homogenized with a motor-driven Potter homogenizer in solubilization buffer (1% IGEPAL, 20 mM Hepes, 8 mM EDTA, 530 mM NaF, 30 mM sodium pyrophosphate, 7 mM sodium vanadate, 2 mM dichloroacetic acid, 9 mM PMSF, 21 mM benzamidine, 5 µM leupeptin, 9 µM pepstatin and 10 µg/ml aprotinin, pH 7.4; final concentrations) at 4 °C. Samples were centrifuged at 10,000 g (4 °C) to remove insoluble material, and protein concentrations measured by BioRad protein assay.

For IRS-1 or -2 immunoblots, solubilized cells (600 or 500 µg protein for IRS-1 or IRS-2 respectively) were incubated overnight with 3 µg anti-IRS-1 or 6 µg anti-IRS-2 antibody, and then added to 50 µl packed agarose beads crosslinked with protein G. Immunoprecipitated proteins were detached with Laemmli buffer, boiled and subjected to SDS/PAGE. For determination of total Akt

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and Thr\textsuperscript{308}-phosphorylated Akt, cell lysates (50 µg protein) were subjected directly to SDS/PAGE. Proteins were transferred to nitrocellulose membranes and detected with specific antibodies against IRS-1, IRS-2, phosphoryl tyrosine, Akt or Thr\textsuperscript{308}-phosphorylated Akt. They were then visualized with horseradish peroxidase-labeled secondary antibodies using enhanced chemiluminescence.

Cell number, viability and DNA content

Cells were counted in a Neubauer chamber, and viability tested with Trypan Blue exclusion. In addition, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used for the measurement of the activity of living cells (Mosmann 1983). This assay is based on the capacity of cellular mitochondrial dehydrogenase enzymes to convert the yellow water-soluble substrate MTT into an insoluble, intracellular purple formazan product. Subsequently, the optical density (OD) of this product was measured by spectrophotometer (test wavelength 570 nm and reference wavelength 630 nm). DNA content of cell extracts was measured using a Versa Fluor fluorometer and the fluorescent reagent Hoechst 33258 (bisbenzimide; Wu et al. 2003).

Data analysis

Bands on the gels were quantified densitometrically with Molecular Analyst software (BioRad). To calculate arbitrary units (AU), the bands of the four corresponding samples (without or with insulin/with or without PI) were always compared. This was done according to Schütt et al. (2000) and Meyer et al. (2002) by dividing the density of the individual band by the mean density of all four bands that were compared. Analysis of variances on ranks (ANOVA) was performed to assess statistical differences between sample means of the insulin-release measurements. The other comparisons were performed with Student’s unpaired t-test after normal distributions had been verified with the Kolmogorov–Smirnov test.

Results

Glucose-stimulated insulin secretion

In cells that had not been preincubated with PIs, glucose increased insulin secretion approximately 2-fold (Fig. 1). The PIs had different effects on insulin secretion: ritonavir, nelfinavir and saquinavir decreased glucose-stimulated insulin secretion at concentrations as low as 2·5, 5 and 5 µM respectively, whereas amprenavir and indinavir had no effect even at the maximally tested 20 and 100 µM. Furthermore, preincubation of the cells with 5 and 10 µM nelfinavir or saquinavir respectively, or with 10 µM ritonavir significantly reduced the basal insulin secretion of the cells. The effects on the insulin secretion were without changes in cell number or viability as assessed by Trypan Blue exclusion, alterations in MTT reduction or alterations of the DNA content/dish (Table 1).

IRS-1, IRS-2 and Thr\textsuperscript{308}-Akt phosphorylation

In cells that had not been preincubated with PIs, insulin stimulation of the cells led to 2-, 5- and 4-fold increases in IRS-1, IRS-2 or Thr\textsuperscript{308}-Akt phosphorylation respectively (Fig. 2a). The PIs that impaired glucose-stimulated insulin secretion (nelfinavir, ritonavir and saquinavir; Fig. 1) also decreased insulin-stimulated IRS-2 phosphorylation whereas this was not the case with amprenavir and indinavir. Insulin-stimulated IRS-1 and Akt-Thr\textsuperscript{308} phosphorylation were also reduced by two (nelfinavir, saquinavir) of the three PIs that inhibited insulin secretion, and there was also a tendency for the third (ritonavir) to decrease IRS-1 phosphorylation. However, this was not statistically significant.

Preincubation of the cells with PIs had no effect on the protein expression of IRS-1, IRS-2 or Akt (Fig. 2b).

Discussion

In this study we describe how a long-term exposure of INS-1 cells to the PIs nelfinavir, saquinavir and ritonavir impaired glucose-stimulated insulin secretion. These effects were observed at non-cytotoxic concentrations and suggest that, in addition to inducing peripheral insulin resistance, long-term (48 h) exposure to PIs can also impair insulin secretion. The notion that PIs affect insulin secretion in vitro has also been suggested by a recent study where PIs acutely (1 h) decreased glucose-stimulated insulin release from mouse islets and MIN6 beta cells (Koster et al. 2003). Moreover, there is clinical evidence that patients treated predominantly with nelfinavir plus nucleoside reverse transcriptase inhibitors have an impaired first-phase insulin release as compared with non-treated healthy subjects (Woerle et al. 2003).

Our data also suggest that the tested PIs differ in their effect on glucose-induced insulin secretion. Thus 2·5 µM ritonavir inhibited glucose-stimulated insulin secretion, whereas amprenavir had no effect even at an eight-times higher concentration. This difference between the two PIs was observed although ritonavir and amprenavir are similarly effective at inhibiting the production of infectious virus from HIV-infected lymphocytes (IC\textsubscript{50} 0·068 and 0·078 µM for ritonavir and amprenavir respectively; Molla et al. 1998). In the presence of binding proteins (50% human serum), a condition that more likely resembles the situation in our experiments (our medium contained 10% fetal calf serum) ritonavir was even less effective at inhibiting virus replication than amprenavir (IC\textsubscript{50} 1·34 and 0·55 µM for ritonavir and amprenavir respectively; Molla et al. 1998). Taken together, this
suggests that, at least in vitro, ritonavir inhibits virus replication only at concentrations similar to or higher than amprenavir, but, after a 2 day incubation period, impairs insulin secretion at considerably lower concentrations than amprenavir.

Different relative effects on the inhibition of glucose-stimulated insulin secretion and the effects on viral replication also appear to exist for indinavir as compared with nelfinavir and saquinavir. Thus we did not find an effect of indinavir even at the maximally tested concentration of 100 µM. In contrast, nelfinavir and saquinavir inhibited the glucose-stimulated insulin secretion at a 20-times lower concentration. This is again in contrast to the inhibitory effects on viral replication, where the free indinavir concentration only needed to be two or three times higher than nelfinavir and saquinavir to attain similar effects (IC_{50} 0·041, 0·025 and 0·014 µM for indinavir, nelfinavir and saquinavir respectively; Molla et al. 1998). Since indinavir has lower serum protein binding and thus results in a higher free indinavir concentration, it was

Figure 1 Effect of PIs on insulin secretion. Cells were treated for 48 h with the indicated PI concentrations. Cells were then incubated without (white bars) or with 20 mM D-glucose (black bars) for 30 min. The insulin concentration in the supernatant was measured by ELISA and normalized for the cellular DNA content. Shown are means ± S.E.M. (n=4). *P<0·01 versus no PI.
**Table 1** Cell number and viability. Cells were incubated in parallel under exactly the same conditions as for the measurements of insulin release and insulin signaling and parameters measured as described in the Materials and Methods section. Shown are means ± S.E.M. (n=4).

<table>
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<th>PI, concentration (µmol/l)</th>
<th>Cell number (10^6/dish)</th>
<th>Trypan blue exclusion (% viable cells)</th>
<th>MTT-reduction (OD570–630 nm)</th>
<th>DNA (µg/dish)</th>
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<td>2.5</td>
<td>6.00 ± 0.65</td>
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<td>1.16 ± 0.9</td>
<td>56.4 ± 2.7</td>
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<td>20</td>
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<td>1.13 ± 0.2</td>
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<td>Indinavir</td>
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<td>1.16 ± 0.3</td>
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effective at even lower concentrations than nelfinavir and saquinavir if 50% human serum was present (IC50 0.92 and 0.37 µM for indinavir, nelfinavir and saquinavir, respectively; Molla et al. 1998). These data for the effects on virus replication and our results, based on a long-term incubation period, therefore suggest that, compared with the effect on virus replication, indinavir causes less impairment of insulin secretion than nelfinavir and saquinavir.

Differences between PIs have previously also been observed for effects in insulin target tissues. Thus, consistent with our observations, long-term (48 h) exposure to nelfinavir, ritonavir or saquinavir, but not to amprenavir or indinavir, increased intracellular triglyceride synthesis of HepG2 cells (Lenhard et al. 2000a), and in C3H10T1/2 stem cell adipocytes reduced lipogenesis and stimulated lipolysis (Lenhard et al. 2000b). Moreover, our finding that long-term exposure to indinavir even at relatively high concentrations does not impair insulin secretion is consistent with a previous study where indinavir was the only PI tested, and 20 µM for 48 h also had no effect on the glucose-stimulated insulin secretion from INS-1 cells (Yarasheski et al. 1999). Our finding that PIs differ in their effect on insulin secretion in INS-1 cells is, however, in contrast to the observations by Koster et al. (2003) who found that amprenavir and indinavir (20 µM), similar to the other PIs, decreased insulin secretion in mouse islets and MIN6 beta cells. Potential reasons for this discrepancy include the fact that, in their study, short-term effects (1 h) were investigated. Mechanisms for the PI-induced effects may differ between short- and long-term exposure. Thus short-term exposure appears to predominantly affect the glucose-transport system (Murata et al. 2000, 2002, Hruz et al. 2002, Ben-Romano et al. 2003) whereas effects on insulin signaling at the level of IRS-1 (Schütt et al. 2000, 2002, Hruz et al. 2002, Ben-Romano et al. 2003) are observed only after a longer exposure.

PI-induced effects on insulin secretion, and especially differences between PIs as mentioned above, may be of clinical importance. It is not clear, however, whether the in vitro observations reflect the in vivo situation. Therapeutically effective concentrations for ritonavir, nelfinavir and saquinavir are 3.6–15.5, 1.4–5.2 and 0.3–9.2 µM respectively (Acosta & King 2003), and therefore within the range that led to an inhibition of glucose-stimulated insulin secretion in INS-1 cells in our study (2.5, 5 and 5 µM respectively). For amprenavir and indinavir, therapeutically effective concentrations are 2.3–12.6 and 1.8–9.5 µM respectively, and no inhibition of the glucose-stimulated insulin secretion from INS-1 cells was observed even at 20 and 100 µM respectively, in our study. Although such in vitro/in vivo comparisons are difficult, our data therefore suggest that differential effects of the PIs on...
Figure 2 (a).
Figure 2 (b). Effects of HIV-1 protease inhibitors on beta-cell function.
insulin secretion may also be present in vivo. So far, no controlled clinical study has investigated whether PIs differ with respect to their effect on insulin secretion. However, there are limited clinical observations that suggest different drug-specific metabolic side effects concerning insulin secretion and/or insulin resistance (Periard et al. 1999, Dube et al. 2002).

To investigate potential mechanisms for the PI-induced impairment of beta-cell function, we also investigated insulin signaling. The role of the beta-cellular insulin signaling system for insulin secretion is not exactly understood and involves a controversially discussed concept, namely the autocrine feedback action of secreted insulin on beta-cell function. Whereas former studies frequently suggested a negative effect of insulin on beta-cell function, more recent studies provide evidence for a positive role of insulin in insulin secretion and beta-cell survival (Leibiger et al. 2002). The notion that insulin signaling may be important for the regulation of glucose-induced insulin secretion has emerged from studies in mice, where knockout of an insulin receptor (Kulkarni et al. 1999b), IRS-1 (Kulkarni et al. 1999a, Kido et al. 2000) or IRS-2 (Kido et al. 2000, Kubota et al. 2000, White et al. 2002) led to insulin-secretion defects. Further evidence comes from the observation that insulin secretion was impaired in isolated human islets carrying the Arg^972->Gln-IRS-1 polymorphism which results in decreased binding of the p85 subunit of PI3-kinase to IRS-1 (Marchetti et al. 2002), and the association of reduced or enhanced insulin secretion with reduced or enhanced PI3-kinase and Akt signaling in hyperglycemic (Hribal et al. 2003) or rosiglitazone-treated (Yang et al. 2001) rats respectively. In our study, the three PIs that impaired glucose-stimulated insulin secretion also reduced insulin-stimulated IRS-2 phosphorylation, two of them also insulin-stimulated IRS-1 and Thr^308-Akt phosphorylation, and no such effects were observed with the PIs that did not inhibit insulin secretion. These observations suggest that alterations in insulin signaling could cause or contribute to the long-term effects of some PIs on glucose-induced insulin secretion.

The impairment of glucose-stimulated insulin secretion from PI-treated INS-1 cells was most clearly associated with a decreased insulin-stimulated IRS-2 phosphorylation. This is consistent with previous studies in IRS-2-knockout mice, where the defect in insulin secretion was associated with severe alterations in beta-cell mass, insulin content and the expression of several gene products that promote beta-cell function, including normal glucose detection (Kido et al. 2000, Kubota et al. 2000, White et al. 2002). The observation in our study — that only insulin secretion was impaired, but INS-1 cell proliferation was unaltered — may be related to the INS-1 tumor cell line where proliferation regulation is abnormal. It also needs to be taken into account that the knockout data relate to a complete loss of the IRS-2 protein (Kido et al. 2000, Kubota et al. 2000, White et al. 2002), whereas in our study only insulin-induced IRS-2 phosphorylation was reduced but protein expression was unaltered. In any case, our findings are consistent with a role of insulin signaling, especially IRS-2 phosphorylation, in the PI-induced impairment of insulin secretion. Potential mechanisms for a contribution of insulin signaling to insulin secretion include insulin-dependent effects on the increase of cytosolic free Ca^{2+}, regulation of the glucokinase and/or gene transcription (Leibiger et al. 2002, Ohsugi et al. 2004). Under our conditions we detected no effect of any of the PIs on 3-O-methylglucose uptake (data not shown). Therefore alterations in glucose uptake appear not to be involved. In general it has, however, to be considered that the importance of such alterations of the insulin signaling system under in vivo conditions, where insulin-induced signal transduction is influenced by a complex crosstalk between various signals, such as neural factors, incretins and other co-secreted hormones, is not clear.

In conclusion our results suggest that a long-term exposure to PIs not only induces peripheral insulin resistance but also impairs glucose-stimulated insulin secretion from beta cells. With respect to the effect on beta-cell function there appear to be differences between the PIs tested, and these differences may be clinically relevant. Finally, the long-term PI effects on insulin secretion were associated with a reduction of the insulin-stimulated phosphorylation of insulin-signaling parameters, suggesting that the induction of insulin resistance in the beta cells may have played a role for the impaired beta-cell function.

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**Figure 2** (A) Effect of PIs on insulin-stimulated IRS-1 and IRS-2 tyrosine phosphorylation (PY-IRS-1, PY-IRS-2), and Thr^308-Akt phosphorylation of Akt (P-Akt), and (B) on protein amounts. Cells were treated for 48 h with the indicated PI concentrations and then incubated with no (white bars) or 100 nM (black bars) insulin for 2 min. Subsequently the incubation was stopped and cells were lysed. IRS-1 (left-hand columns) or IRS-2 (middle columns) were first immunoprecipitated, subjected to SDS/PAGE and then immunoblotted with anti-phosphotyrosine antibody as described in the Materials and Methods section. For Akt immunoblots (right-hand column), complete cell extracts were subjected to SDS/PAGE and immunoblots performed with anti-phospho-Thr^308-Akt antibody. Protein amounts were measured as described in the Materials and Methods section. Data represent means ± S.E.M. (n=4). *P<0.01 versus no PI.
References


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