Short communication

The HIV-1 protease inhibitor indinavir impairs insulin signalling in HepG2 hepatoma cells

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Abstract

Aims/hypothesis. Patients treated with human immunodeficiency virus-1 protease inhibitors often develop impaired glucose tolerance or diabetes, most likely due to an induction of insulin resistance. We therefore investigated whether the protease inhibitor indinavir alters insulin signalling.

Methods. We incubated HepG2 cells for 48 h without or with indinavir (100 µmol/l). Subsequently ¹²⁵I-insulin binding to the cells and the effects of insulin stimulation on insulin-receptor substrate-1-phosphorylation, association of phosphatidylinositol 3-kinase with insulin-receptor substrate-1 and Akt-Thr³⁰⁸-phosphorylation were measured.

Results. In cells not exposed to indinavir, insulin (100 nmol/l) led to rapid increases of insulin-receptor substrate-1-phosphorylation, association of phosphati-

dylinositol 3-kinase with insulin-receptor substrate-1 and Akt-phosphorylation during the first 75 s, followed by subsequent decreases. In indinavir-treated cells, these insulin-stimulated increases during the first 75 s were reduced by 30–60 % and this was not associated with alterations in cell number or viability, insulin binding to the cells or cellular insulin-receptor substrate-1-content.

Conclusion/interpretation. Effects of indinavir on initial insulin signalling could cause, or contribute to, the metabolic effects of human immunodeficiency virus-1 protease inhibitors. [Diabetologia (2000) 43: 1145–1148]

Keywords HIV protease inhibitors, lipodystrophy syndrome, diabetes mellitus, insulin resistance, insulin signalling.

Patients treated with human immunodeficiency virus-1 (HIV-1) protease inhibitors frequently develop IGT or diabetes [1]. The underlying cause seems to be a protease inhibitor-induced insulin resistance [1]. We investigated potential molecular mechanisms by which the protease inhibitor indinavir might alter insulin signalling and in this way trigger insulin resistance.

Cellular insulin signalling is initiated by binding to its specific cell surface receptors, followed by rapid

phosphorylation of tyrosine residues on the receptor β-subunit [2]. This results in activation of the insulin-receptor kinase towards tyrosine-containing sites of a number of intracellular target proteins. Phosphorylation of these insulin-receptor substrates is thought to play a major part in the transduction of the insulin signal [2]. An important substrate is IRS-1. Once it is phosphorylated by the insulin receptor, several other intracellular proteins bind to IRS-1 and further propagate the insulin signal [2]. Among these is phosphatidylinositol 3-kinase (PI 3-kinase), a lipid kinase, which upon binding to IRS-1 is activated and which seems to be essential for the signalling that leads to the initiation of glucose transport [2]. It is likely that this downstream signalling involves activation of Akt by Thr³⁰⁸/Ser⁴⁷³-phosphorylation [2]. To investigate whether indinavir alters insulin signalling, we

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Abbreviations: HIV-1, Human immunodeficiency virus-1; PI 3-kinase, phosphatidylinositol 3-kinase.

measured insulin-stimulated IRS-1-phosphorylation, association of PI 3-kinase with IRS-1 and Akt-Thr³⁰⁸-phosphorylation in HepG2 hepatoma cells treated without or with indinavir.

Materials and methods

Materials. Fetal calf serum (FCS) was purchased from Biochrom (Berlin, Germany), Leibovitz-L15 medium from Gibco (Karlsruhe, Germany) and [¹²⁵I-Tyr-A¹⁴]-monoiodoinsulin from Amersham (Braunschweig, Germany). Antibodies against IRS-1 and PI 3-kinase 85000 M_r-regulatory subunit were from Upstate Biotechnology (Lake Placid, N. Y., USA), anti-phosphotyrosine antibody from Transduction Laboratories (Lexington, Ky., USA) and Akt and Thr³⁰⁸-phosphorylated Akt antibodies from New England BioLabs (Beverly, Mass., USA). Peroxidase-labelled antibodies came from Dako (Glostrup, Denmark); other chemicals were from Sigma (Deisenhofen, Germany) or Roche (Mannheim, Germany). Indinavir was from Merck&Co. (Rahway, N. J., USA).

Preparation of indinavir stock solution and cell incubation. To prepare indinavir stock solution, 35.6 mg indinavir powder (purity ≥ 99.5% according to manufacturer) were dissolved in 1 ml 6.25% HCl. This was added to Leibovitz-15 medium and the pH adjusted to 7.4 with NaHCO₃ (final volume 100 ml). Stock solution without indinavir was similarly prepared, except that the HCl did not contain indinavir. HepG2 cells that had grown to near confluency (Leibovitz-15 medium supplemented with 10% FCS, 5 mmol/l glucose, 2.5 µl/ml transferrin, 60 µg/ml fetuin and 20 µg/ml gentamicin at 37 °C) were then incubated for 24 h with 16 ml fresh Leibovitz-15 medium to which 4 ml of the stock solutions without or with indinavir (final concentration 100 µmol/l) had been added. This medium was replaced by fresh medium that was similarly prepared, except that it now contained only 1% FCS, and the incubation continued for 24 h. We chose 48 h of incubation without or with 100 µmol/l indinavir because preceding experiments had shown that indinavir more strongly inhibited insulin-stimulated IRS-1-phosphorylation after 48 h (35%) than after 24 (24%) and 8 h (17%) and that 100 µmol/l indinavir was more potent (33%) than 10 µmol/l (15%; data not shown).

Cell number and viability. Cells were counted in a Neubauer chamber and viability tested with trypan blue exclusion.

Insulin binding. Insulin binding to the cells was measured as described previously [3]. Briefly, cells were preincubated for 15 min with incubation buffer (20 mmol/l HEPES, 130 mmol/l NaCl, 4.8 mmol/l KCl, 1.3 mmol/l KH₂PO₄, 1 g/l D-glucose, 1.3 mmol/l MgSO₄, 1.2 mmol/l CaCl₂, 2% BSA, pH 7.4) that contained 0 or 100 µmol/l indinavir. This buffer was then replaced by fresh incubation buffer without and with indinavir, and after another preincubation for 15 min 0.17 nmol/l (final concentration) [¹²⁵I-Tyr-A¹⁴]-monoiodoinsulin (185 · 10⁶ Bq⁻¹ · mmol⁻¹) and different concentrations of unlabelled insulin were added. After different time periods the buffer was removed and dishes washed with a solution that contained 154 mmol/l NaCl and 10 mmol/l TRIS, pH 7.4. Cells were detached with 1% Triton X-100, collected and associated radioactivity detected.

Insulin stimulation and solubilization of cells. We preincubated cells as described for insulin binding. We then added 0 or 100 nmol/l insulin (final concentration) and incubated cells for different times at 37 °C. Subsequently, the buffer was removed

and the dishes with the cells rapidly frozen in liquid nitrogen. The thin ice layer with the cells was scraped off from the dishes at -20 °C and homogenized with a motor-driven Potter homogenizer in 1% Triton X-100, 5 mmol/l phenylmethylsulphonyl-fluoride, 800 U/ml aprotinin, 8 mmol/l EDTA, 30 mmol/l benzamidine, 2.5 µg/ml pepstatin, 2.5 µg/ml leupeptin, 160 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 0.2 mmol/l sodium vanadate, 2 mmol/l dichloroacetic acid and 20 mmol/l HEPES, pH 7.4 (final concentrations). Samples were then centrifuged at 10⁵ g (4 °C) to remove insoluble material and protein concentrations measured.

Immunoblots. We incubated solubilized cells (300 µg protein) overnight with 1 µg anti-IRS-1-antibody and then added them to 30 µl packed agarose beads cross-linked with protein G. These were shaken 30 s every 5 min for 12 h and then washed. Bound proteins were detached with Laemmli-buffer, boiled and subjected to SDS-PAGE. For measurement of Thr³⁰⁸-phosphorylated Akt, cell lysates (100 µg protein) were directly subjected to SDS-PAGE (7.5%). Proteins were then transferred to nitrocellulose (phosphotyrosine, p85, Akt and phospho-Akt blots) or polyvinylidene difluoride-membranes (IRS-1 blots). Membranes were blocked in 10% block mix (Boehringer Mannheim, Germany) or 5% skim, milk, respectively and incubated with the specific antibodies for 2 h. Bound antibodies were detected by peroxidase-labelled antibodies and chemiluminescence.

Data analysis. In every western blot we compared eight bands (four time points ± indinavir) by scanning densitometry. Arbitrary units represent the ratio between the intensity of the single bands and the mean intensity of all eight bands in the same blot. Differences were tested by *t* statistics for paired data. We considered a *p* value of 0.05 or less to be significant.

Results

Cell number and viability. Indinavir had no effect on cell number ($1.7 \times 10^6 \pm 0.16$ and $1.7 \times 10^6 \pm 0.13$

Fig. 1. ¹²⁵I-Insulin binding. Cells were incubated without (□, ○, △) or with (■, ●, ▲) 100 µmol/l indinavir for 48 h as described. Subsequently, insulin binding was measured as described with 0.17 nmol/l ¹²⁵I-insulin and 0 (□, ■), 3.3 (○, ●), or 3300 nmol/l (▲, △) unlabelled insulin. After the indicated times cells were washed and transferred for gamma-counting. Data represent means ± SEM, *n* = 3

Fig. 2A–D. Effect of indinavir on IRS-1-phosphorylation (A), association of PI 3-kinase with IRS-1 (B), IRS-1-amount (C) and Akt-phosphorylation (D). Cells were incubated without (○) or with (●) 100 μmol/l indinavir for 48 h and then stimulated with 0 or 100 nmol/l insulin for the indicated times. They were then solubilized and IRS-1 immunoprecipitated with anti-IRS-1-antibody bound to protein G agarose beads. The beads were washed, bound proteins detached with Laemmli-buffer and immunoblots done as described in *Methods*. Thr³⁰⁸-phosphorylated Akt in cell lysates was measured by immunoblot with specific anti-phospho-Akt antibody. AU were calculated as described. Shown are means ± SEM (*n* = 5), * *p* ≤ 0.05. Insets show representative blots ± indinavir at the respective time points

cells/dish treated without or with indinavir, respectively) or cell viability (9.7 ± 0.4 and 9.7 ± 0.4 % trypan positive cells/dish, respectively). Moreover, there were no detectable morphological changes between cells treated without or with indinavir (data not shown).

¹²⁵I-Insulin binding. Insulin association with the cells at the trace insulin concentration (0.17 nmol/l ¹²⁵I-insulin) and at a concentration that resulted in approximately 50 % competition (3.3 nmol/l) was not affected by indinavir (Fig. 1). Non-specific insulin binding (3300 nmol/l) was also not different.

IRS-1-phosphorylation, association of PI 3-kinase with IRS-1 and Akt-phosphorylation. In cells not pre-incubated with indinavir insulin rapidly increased IRS-1-phosphorylation, association of PI 3-kinase with IRS-1 and Thr³⁰⁸-phosphorylation of Akt (Fig. 2). The greatest effects were observed after 75 s, followed by subsequent decreases. Preincubation with indinavir reduced insulin-stimulated IRS-1-phosphorylation and association of PI 3-kinase with IRS-1 at the 25 and 75 s points by 30–60 %. A similar reduction was observed for Akt-phosphorylation, except that the difference at 75 s did not reach statistical significance. At 225 s no effects were observed between cells treated without or with indinavir. Indinavir had no effects on the amounts of IRS-1 (Fig. 2) or Akt (data not shown).

Discussion

We show that incubation of HepG2 cells with the HIV-1 protease inhibitor indinavir for 48 h resulted in reduced insulin effects on initial IRS-1-tyrosine-phosphorylation, association of PI 3-kinase with IRS-1 and Thr³⁰⁸-phosphorylation of Akt. Such indinavir effects on important steps of the initial insulin signalling cascade could represent a mechanism that could, at least partially, be responsible for the protease inhibitor-induced insulin resistance.

We chose HepG2 cells because they represent an established human cell model and allow the investigation of long-term effects [3]. Insulin stimulation of cells that had not been exposed to indinavir resulted in rapid increases of IRS-1-phosphorylation, association of PI 3-kinase with IRS-1 and Thr³⁰⁸-phosphorylation of Akt followed by decreases to approximately 40–60% of the peak levels. Such biphasic kinetics of insulin signalling have also been described in previous studies with HepG2 cells and other cell systems [4,5] and could be caused, among other things, by feed-back mechanisms or compartmentalization and trafficking of signalling molecules or both within the cells [2,5].

It is not clear whether an indinavir-induced reduction of insulin-stimulated IRS-1-phosphorylation, association of PI 3-kinase with IRS-1 and Thr³⁰⁸-phosphorylation of Akt in the initial phase of insulin signalling, as we observed, could by itself lead to insulin resistance. It is, however, possible that the “initial burst” in signalling by the early insulin signalling elements is important for the signalling to more long-lasting downstream intermediates [6]. Therefore the indinavir-induced impairment of the initial rise in insulin signalling that we observed could result in sustained reductions in later signals and downstream biological effects. In any case, our finding that indinavir interferes at all with the activation of initial elements of the insulin signalling cascade implies that such interference could cause, or contribute to, the metabolic effects of indinavir.

It is not clear by which mechanism indinavir might exert its effects on insulin signalling. Our finding that insulin-binding to the cells was not altered by indinavir suggests that indinavir influences IRS-1-phosphorylation, association of PI 3-kinase with IRS-1 and Akt-phosphorylation by mechanisms on the post-binding level. Potential mechanisms include a HIV-1 protease inhibitor-mediated inhibition of the proteolysis of certain phosphotyrosine-phosphatases or serine kinases that are involved in the regulation of insulin signalling [2]. A high specificity of indinavir for the HIV-1 protease has, however, been shown [7]. Alternatively, indinavir, which is approximately 60% homologous with regions of the cytoplasmic retin-

oid-acid binding protein type-1, might lead to decreased stimulation of the retinoid X receptor (RXR) [8]. This receptor, together with the peroxisomal proliferator-activated receptor gamma (PPAR γ) has been shown to participate in the regulation of insulin sensitivity [9] and RXR/PPAR γ -stimulation has been found to be associated with increased insulin-stimulated IRS-1-phosphorylation and PI 3-kinase activity [10].

Although the mechanism is still not known, we provide evidence that treatment with the protease inhibitor indinavir impairs initial insulin signalling. Such an interference of indinavir with insulin signal transduction could, at least in part, represent a molecular basis for its metabolic effects.

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