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In Vitro and In Vivo Prevention of HIV Protease Inhibitor-Induced Insulin Resistance by a Novel Small Molecule Insulin Receptor Activator

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Abstract Protease inhibitor (PI) therapy for the treatment of patients infected with human immunodeficiency virus is frequently associated with insulin resistance and diabetic complications. These adverse effects of PI treatment result to a large extent from their inhibition of insulin-stimulated glucose transport. Insulin receptor (IR) activators that enhance the insulin signaling pathway could be effective in treating this resistance. However, there are no agents reported that reverse inhibition of insulin action by PIs. Herein, we describe the effects of TLK19781. This compound is a non-peptide, small molecule, activator of the IR. We now report in cultured cells, made insulin resistant HIV by PI treatment, that TLK19781 both increased the content of insulin-stimulated GLUT4 at the plasma membrane, and enhanced insulin-stimulated glucose transport. In addition, oral administration of TLK19781 with the PI, indinavir improved glucose tolerance in rats made insulin resistant. These results suggest, therefore, that IR activators such as TLK19781 may be useful in treating the insulin resistance associated with PIs. J. Cell. Biochem. 92: 1234–1245, 2004.

Key words: glucose; insulin receptor; tyrosine kinase; activator; diabetes

Pharmacological agents that inhibit the protease activity of human immunodeficiency virus type-1 (HIV) are key components of drug therapy for patients infected with HIV. Protease inhibitor (PI) therapy has significantly reduced morbidity and mortality in this disease [Eron, 2000; Flexner, 2000; Tavel, 2000]. However, PI therapy produces metabolic abnormalities that include peripheral insulin resistance, hyperglycemia, hypertriglyceridemia, hypercholesterolemia, fat wasting, and central adiposity [Carr et al., 1998a,b, 1999; Safrin and Grunfeld, 1999; Mynarcik et al., 2000; Distler et al., 2001]. The insulin resistance caused by the PIs [Hruz et al., 2001; Murata et al., 2002] can be observed after only a single dose of a PI and is believed to be due to the decreased activity of GLUT4, the key insulin-regulated glucose transporter. This inhibition of GLUT-4 by PIs is consistent with the development of insulin resistance and its complications [Carr et al., 1998a,b, 1999; Safrin and Grunfeld, 1999; Mynarcik et al., 2000; Distler et al., 2001]. Thus, an adjunctive pharmacological approach, directed at overcoming PI-induced insulin resistance, should prove beneficial in either the prevention or reversal of these adverse effects.

Small molecules, that act on the insulin receptor (IR) and enhance insulin signaling, are a potential new class of drugs for the treatment of insulin resistance and type 2 diabetes [Zhang et al., 1999; Liu et al., 2000; Zhang and Moller, 2000; Vara Prasad et al., 2001]. Previously, we have reported the effects of a family of small non-peptide molecules, such as TLK16998, which activate IR tyrosine kinase activity, and

Abbreviations used: HIV, human immunodeficiency virus; IR, insulin receptor; IRS, insulin receptor substrate-1; CKD, cytoplasmic kinase domain; TK, tyrosine kinase; IGF-1R, insulin-like growth factor-1 receptor; EGFR, epidermal growth factor receptor; PI, protease inhibitor.

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lower blood glucose in diabetic mice [Li et al., 2001; Vara Prasad et al., 2001]. Herein, we present the biological activity of TLK19781, a more potent and lower molecular weight analog. This agent increases the phosphorylation of IR tyrosine kinase domain (IR-CKD), enhances IR signaling, and results in increased GLUT4 content at the plasma membrane and enhanced glucose transport. In cultured cells, made insulin resistant with PIs, TLK19781 enhances insulin action and reverses insulin resistance. Moreover, in normal rats made insulin resistant with the PI, indinavir, oral administration of TLK19781 reverses this resistance.

MATERIALS AND METHODS

Autophosphorylation of the IR (Cytoplasmic Kinase Domain) CKD

In total, 200 ng of IR-CKD, the entire cytoplasmic domain of the IR without either the extracellular or transmembrane domain residues (Stratagene, La Jolla, CA), were dissolved in 30 μl of 50 mM Tris (pH 7.4), 2 mM $MnCl_2$, 10 mM $MgCl_2$ and combined with increasing concentrations of TLK19781. Reactions were started with the addition of 50 μM ATP ([γ-^{32}P]-ATP 10 μCi), and incubated for 10 min at 25°C. For gel analysis, samples were boiled in SDS–PAGE sample buffer for 5 min, electrophoresed on 10% SDS–PAGE gels, and visualized by autoradiography. Radioactivity was measured by scintillation counting of the labeled bands.

Immunoprecipitation and Immunoblotting

Assessment of TLK19781 activity on the insulin signaling pathway was performed on 3T3-HIR cells. Cells were grown in 6-well plates and serum-starved for 16 h in Dulbecco's Modified Essential Medium (DMEM) supplemented with 0.1% (w/v) bovine serum albumin (BSA). Cells were stimulated with TLK19781, in the presence or absence of 2.5 nM insulin (determined to be a sub-maximal dose in preliminary experiments), for 15 min at 37°C, washed with cold phosphate-buffered saline (PBS), and solubilized by adding lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.25% (w/v) sodium deoxycholate, 1% (v/v) NP-40, 1 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, and 1 μg/ml each of aprotinin, leupeptin, pepstatin). Lysates were clarified by centrifugation (14,000 × g), and approximately 250 μg of protein was immunoprecipitated with either the anti-IR-β polyclonal antibody (C-19; sc-711) or the IRS-1 polyclonal antibody (C-20; sc-559) (Santa Cruz Biotechnology, Santa Cruz, CA). The immune complexes were adsorbed onto protein G-agarose beads (GIBCO-BRL, Bethesda, MD) for 2 h at 4°C, washed with lysis buffer, resolved by 7.5% SDS–PAGE and transferred to Immobilon-P membrane (Schleicher & Schuell, Keene, NH) by electroblotting. The membrane was blocked, incubated with PY20 anti-phosphotyrosine antibody (BD Transduction Laboratories, Lexington, KY) overnight at 4°C, washed extensively in PBS, and incubated with peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using enhanced chemiluminescence. For re-probing, membranes were stripped (62.5 mM Tris, pH 6.7, 100 mM β-mercaptoethanol, 2% (w/v) SDS, for 30 min at 50°C), washed, and incubated with either anti-IR polyclonal antibody or anti-IRS-1 polyclonal antibody.

Receptor Specificity Studies

HepG2 cells (ATCC no. HB8065) were grown in 6-well plates, and serum-starved in Minimal Essential Medium (MEM) (GIBCO-BRL) supplemented with 0.1% BSA for 16 h prior to stimulation with TLK19781 in the presence or absence of increasing concentrations of IGF-1 for 15 min at 37°C. Cell lysates were prepared, and immunoprecipitation reactions using anti-insulin-like growth factor-1 (IGF-1) receptor polyclonal antibody (Santa Cruz Biotechnology) and immunoblotting was carried out as described above.

A431 cells (ATCC no. CRL-1555) were grown in 6-well plates and serum starved for 16 h prior to the experiment in DMEM supplemented with 0.1% BSA. The A431 cells were then treated with TLK19781 with or without different concentrations of EGF (CalBiochem, La Jolla, CA). Lysates were prepared as described above, and immunoblotting was performed using an anti-phospho specific EGF receptor monoclonal antibody (BD Transduction Laboratories).

2-Deoxy-D-Glucose Transport

3T3-L1 fibroblasts (ATCC no. 173-CL) were induced to differentiate into adipocytes as described previously [Frost and Lane, 1985]. Adipocytes were serum-starved for 16 h in medium containing 0.1% BSA, stimulated with

increasing concentrations of TLK19781 in the presence or absence of insulin (where indicated) for 30 min at 37°C, and then incubated with 0.5 μCi/ml of 2-deoxy-D-[^{14}C] glucose (50 μM final concentration) for additional 30 min at 37°C. For experiments involving PI, the cells were pre-incubated with indicated concentrations of indinavir or amprenavir for 6 min followed by stimulation with insulin or insulin plus 56 μM TLK19781 for 30 min at 37°C followed by labeling with 2-deoxy-D-[^{14}C] glucose as described above. Cell monolayers were washed with a cold solution of 20 mM glucose in PBS, and lysed in 1% (v/v) Triton X-100 (dissolved in 50 mM HEPES, pH 7.6). Radioactivity was quantified by scintillation counting. For TNF-α experiments, fully differentiated adipocytes were treated with TNF-α (3 ng/ml) for 4 days. The cells were then serum starved for 16 h in medium containing 0.1% BSA, stimulated with 30 μmol/L TLK19781 in the presence of different concentrations of insulin (0–1,000 nM) for 30 min at 37°C, and then incubated with 0.5 μCi/ml of 2-deoxy-D-[^{14}C] glucose (50 μM final concentration) for an additional 30 min at 37°C. Cell monolayers were washed with a cold solution of 20 mM glucose in PBS, and lysed in 1% (v/v) Triton X-100 (dissolved in 50 mM HEPES, pH 7.6). Radioactivity was quantified by scintillation counting.

Plasma Membrane Preparation and GLUT4

3T3-L1 fibroblasts (ATCC no. 173-CL) were induced to differentiate into adipocytes as described previously [Frost and Lane, 1985]. Prior to the experiment, adipocytes were serum-starved for 16 h in medium containing 0.1% BSA. The cells were pre-incubated with 100 μmol/L indinavir for 6 min and then stimulated with 50 nM insulin with or without 56 μmol/L of TLK19781 for 20 min at 37°C. Plasma membranes were prepared as described previously [Clancy and Czech, 1990]. Briefly, the cells were washed with cold PBS twice and cell pellets were resuspended in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and incubated on ice for 20 min. The samples were homogenized in a Dounce homogenizer and centrifuged at 16,000 × g for 20 min at 4°C and the pellets were resuspended in 0.5 ml of lysis buffer in a Dounce homogenizer and applied to sucrose cushions (1.12 M sucrose in lysis buffer) and centrifuged at 100,000 × g for 1 hr at 4°C. Plasma membranes were removed from the top of the sucrose cushion and centrifuged at 30,000 × g for 30 min at 4°C. The pellets were resuspended in lysis buffer and equal quantities of protein were resolved using SDS–PAGE (10% gels), and immunoblotted onto a nitrocellulose membrane and incubated with anti-GLUT4 goat polyclonal antibody (Santa Cruz, CA), washed extensively and incubated with peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using enhanced chemiluminescence. For re-probing, membranes were stripped (62.5 mM Tris, pH 6.7, 100 mM β-mercaptoethanol, 2% (w/v) SDS, for 30 min at 50°C), washed, and incubated with anti-actin goat polyclonal antibody (Santa Cruz, CA). Quantitation of individual protein bands was carried out with the Storm phosphorimager with Imagequant software (Molecular Dynamics, Sunnyvale, CA).

Oral Glucose Tolerance Test in Normal Rats

All animal studies were conducted using protocols approved by the Telik Institutional Animal Care and Use Committee. These protocols were in accordance with accepted standards of humane animal care, as outlined in the Ethical Guidelines. Normal male CD rats (Charles River, Hollister, CA) or Wistar rats (Harlan, Indianapolis, IN) were maintained according to NIH guidelines. The rats were housed three per cage, and fed standard laboratory chow (Tekland Laboratory Diets, James Grain, San Jose, CA). Prior to use, animals were fasted for 16 h (free access to water). In the morning, the animals were treated with vehicle, indinavir (100 mg/kg), indinavir (100 mg/kg) plus TLK19781 (10 or 1 mg/kg) by oral gavage or intraperitoneal routes. After 30 min, animals were given an oral bolus of glucose (2.5 g/kg) by gavage tube. At the indicated time points, blood was taken using the tail-cap method. Blood glucose was measured using the Glucometer Elite (Bayer Corporation, Mishawaka, IN). Plasma insulin was measured by ELISA (ALPCO Diagnostics, Windham, NH) according to the manufacturer's instructions.

Statistical Analyses

Results were statistically evaluated by the Student's *t*-test or ANOVA followed by the appropriate post hoc test using Graphpad Prism

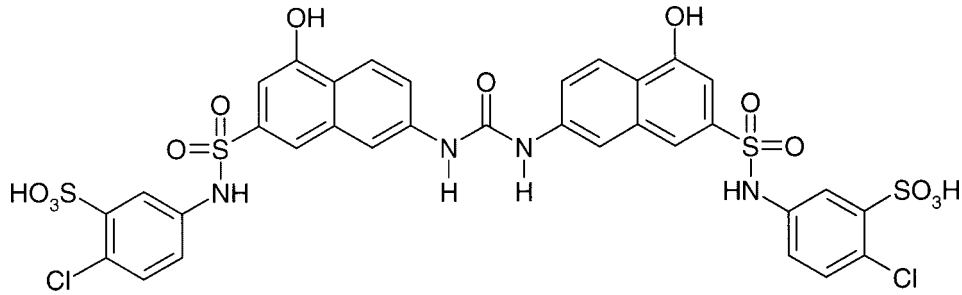

Fig. 1. Chemical structure of TLK19781.

(MS Windows version 3.02), GraphPad Software, San Diego, CA.

RESULTS

TLK19781 Increases Autophosphorylation of the CKD of the IR

TLK19781 is a member of a unique chemical class of compounds that activate autophosphorylation of the purified human IR (Fig. 1). Previous studies established that the prototype member of this class, TLK16998, acted directly on the β subunit of the IR [Clancy and Czech, 1990]. Consistent with those data, TLK19781 directly activated CKD of the human IR (Fig. 2). An effect on this function was readily observed at concentrations as low as 10 nM, and reached a maximum at 1,000 nM.

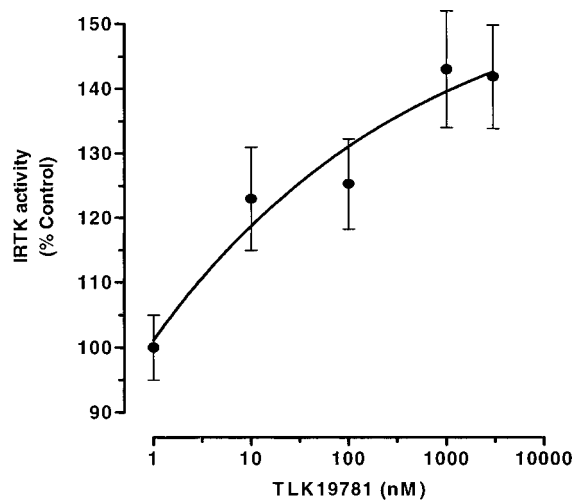

Fig. 2. TLK19781 increases autophosphorylation of the isolated IR cytoplasmic kinase domain. The isolated cytoplasmic tyrosine kinase domain of the cloned human IR (IR CKD) was incubated with 50 μM ATP and 10 μCi γ-[^{32}P] ATP plus TLK19781 for 10 min at 25°C, and radioactivity incorporated into the IR-CKD was quantified by SDS–PAGE analysis and followed by autoradiography. Radiolabeled bands were counted by a liquid scintillation counter. Data represent mean ± SEM of three independent experiments expressed as a percent of control.

The IR Signaling Pathway Is Enhanced in Whole Cells

We investigated the ability of TLK19781 to increase the tyrosine kinase activity of the IR in murine 3T3-HIR cells that overexpress the human IR. In the absence of insulin, 18, 32, 56 μM of TLK19781 increased tyrosine phosphorylation of both the IR and IRS-1. In the presence of a sub-maximal dose of insulin (2.5 nM), TLK19781 at a concentration of 10 μM and above significantly potentiated the effects of insulin by increasing tyrosine phosphorylation of the IR and IRS-1 (Fig. 3). The same pattern of effects was seen on the phosphorylation of the serine kinase, PKB/Akt (data not shown). Similar results on the IR and IRS-1 were obtained in 3T3-L1 adipocytes also (data not shown).

TLK19781 Does Not Activate IGF-1 Receptor and Epidermal Growth Factor Receptor (EGFR) Tyrosine Phosphorylation

To evaluate the selectivity of TLK19781, we assessed its ability to affect the tyrosine phosphorylation of the related receptors, the IGF-1R and EGFR. Because HepG2 cells (human hepatoma) express a relatively high amount of IGF-1 receptors, we used these cells to evaluate the effect of TLK19781 on the IGF-1R. In the presence and absence of IGF-1 (1–1,000 ng/ml), TLK19781 (30 μM) had no effect on the phosphotyrosine content of the IGF-1R (Fig. 4).

Next, we employed A431 cells (human epidermoid carcinoma cells with a high EGFR content) to evaluate the effect of TLK19781 on the EGFR. In the presence and absence of EGF stimulation (1–100 ng/ml), TLK19781 (30 μM) had no significant effects on the phosphotyrosine content of the EGFR (Fig. 4).

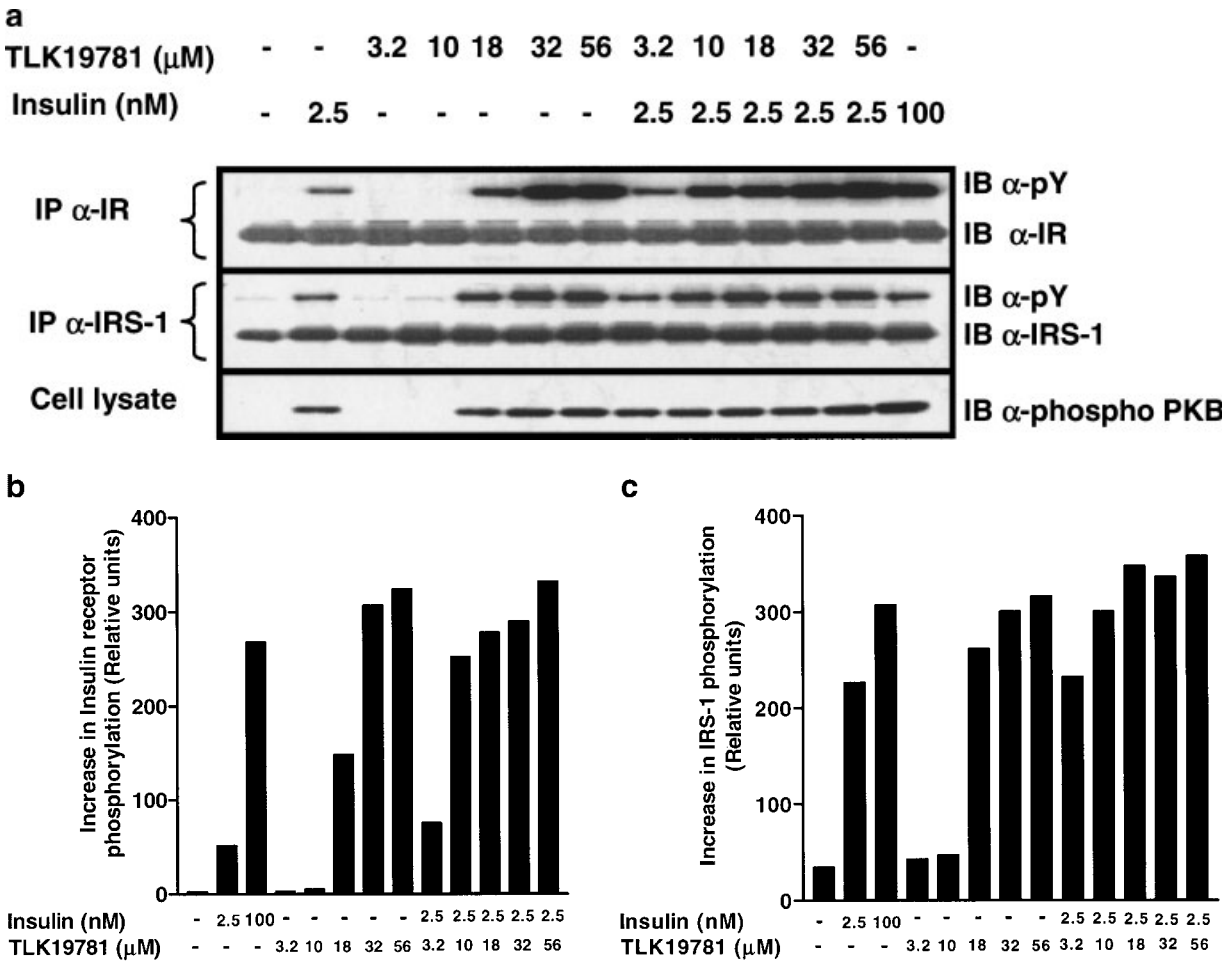

Fig. 3. TLK19781 enhances insulin-stimulated IR signaling. NIH 3T3 cells overexpressing human IR were treated with TLK19781 in the absence or presence of insulin at 37°C for 15 min. The cells were washed and lysed. An equal amount of lysate protein was immunoprecipitated with either anti insulin receptor antibody (α-IR) or anti IRS-1 antibody (α-IRS-1). Immunoprecipitates were analyzed by SDS–PAGE followed by immunoblotting with an anti phosphotyrosine antibody (α-pY). Blots were stripped and reprobed with the α-IR or α-IRS-1. **a**: Western blot. **b**: Scan of blots. **c**: Scan of blots.

Glucose Transport in 3T3-L1 Adipocytes

To determine if stimulation the insulin signaling pathway by TLK19781 would result in enhanced glucose transport, we evaluated the effect of TLK19781 on this function in murine 3T3-L1 adipocytes [Garcia de Herreros and Birnbaum, 1989; Vara Prasad et al., 2001]. In the absence of insulin, relatively high concentrations of TLK19781 (30 and 56 μM) increased glucose transport by 290 and 500%, respectively (Fig. 5a). At 10 μM TLK19781, there was no direct effect on glucose transport but TLK19781 treatment shifted the insulin concentration-response curve to the left, indicative of an IR activating effect (Fig. 5b). The EC_{50} values for insulin-stimulated transport were 11.3 nM insulin in the absence of TLK19781, 1.2 nM ($P < 0.03$ vs. control) in the presence of 10 μM TLK19781, and 0.35 nM ($P < 0.02$ vs. control) in the presence of 30 μM TLK19781.

Reversal of PI-Induced Insulin Resistance in 3T3-L1 Adipocytes

To demonstrate the effects of TLK19781 on PI-induced insulin resistance, we studied glucose transport in 3T3-L1 adipocytes. After incubation with the PIs, indinavir and amprenavir, cells were then stimulated with either insulin or insulin plus TLK19781. Glucose transport was evaluated by measuring 2-deoxy-D-[^{14}C] glucose uptake. At concentrations of up to 100 μM both indinavir and amprenavir had no significant effects on basal glucose transport (Fig. 6a,b).

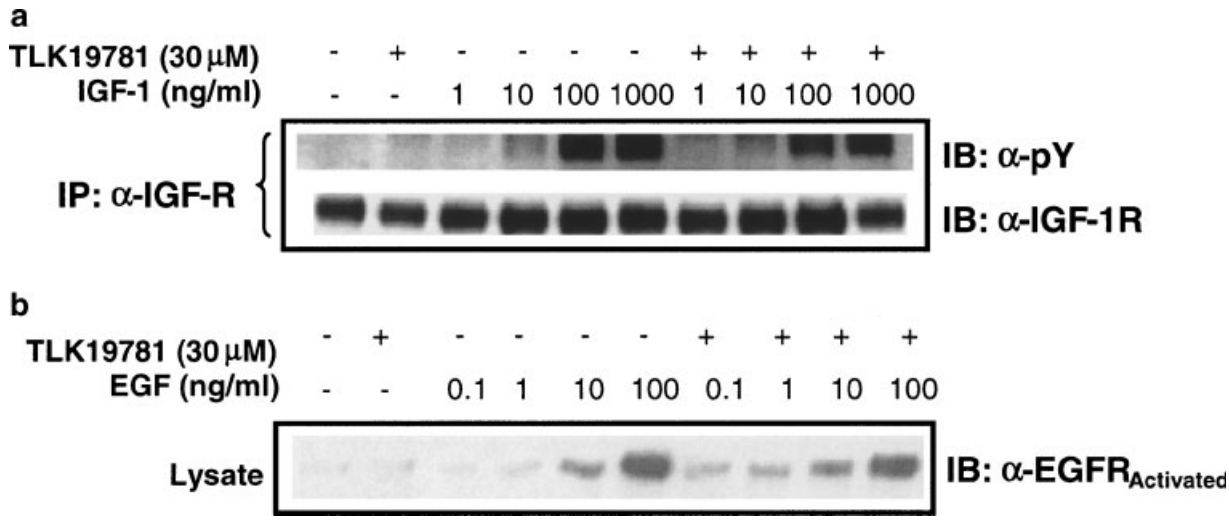

Fig. 4. TLK19781 is specific for IR phosphorylation. **a**: HepG2 cells were treated with the indicated concentrations of IGF-1 in the absence or presence of TLK19781 at 37°C for 15 min. The cells were washed, lysed, and an equal amount of lysate protein was immunoprecipitated with anti IGF-1 receptor antibody (α-IGF-1R). Immunoprecipitates were analyzed by SDS–PAGE followed by immunoblotting with α-pY (**upper image**). Blots were stripped and reprobed with α-IGF-1R (**lower image**). **b**: A431 cells were treated with the indicated concentrations of EGF in the absence or presence of TLK19781 at 37°C for 15 min. The cells were washed, lysed, and an equal amount of lysate protein was analyzed by SDS–PAGE followed by immunoblotting with an antiphospho-EGFR monoclonal antibody (α-$EGFR_{activated}$).

Indinavir and amprenavir have been reported to significantly inhibit insulin-stimulated glucose transport [Murata et al., 2002]. We observed that at 50 μM indinavir and amprenavir inhibited insulin-stimulated glucose transport by 25 and 37%, respectively. At 100 μM, indinavir and amprenavir inhibited insulin-stimulated glucose transport by 45 and 57%, respectively (Fig. 6a,b).

Next, 3T3-L1 adipocytes were treated with TLK19781 to determine if TLK19781 would reverse PI-induced insulin resistance. In the absence of PI, TLK19781 enhanced insulin-stimulated glucose transport by approximately

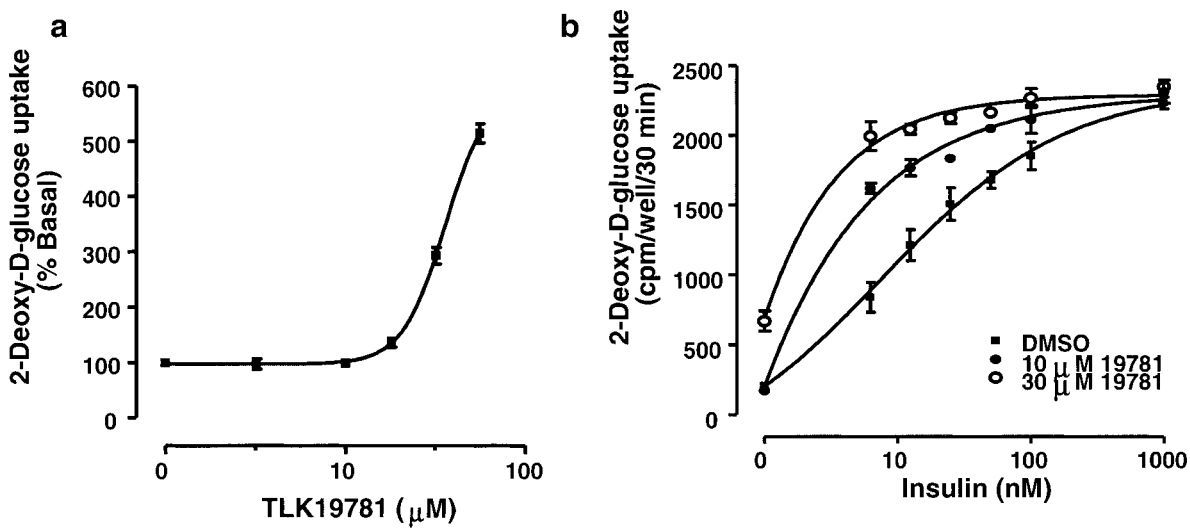

Fig. 5. TLK19781 increases both basal and insulin-stimulated glucose transport in adipocytes. **a**: 3T3-L1 adipocytes were stimulated with increasing concentrations of TLK19781 in the absence of insulin at 37°C for 30 min, and 2-deoxy-D-glucose uptake was measured. **b**: 3T3-L1 adipocytes were stimulated with increasing concentrations of insulin alone in either DMSO buffer or the presence of TLK19781 (10, 30 μM) at 37°C for 30 min and 2-deoxy-D-glucose transport was measured. Data represent mean ± SEM of three independent experiments. Concentration of TLK19781 in μM and insulin in nM is shown as log scale.

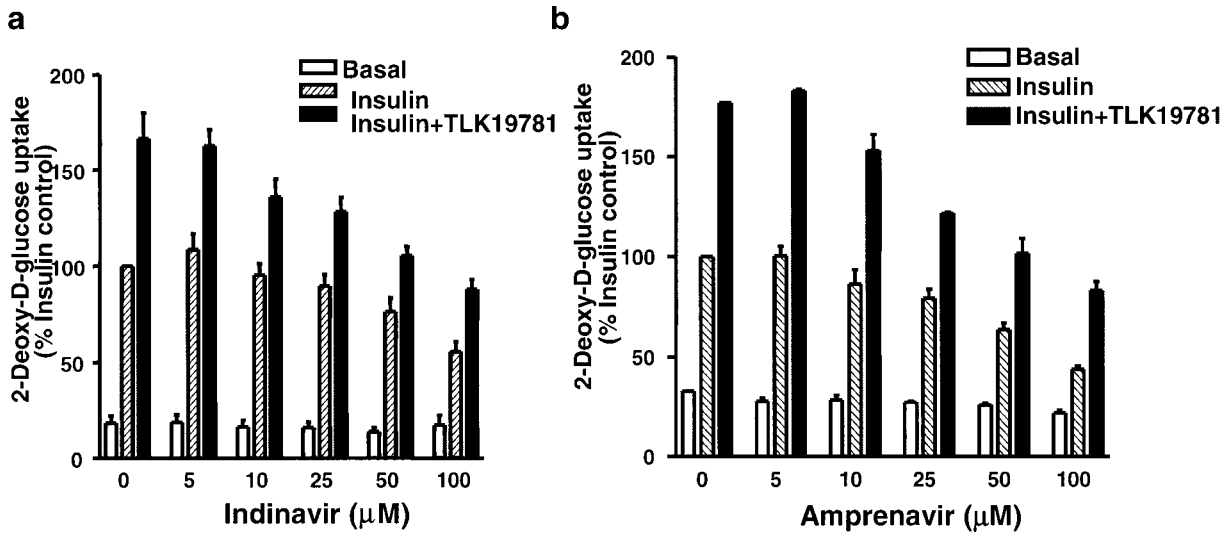

Fig. 6. TLK19781 improves PI-mediated inhibition of glucose transport in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pre-treated with the indicated concentrations of indinavir (**a**) or amprenavir (**b**) followed by stimulation with 50 nM insulin plus 10 μM TLK19781 at 37°C for 30 min. 2-deoxy-D-glucose transport was then measured. Results are means ± SEM of three independent experiments.

60–70% (Fig. 6a,b). At concentrations of 5, 10, and 25 μM of the PIs indinavir and amprenavir, TLK19781 was demonstrated to enhance insulin-stimulated glucose transport. In the presence of 50 and 100 μM of either indinavir or amprenavir, TLK19781 restored insulin-stimulated glucose transport to a level similar to that observed in the absence of either PI (Fig. 6a,b). Thus, we conclude that following treatment with increasing concentrations of PI, TLK19781 relieved indinavir and amprenavir induced insulin resistance. These results demonstrate that TLK19781-activation of IR tyrosine kinase activity can augment insulin-dependent intracellular signaling and overcome the insulin resistance induced by PIs.

Increase GLUT4 Glucose Transporter Translocation to the Plasma Membrane

Next, we investigated whether TLK19781 reverses the effect of indinavir on glucose transport by increasing GLUT4 content at the plasma membrane. We analyzed purified plasma membranes from 3T3-L1 adipocytes untreated or treated with 100 μM indinavir and then stimulated with 50 nM insulin (with or without TLK19781). In TLK19781-treated cells, GLUT4 content was increased by 85% with insulin stimulation (Fig. 7a,b). In indinavir-treated cells the insulin stimulated GLUT4 content was decreased by approximately 50% while the TLK19781 treatment in the presence of indinavir resulted in an approximately 100% increase in GLUT4 at the plasma membrane. These results suggested that TLK19781 reversed indinavir's effects on glucose transport by increasing insulin-stimulated GLUT4 content at the plasma membrane.

Reversal of TNF-α Induced Insulin Resistance in 3T3-L1 Adipocytes

It has been reported that there is an increase in the level of tumor necrosis factor-α in HIV-infected patients [Alonso et al., 1997; Resino et al., 2000]. TNF-α is associated with insulin resistance in obesity and has been causally linked to its etiology [Hotamisligil and Spiegelman, 2000]. TNF-α increases the serine phosphorylation of IRS-1, which in turn attenuates insulin action [Hotamisligil et al., 1996]. Earlier studies with cultured cells indicated that compounds of the TLK19781 class restored insulin signaling that had been inhibited by TNF-α [Li et al., 2001]. Accordingly, we determined whether TLK19781 could also overcome TNF-α-induced insulin resistance. Chronic treatment of differentiated 3T3-L1 adipocytes with TNF-α resulted in a right-shift in the insulin dose-response curve, along with 30% inhibition of glucose transport at a maximally effective concentration of insulin (Fig. 8). Treatment with TLK19781 (10 μM) reversed this insulin resistance as evidenced by the shift to the left in the insulin dose-response curve (compared to

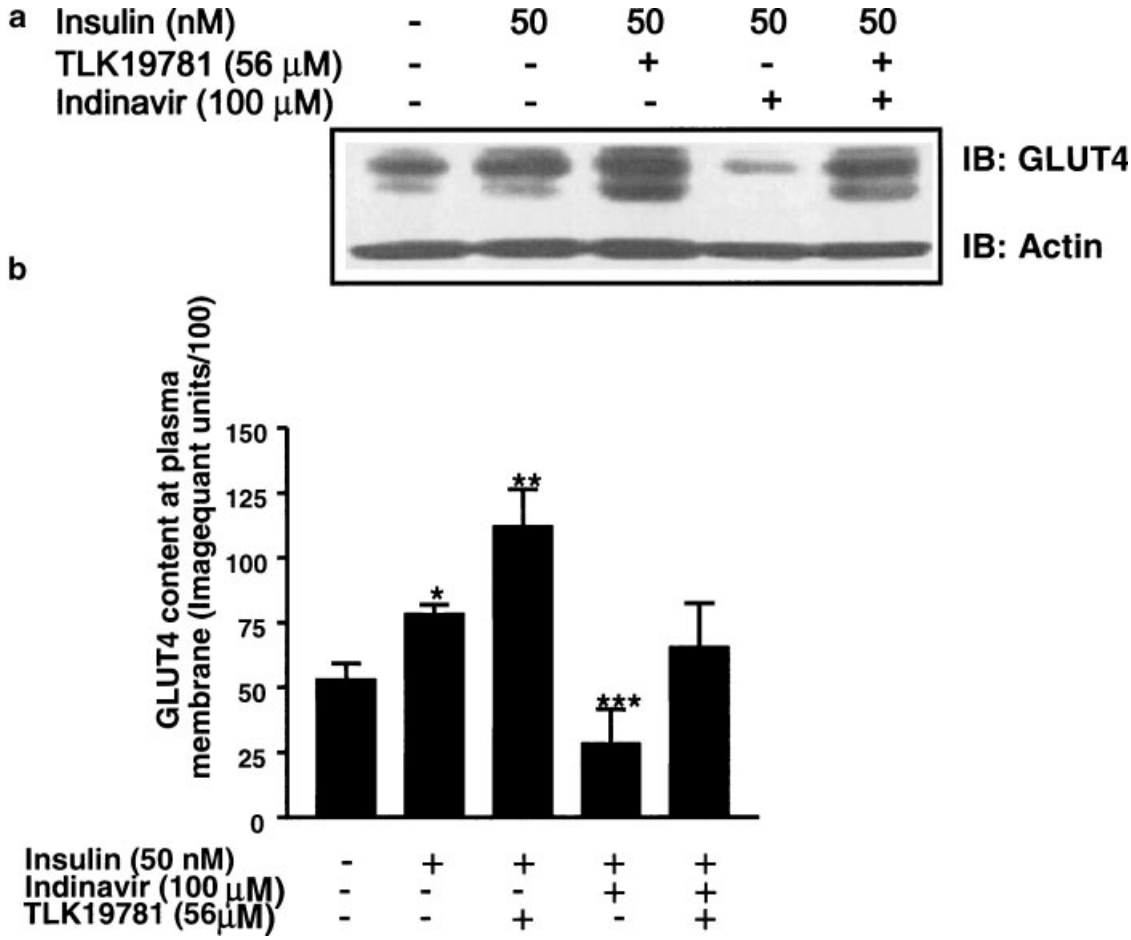

Fig. 7. TLK19781 increases GLUT4 content at the plasma membrane. 3T3-L1 adipocytes were treated with insulin and indinavir in the absence or presence of TLK19781 for 20 min at 37°C. Plasma membranes were purified on sucrose cushions and of samples were analyzed on SDS–PAGE. **a**: The immunoblots were probed with anti-GLUT4 antibody (α-GLUT4). **b**: The blots were stripped and re-probed with anti actin antibody (α-actin). Values are the means of three independent experiments.

both untreated control and TNF-α-treated adipocytes). These results suggested that the enhancement of IR tyrosine kinase activity can overcome insulin resistance caused by agents acting by different mechanisms

Oral TLK19781 Prevents Indinavir-Induced Insulin Resistance in Rats

To investigate, if indinavir induced acute insulin resistance in vivo, normal rats of the CD strain were treated with a single oral dose of indinavir (100 mg/kg) followed by an oral glucose tolerance test. In the vehicle-only treated group, maximal blood glucose levels after oral glucose administration increased to 285% over basal (Fig. 9a). Indinavir treatment resulted in a significant increase in maximal blood glucose levels to 400% over basal.

To address whether indinavir-induced insulin resistance could be overcome by TLK19781, we co-administered indinavir (100 mg/kg) and TLK19781 (10 mg/kg) followed by an oral glucose tolerance test. TLK19781 significantly improved glucose tolerance in the presence of indinavir; the effect was observed as early as 10 min and continued at all time points examined ($P < 0.01$; Fig. 9a). A similar result was obtained using Wistar rats (data not shown).

To determine if oral TLK19781 interfered with the absorption of indinavir, we treated rats orally with indinavir, and intraperitoneally with TLK19781. TLK19781 also significantly improved glucose tolerance under these conditions ($P < 0.05$; Fig. 9b).

Plasma insulin levels were also measured after administering indinavir or indinavir plus TLK19781. Indinavir significantly increased

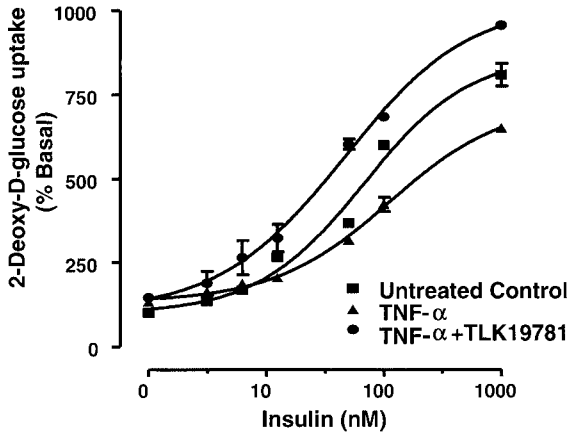

Fig. 8. Effect of TLK19781 on TNF-α induced insulin resistance in 3T3-L1 adipocytes. 3T3-L1 adipocytes were untreated or treated with 3 ng/ml TNF-α and then stimulated with insulin with or without 10 μM TLK19781 at 37°C for 30 min and 2-deoxy-D-glucose transport measured. Results are mean ± SEM of three independent experiments using triplicate incubations.

plasma insulin levels by 200% when compared to the vehicle treated group (Fig. 9c; $P < 0.01$) indicating that indinavir caused acute insulin resistance. The addition of TLK19781 caused a significant decrease in the plasma insulin levels of animals treated with indinavir along with a significant reduction in the area under the insulin curve ($P < 0.001$; Fig. 9c).

DISCUSSION

PI therapy provides a significant beneficial effect in HIV-infected patients. Recently, it has been recognized that prolonged therapy with these inhibitors can result in metabolic abnormalities such as insulin resistance, lipodystrophy, and diabetic complications [Carr et al., 1998a,b, 1999; Mynarcik et al., 2000]. It has been reported that acute treatment with indinavir for four weeks in healthy HIV negative volunteers resulted in marked insulin resistance and impaired glucose tolerance [Noor et al., 2001], and these effects are attributed to the inhibition of insulin-responsive glucose transporter activity [Murata et al., 2002].

Previously, we characterized TLK16998, a small molecule, IR kinase activator that had IR activating activity, both in vitro and in vivo [Li et al., 2001; Vara Prasad et al., 2001]. This agent, and others of its class acted rapidly with insulin to increase the number of active, tyrosine-phosphorylated IRs in target cells [Pender et al., 2002]. Moreover, they enhanced IR function in insulin resistant cells [Li et al., 2001, 2002]. In the present investigation, we report that a new member of this class of compounds, TLK19781, activates the IR. The effects exhibited by TLK16998 and TLK19781 on the IR, along with those previously reported for a structurally different class of IR activators [Zhang et al., 1999], validate the concept that the β-subunit of the IR is an important target for the development of new agents to treat insulin resistance [Liu et al., 2000; Zhang and Moller, 2000; Li et al., 2001; Vara Prasad et al., 2001].

In the present study, we observed that TLK19781, at nanomolar concentrations, acted on the CKD of the IR, resulting in increased tyrosine kinase activity. This observation indicated that the IR β-subunit is the site of action of TLK19781. In 3T3-HIR cells, TLK19781 had no direct activating effect on IR tyrosine kinase activity at 10 μM or lower. However, TLK19781, at concentrations as low as 3.2 μM, potentiated the effects of insulin on IR tyrosine kinase activity. At concentrations greater than 10 μM, TLK19781 acted as a direct IR agonist.

As a consequence of its ability to activate IR tyrosine kinase activity in whole cells, TLK19781 activated the proximal steps of the IR signaling pathway. Specifically, TLK19781 enhanced insulin-stimulated phosphorylation of IRS-1 and Akt. In contrast, TLK19781 exhibited no significant effects on the tyrosine phosphorylation of other related receptors such as either the IGF-1R or the EGFR.

In cultured 3T3-L1 adipocytes, TLK19781 enhanced insulin-stimulated glucose transport. This enhancement of glucose transport was due to increased GLUT4 glucose transporter content at the plasma membrane. Therefore, TLK19781 is a specific activator of the IR that enhances insulin action.

In cells treated with PIs, resistance to insulin-stimulated glucose transport was observed, TLK19781 overcame this resistance. This improvement in glucose transport by TLK19781 was due to enhancement of IR signaling via an increase in GLUT4 content. TLK19781 was also effective on TNF-α mediated insulin resistance suggesting that TLK19781 augments the insulin signaling pathway in several forms of insulin resistance.

This prevention of indinavir-mediated insulin resistance by TLK19781 was observed in animals. In rats treated orally with indinavir, decreased glucose tolerance and increased plasma insulin levels was observed [Hruz et al., 2002]. In animals treated with indinavir the

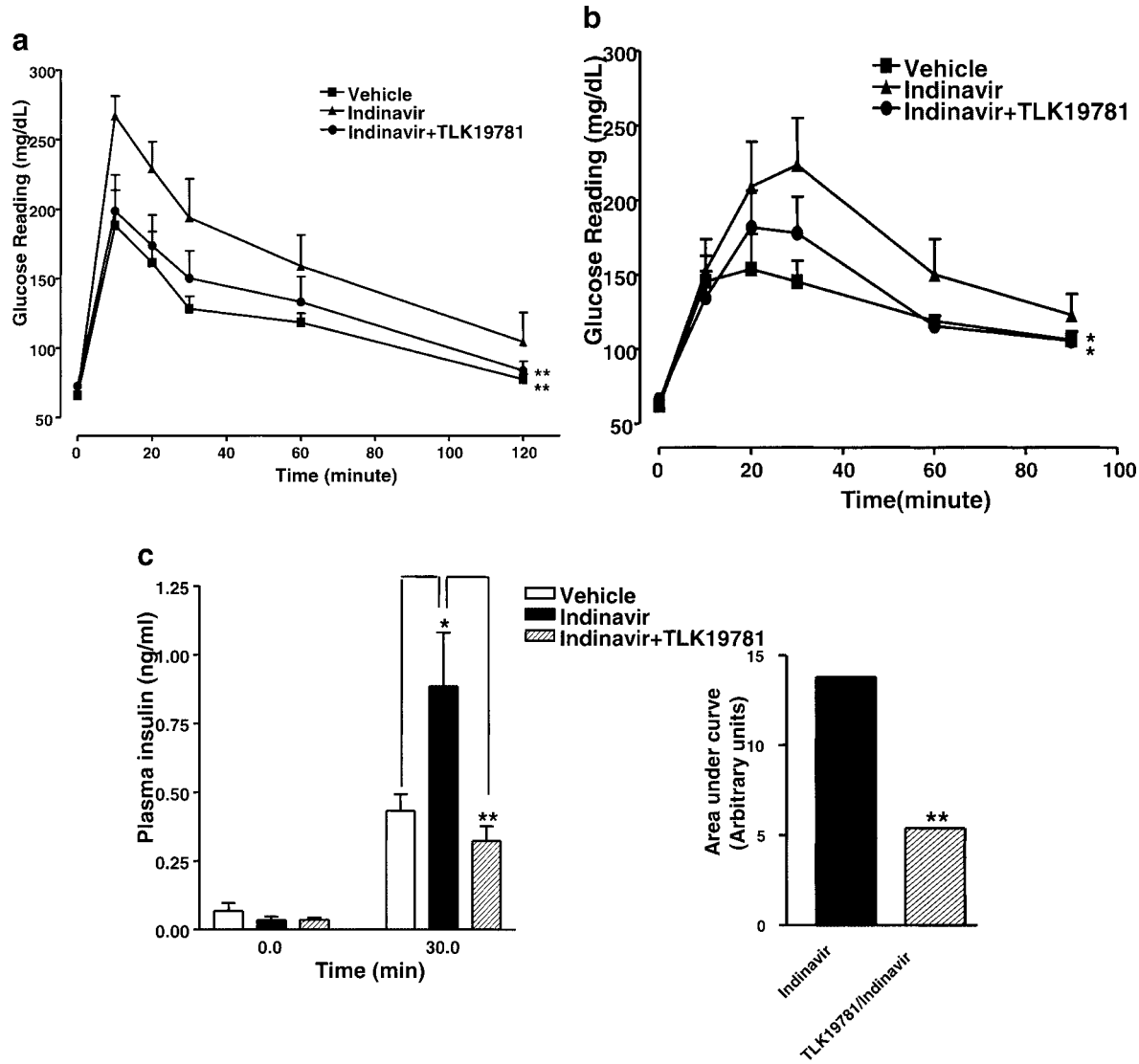

Fig. 9. In rats, TLK19781 improves the insulin resistance induced by indinavir. **a**: Effects of oral TLK19781. Normal CD rats were treated with either vehicle, a single oral dose of indinavir (100 mg/kg), or indinavir (100 mg/kg) plus TLK19781 (10 mg/kg). After 30 min, animals were given oral glucose (2.5 g/kg) and at the indicated time points. $^{**}P < 0.01$ compared to the value of indinavir treated group by repeated measures ANOVA followed by Newman-Keuls multiple comparison test. **b**: Effects of intraperitoneal TLK19781. Animals were treated with vehicle, a single oral dose of indinavir (100 mg/kg) in the presence or absence of TLK19781 (1 mg/kg) intraperitoneally. Then, 30 min later, animals were given oral glucose (2.5 g/kg). $^{*}P < 0.05$ compared to the value of indinavir treated group by repeated measures ANOVA followed by Newman–Keuls multiple comparison test. **c**: TLK19781 lowers elevated insulin levels due to PI. Blood samples were collected at indicated time points and plasma insulin was measured by using insulin ELISA kit. Animals were given indinavir (100 mg/kg) or indinavir (100 mg/kg) plus TLK19781 (10 mg/kg). All treatments were given by oral administration. Blood samples were collected at indicated time points and plasma insulin was measured by using insulin ELISA kit. Vehicle Values are the mean ± SEM, $^{*}P < 0.01$, $^{**}P < 0.001$ ANOVA and Newman–Keuls post hoc test. Inset: Area under the curve was used to assess statistical significance using one way ANOVA versus indinavir treated group.

concomitant administration of oral TLK19781 significantly improved glucose tolerance. In addition, plasma insulin levels were also decreased in TLK19781-treated animals suggesting that the compound improved IR signaling and decreased insulin resistance. That oral TLK19781 did not interfere with the absorption of indinavir was suggested by its effectiveness after intraperitoneal administration.

Due to the increasing use of PIs in chronic HIV therapy, it is likely that the development of insulin resistance, metabolic syndrome, and its resultant pathological consequences will become more prevalent. The future manage-

ment of this problem could be improved by the identification of new PIs that do not affect glucose transporter function. Alternatively, new agents that override PI-inhibition of glucose transporter activity would be of benefit. In this regard, compounds that activate the IR tyrosine kinase, including TLK19781, qualify as candidates that could prove clinically useful in the amelioration or even prevention of PI-induced insulin resistance, hyperglycemia, and other metabolic complications derived from impaired insulin signaling. Moreover, in light of the heterogeneous nature of insulin resistance in vivo, pharmacological enhancement of IR tyrosine kinase activity could evolve as a more generalized strategy directed at overcoming insulin resistance independent of the causative element(s).

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