A Novel Small Molecule That Directly Sensitizes the Insulin Receptor In Vitro and In Vivo

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Insulin resistance, an important feature of type 2 diabetes, is manifested as attenuated insulin receptor (IR) signaling in response to insulin binding. A drug that promotes the initiation of IR signaling by enhancing IR autophosphorylation should, therefore, be useful for treating type 2 diabetes. This report describes the effect of a small molecule IR sensitizer, TLK16998, on IR signaling. This compound activated the tyrosine kinase domain of the IR β-subunit at concentrations of 1 μmol/l or less but had no effect on insulin binding to the IR α-subunit even at much higher concentrations. TLK16998 alone had no effect on IR signaling in mouse 3T3-L1 adipocytes but, at concentrations as low as 3.2 μmol/l, enhanced the effects of insulin on the phosphorylation of the IR β-subunit and IR substrate 1, and on the amount of phosphatidylinositol 3-kinase that coimmunoprecipitated with IRS-1. Phosphopeptide mapping revealed that the effect of TLK16998 on the IR was associated with increased tyrosine phosphorylation of the activation loop of the β-subunit tyrosine kinase domain. TLK16998 also increased the potency of insulin in stimulating 2-deoxy-D-glucose uptake in 3T3-L1 adipocytes, with a detectable effect at 8 μmol/l and a 10-fold increase at 40 μmol/l. In contrast, only small effects were observed on IGF-1–stimulated 2-deoxy-D-glucose uptake. In diabetic mice, TLK16998, at a dose of 10 mg/kg, lowered blood glucose levels for up to 6 h. These results suggest, therefore, that small nonpeptide molecules that directly sensitize the IR may be useful for treating type 2 diabetes. *Diabetes* 50: 824–830, 2001

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BSA, bovine serum albumin; CKD, cytoplasmic kinase domain; DEX, dexamethasone; EGF, epidermal growth factor; HFD, high-fat diet; IBMX, isobutylmethylxanthine; IR, insulin receptor; IRS-1, insulin receptor substrate 1; PBS, phosphate-buffered saline; PI, phosphatidylinositol; TLC, thin-layer chromatography.

Diabetes is a growing medical problem in developed countries (1). It is estimated that diabetes affects 6% of the population in the U.S. and 3% of the population in northern Europe, with type 2 diabetes representing 90% of the cases (1). Two metabolic defects that seem to be central to the pathology of type 2 diabetes are impaired insulin secretion and the reduced ability of insulin to act on the major insulin-sensitive tissues (2–5). The combination of these defects results in an inability of the body to maintain glucose homeostasis leading to hyperglycemia and other metabolic disturbances (2). The decreased responsiveness to insulin, termed "insulin resistance," is due to a reduced ability of insulin to activate its signaling pathways (3).

Insulin acts by binding to the α-subunits of the heterotetrameric (α,α,β,β) insulin receptor (IR) and inducing autophosphorylation of the β-subunits. This autophosphorylation is performed by the cytoplasmic kinase domain (CKD) regions of the β-subunits. Autophosphorylation of the IR CKD occurs in its juxtamembrane, activation loop, and COOH-terminus subdomains (6). CKD autophosphorylation initiates a cascade of signaling events that begin with the recruitment of specific substrates, such as insulin receptor substrate 1 (IRS-1), and includes activation of second messenger systems, such as phosphatidylinositol (PI) 3-kinase, and translocation of glucose transporters (GLUT4) to the cell surface (3,7–10). In insulin-resistant tissues or cells, deficiencies have been demonstrated in several steps in this pathway, including autophosphorylation of the IR in response to insulin binding (2–5).

Small-molecule drugs that act by producing insulin-dependent (11) activation of the IR tyrosine kinase domains are potentially attractive for the treatment of type 2 diabetes. Moreover, agents with insulin-dependent activity might offer unique advantages in the control of hyperglycemia by modulation of their effects as insulin levels change in response to physiological stimuli. We have now observed that a nonpeptide molecule, TLK16998, increases IR autophosphorylation in the presence of insulin and also enhances downstream signaling events, including phosphorylation of IRS-1 and GLUT4 translocation. In addition, this compound significantly lowers blood glucose levels in two animal models of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Autophosphorylation of IR CKD. In this study, 200 ng of IR CKD,"β-insulin receptor kinase," which is the entire cytoplasmic domain of the IR without any

extracellular or transmembrane domain residues (Stratagene, San Diego, CA) were dissolved in 30 μl of 50-mmol/l Tris pH 7.4, 2 mmol/l $MnCl_2$, and 10 mmol/l $MgCl_2$ and combined with different concentrations of TLK16998, 50 μmol/l ATP, and [γ-^{32}P]ATP (10 μCi) and incubated for 10 min at 25°C. For gel analysis, the samples were boiled in SDS-PAGE sample buffer for 5 min, electrophoresed on 10% SDS-PAGE gels, and visualized by autoradiography. Radioactivity was quantified by scintillation counting of the excised, labeled bands. For analysis by blotting, the samples were precipitated with trichloroacetic acid, spotted on a 96-well plate (Multiscreen; Millipore, Bedford, MA), washed three times with cold 75 mmol/l phosphoric acid, air-dried, and quantified by scintillation counting.

[^{125}I]insulin binding. IM-9 cells (ATCC no. CCL-159, 1.2×10^6 cells/ml) were incubated with insulin or compound at room temperature for 15 min in 1 ml phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Then, [^{125}I]insulin (0.2 μCi/ml; New England Nuclear, Wilmington, DE) was added and incubation was continued at 16°C for 90 min. After incubation, the cells were chilled, centrifuged at 2000 rpm for 10 min at 4°C, washed twice with ice-cold 10 mmol/l Tris, pH 7.6, 150 mmol/l NaCl, and counted for radioactivity.

Whole-cell IR autophosphorylation. Either differentiated 3T3-L1 adipocytes or CHO cells expressing wild type and a mutant IR Y960F (T. Kadowaki, University of Tokyo, Japan) were used in these experiments. 3T3-L1 fibroblasts (ATCC no. CL-173) were induced to differentiate into adipocytes by incubation in medium containing 1 μmol/l dexamethasone (DEX), 0.5 mmol/l isobutylmethylxanthine (IBMX), and 1.7 μmol/l insulin for 72 h. The cells were shifted to a medium containing 1.7 μmol/l insulin but without DEX or IBMX for 48 h. Finally, the cells were returned to normal serum-supplemented medium for 4 days. The cells were serum-starved (Dulbecco's modifed Eagle's medium supplemented with 0.1% BSA) for 16 h and then stimulated with TLK16998 with or without insulin (5.6 nmol/l for 3T3-L1 adipocytes and 2.5 nmol/l for CHO HIR and CHO Y960F mutant) for 15 min at 37°C, washed with cold PBS, lysed, and clarified by centrifugation; ~250 μg of total protein was immunoprecipitated with either the anti-IR polyclonal antibody (C-19), or the IRS-1 polyclonal antibody (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA). The protein was adsorbed onto protein G-agarose beads (Gibco-BRL, Gaithersburg, MD) for 2 h at 4°C, washed with the 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 antiphosphotyrosine antibody (4G10) overnight at 4°C, washed extensively, incubated with peroxidase-conjugated secondary antibody, and processed for enhanced chemiluminescence to visualize the immunoreactive bands. For reprobing, membranes were stripped, washed, and incubated with either anti-IR polyclonal antibody or anti–IRS-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

IR phosphorylation mapping. Partially purified native IR was autophosphorylated for 10 min in 50 mmol/l Tris HCl, pH 7.0, 5 mmol/l manganese acetate, 10 μmol/l [^{32}P]ATP. The incubations also contained 0.1 μmol/l insulin with or without 20 μmol/l TLK16998. The reactions were terminated by boiling with SDS-sample buffer, the products were separated by SDS-PAGE, and the autophosphorylated IR β-subunits were identified in the wet unfixed gel by autoradiography. The [^{32}P]-labeled β-subunit was excised, along with a similarly sized gel segment not containing a distinct band for the blank. Radioactivity incorporated was quantified by Cerenkov counting. The gel segments were soaked in water to remove background salts, crushed in 0.5 ml of 50 mmol/l ammonium bicarbonate and incubated with 1 μg of endoproteinase Lys-C (Wako Bioproducts, Richmond, VA). The samples were digested for 12 h with constant mixing at room temperature. A second 1 μg of Endo Lys-C was added and the digestion was continued for another 6 h. The supernatant from the digest was separated from the residual crushed gel pieces by centrifugation, lyophilized, resuspended in 200 μl water, and relyophilized six times. Two-dimensional phosphopeptide mapping for determination of autophosphorylation sites was performed as described previously (12). The thin-layer chromatography (TLC) plates were subjected to electrophoresis at 16°C in 20% acetic acid and 5% butanol for 1 h at 1,000 V with 19-cm separation between the electrodes. Ascending chromatography was performed in butanol: pyridine:acetic acid:water (15:10:3:12 vol/vol). Radiolabeled phosphopeptides were visualized by autoradiography using Kodak XAR X-ray film.

Measurement of 2-deoxy-D-[^{14}C]glucose uptake. 3T3-L1 fibroblasts (ATCC no. 173-CL) were induced to differentiate into adipocytes, as described above, and the cells were serum-starved in medium containing 0.1% BSA (Sigma, St Louis, MO) for 16 h, stimulated with various concentrations of TLK16998 plus different concentrations of insulin or IGF-1 (Sigma) for 30 min at 37°C, and then incubated with 0.5 μCi/ml of 2-deoxy-D-[^{14}C]glucose (New England Nuclear, Boston, MA) for 30 min at 37°C. The cells were rinsed with cold PBS and 20 mmol/l glucose, and lysed. The radioactivity was quantified by scintillation counting. The cells were pretreated with wortmannin (100 nmol/l; CalBiochem, San Diego, CA) or cytochalasin B (10 μmol/l; Sigma) for 30 min to examine the specificity of the signaling pathway.

Measurement of GLUT4 distribution. 3T3-L1 adipocytes were differentiated as above on a microscope chamber slide. The cells were serum-starved for 16 h and stimulated with TLK16998, insulin, or a combination of the two for 1 h at 37°C. The cells were permeabilized by treating with 3.5% paraformaldehyde (Sigma), incubated with a mouse anti-GLUT4 antibody (R&D Systems, Minneapolis, MN) for 30 min, washed, incubated with a fluorescein isothiocyanate–conjugated anti-mouse (Vector Laboratories, Burlingame, CA) antibody for an additional 30 min, washed again, and mounted for examination by confocal microscopy.

Epidermal growth factor receptor autophosphorylation in A431 cells. A431 epidermoid carcinoma cells (ATCC no. CRL-1555) were serum-starved overnight, incubated with compound for 2 h followed by treatment with 50 nmol/l epidermal growth factor (EGF) (Oncogene Science, Uniondale, NY) for an additional hour at 37°C. The cells were washed with cold PBS and lysed. Lysates were diluted with an equal volume of PBS, centrifuged, and transferred to anti-EGF receptor antibody–coated (AB-1; Oncogene Science, Uniondale, NY) microtiter plates. The plates were incubated for 2 h at room temperature, washed, incubated for an additional hour with horseradish peroxidase–conjugated anti-phosphotyrosine antibody PY20 (Zymed Laboratories, South San Francisco, CA), washed again, and incubated with 100 μl tetramethyl benzidine (TMB) substrate (Kierkegaard and Perry Laboratories, Gaithersburg, MD). The plate was read kinetically on a Vmax reader (Molecular Devices, Sunnyvale, CA) at 650 nm.

In vivo antidiabetic activity

db/db Mice. Male 8-week-old *db/db* mice (C57BL6/J background, Jackson Labs, Bar Harbor, ME) were maintained according to National Institutes of Health guidelines, housed five animals per cage, and fed standard lab diet (Tekland Laboratory Diets; James Grain, San Jose, CA). At the end of the 12-h light/dark cycle and just before experiment, the animals were moved to new cages in which no food was available. Because the animals were studied at a time when their insulin levels were rapidly declining (13), they were given 0.01 U of human insulin just before administration of TLK16998. The animals had glucose values in the range of 300–400 mmol/l at the initiation of the experiment.

STZ/high-fat diet (HFD) mice. Male C57Bl6/J mice (Jackson Laboratories, Bar Harbor, ME) were maintained according to National Institutes of Health guidelines. The mice were housed five animals per cage and fed standard laboratory diet (Tekland Laboratory Diets; James Grain, San Jose, CA) supplemented with chocolate bars, cookies, and potato chips such that their final diet contained 30% fat by weight (HFD). After 2 weeks on this diet, the animals were given five daily injections of freshly prepared streptozotocin (35 mg/kg i.p.) and continued on the HFD. Animals that achieved glucose levels of 300–500 mmol/l were used in this study. At the end of the 12-h light/dark cycle and just before experiment, animals were moved to new cages in which no food was available.

For both types of mice, either TLK16998 or vehicle (PBS) was given intraperitoneally, and blood was sampled by approved International Animal Care and Use Committee protocol using tail-cap method. Glucose levels were determined using the Glucometer Elite (Bayer, Elkhart, IN).

RESULTS

Site of action of TLK16998. TLK16998 (Fig. 1) was originally identified by its ability to increase autophosphorylation of the isolated, naturally expressed human IR (data not shown) using the drug discovery technology known as TRAP or target-related affinity profiling (14). Two types of studies were then undertaken to determine the site of action of TLK16998. First, the ability of TLK16998 to activate the cloned human IR CKD (15) was examined. TLK16998 significantly stimulated phosphorylation of the CKD (Fig. 2). This effect was detectable at concentrations of 1 μmol/l or less and reached a maximum at 5.6 μmol/l. Increasing the concentration of TLK16998 to 10 μmol/l diminished this activation. Second, the effect of TLK16998 on insulin binding was measured. TLK16998, at concentrations up to 100 μmol/l, had little or no effect on binding (Fig. 3). Insulin, in contrast, produced a progressive decrease in binding at concentrations between 1 and 100 nmol/l. These data indicate that TLK16998 acted on the IR β-subunit.

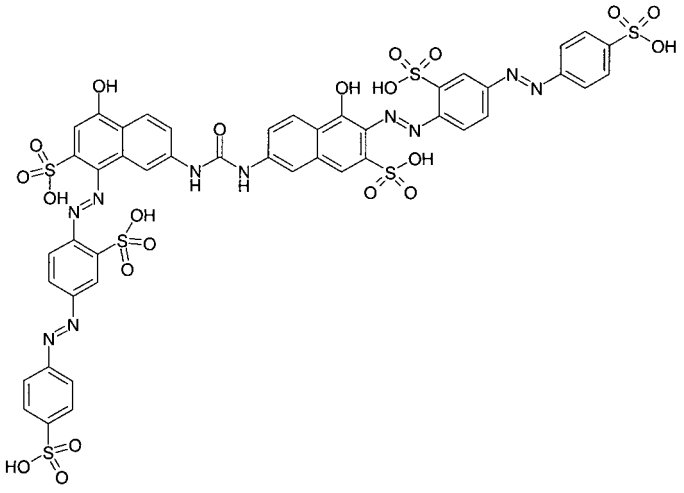

FIG. 1. Structure of TLK16998.

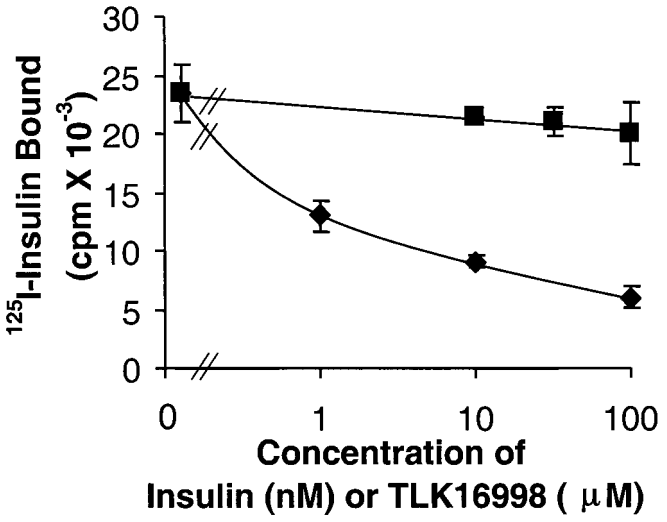

FIG. 3. Lack of inhibition of [^{125}I]insulin binding to cultured IM-9 cells by TLK16998. IM-9 cells (1.2×10^6 cells/ml) were incubated with unlabeled insulin (♦) or TLK16998 (■) for 15 min at 25°C, [^{125}I]insulin was added and incubated at 16°C for 90 min, and specific binding was determined. Results are means ± SE of three experiments.

TLK16998 increases IR signaling in 3T3-L1 cells. To explore the effect of TLK16998 on insulin action in cells, differentiated 3T3-L1 adipocytes were treated with the compound. Insulin, at a concentration of 5.6 nmol/l, increased the phosphorylation of the IR β-subunit and IRS-1, and the amount of PI 3-kinase that coimmunoprecipitated with IRS-1 (Fig. 4). TLK16998 by itself had no significant effect on these parameters. TLK16998, in combination with 5.6 nmol/l insulin, however, caused a greater increase in the phosphorylation of both proteins and in PI 3-kinase coimmunoprecipitation than 5.6 nmol/l insulin alone (Fig. 4*A*). The effect of the compound was observable at 3.2 μmol/l and was maximal at 10 μmol/l. Higher concentrations of TLK16998 were progressively less effective. TLK16998, at concentrations up to 40 μmol/l, did not increase the autophosphorylation of the EGF receptor in either the presence or absence of EGF (data not shown).

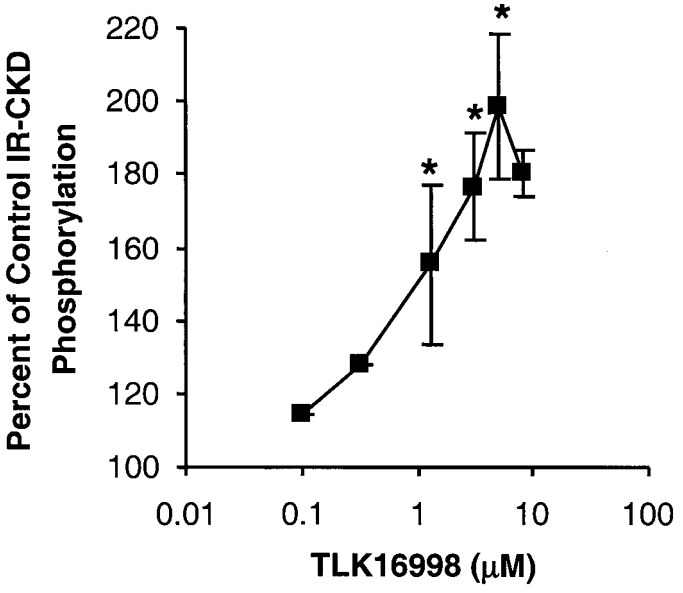

FIG. 2. Effect of TLK16998 on autophosphorylation of the isolated IR CKD. IR CKD (200 ng) was incubated with 50 μmol/l ATP and 10 μCi γ-[^{32}P]ATP plus metals for 10 min at 25°C, and radioactivity incorporated into the IR CKD was quantified by SDS-PAGE analysis and followed by either autoradiography or trichloroacetic acid precipitation and counting. Results are means ± SE of three experiments. Asterisks indicate significant difference from vehicle control; one-tailed Student's *t* test corrected for multiple comparisons ($P < 0.05$).

Effect of TLK16998 on autophosphorylation sites. To study in more detail the effect of TLK16998 on IR autophosphorylation, [^{32}P]phosphopeptide maps of the phosphorylated IR were generated (Fig. 5). After stimulation of IR purified from 3T3-L1 adipocytes with 100 nmol/l insulin, autophosphorylation was observed in regions previously shown to correspond to the juxtamembrane (J), activation loop (A1–A3), and the COOH-terminus (C1, C2) domains of the IR CKD (16). Exposure of the IR to 20 μmol/l TLK16998 with 0.1 μmol/l insulin increased the tris-phosphorylation of the activation loop (A3) and bisphosphorylation of the COOH-terminus (C2). Surprisingly, the juxtamembrane region (J) was less phosphorylated in cells that were treated with both insulin and TLK16998 than in the cells treated with insulin alone.

To confirm the ability of TLK16998 to enhance insulin-induced activation of the IR in the absence of juxtamembrane phosphorylation, an IR mutant in which the juxtamembrane tyrosine 960 was replaced with phenylalanine was expressed in CHO cells (CHO Y960F). As reported (17), this mutant IR was less effective in stimulating IRS-1 phosphorylation in response to insulin than the wild-type IR (CHO-HIR) (Fig. 6). TLK16998, however, was still able to enhance the insulin-induced IRS-1 phosphorylation in CHO cells expressing this mutant IR.

Effect of TLK16998 on insulin and IGF-1 stimulation of glucose transport in 3T3-L1 adipocytes. To establish that the enhancement of IR autophosphorylation by TLK16998 has functional significance, the effect of the compound on insulin-stimulated glucose transport was explored. In 3T3-L1 adipocytes, insulin (1 nmol/l to 1 μmol/l) was able to simulate the uptake of 2-deoxy-D-glucose (Fig. 7). TLK16998, at concentrations ranging from 8 to 40 μmol/l, increased the sensitivity to insulin in stimulating 2-deoxy-D-glucose but did not increase the maximum response. At a concentration of 40 μmol/l, TLK16998 increased the sensitivity to insulin by 10-fold as indicated

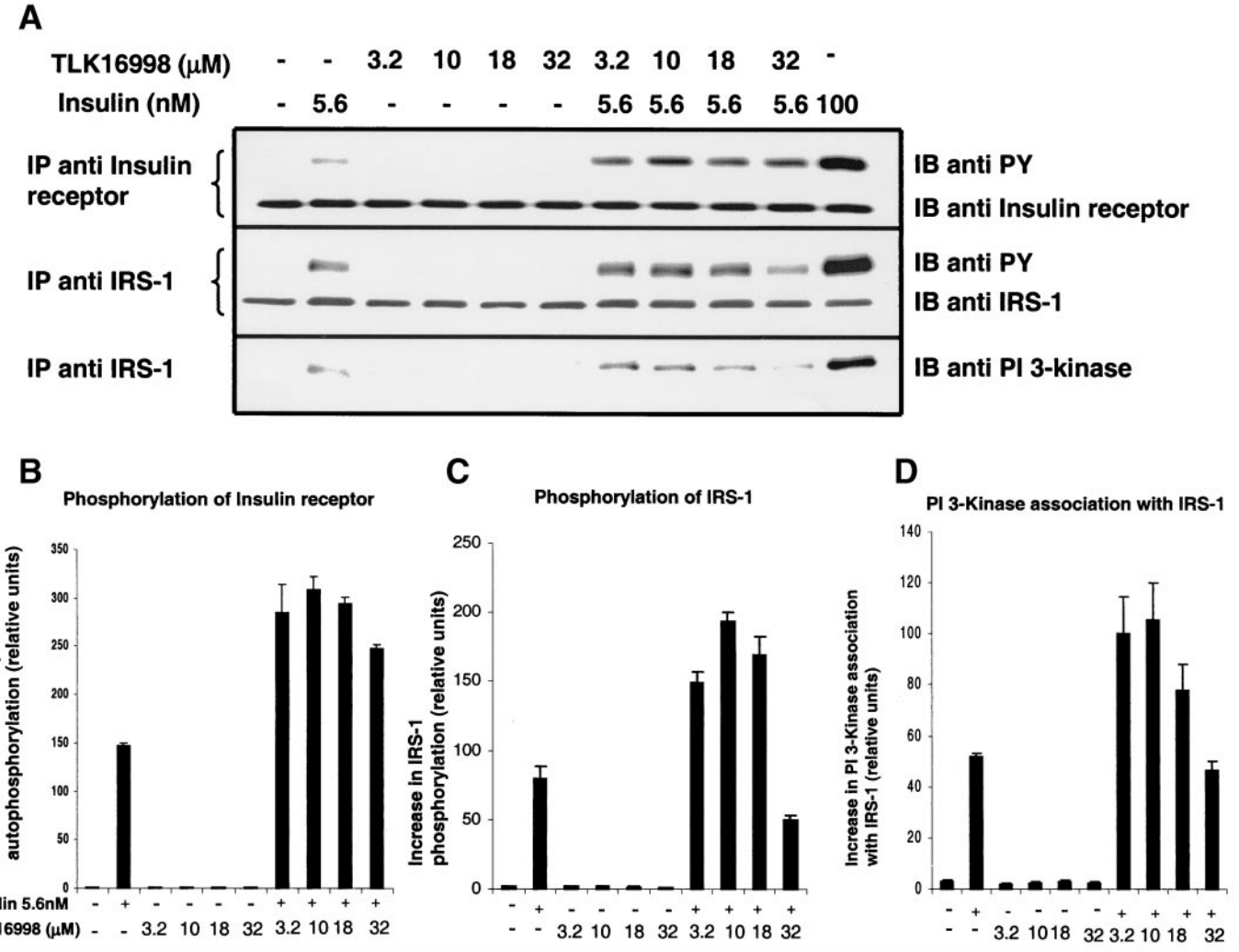

FIG. 4. Effect of TLK16998 on insulin-induced IR autophosphorylation, IRS-1 phosphorylation, and PI 3-kinase association to IRS-1. Differentiated 3T3-L1 adipocytes were incubated with insulin and TLK16998 at 37°C for 15 min. The cells were washed, lysed, immunoprecipitated with specific antibodies, subjected to SDS-PAGE followed by Western blotting with specific antibodies, and developed by enhanced chemiluminescence. *A*: Immunoblot. *B*: Quantitation of the autophosphorylation of the IR. *C*: Phosphorylation of IRS-1. *D*: PI 3-kinase association with IRS-1. Values in (*B*) through (*D*) were measured with a Storm phosphorimager with Imagequant software (Molecular Dynamics, Sunnyvale, CA) and are expressed in Imagequant relative units.

by a 1-log leftward shift of the concentration-response curve for insulin. TLK16998 had little or no effect without insulin (data not shown). In contrast to its sensitization of insulin-induced 2-deoxy-D-glucose uptake, only the highest concentration of TLK16998 (40 μmol/l) had an effect on IGF-1–induced 2-deoxy-D-glucose uptake in 3T3-L1 adipocytes.

We believe that these data show a high degree of specificity of TLK16998 on the IR. The small effect of TLK16998 on the IGF-1 receptor may be due to either a small intrinsic effect on this receptor or to action on IR/IGF-1 receptor hybrids. The effects of TLK16998 on 2-deoxy-D-glucose uptake were inhibited by wortmannin and cytochalasin B, two agents that block the insulin-dependent glucose entry into cells (2,9) (data not shown).

Effect of TLK16998 on GLUT4 translocation. To further link the effects of TLK16998 on IR autophosphorylation with those on glucose uptake, translocation of the GLUT4 glucose transporter (10) was studied (data not shown). In unstimulated 3T3-L1 adipocytes, GLUT4 immunofluorescence was broadly distributed within the cells as visualized by confocal microscopy. Insulin (5.6 nmol/l) alone had only

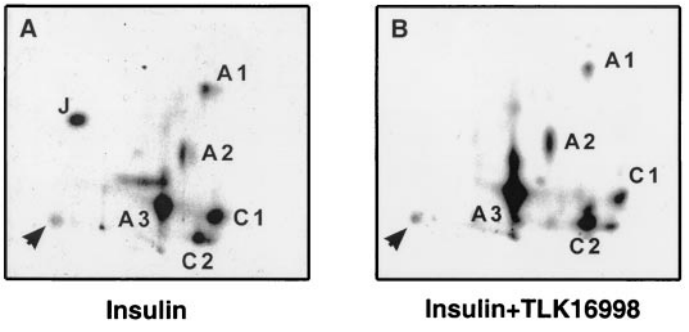

FIG. 5. [^{32}P]phosphopeptide maps of IR autophosphorylated by insulin and TLK16998 in vitro. Partially purified IR from 3T3-L1 adipocytes was autophosphorylated with insulin (100 nmol/l) in the absence or presence of 20 μmol/l TLK16998. After SDS-PAGE, the β-subunit of the IR was excised and analyzed by two-dimensional phosphopeptide mapping as described in RESEARCH DESIGN AND METHODS. Autoradiograms show the two-dimensional resolution of phosphopeptides. Electrophoresis was from left to right and chromatography was from bottom to top. The origins are marked by the arrowheads, and phosphopeptides were identified by comparison with published maps: J = juxtamembrane; A1, A2, and A3 = mono-, bis-, and tris-phosphorylated activation loop, respectively; C1 and C2 = mono- and bis-phosphorylated COOH-terminus, respectively.

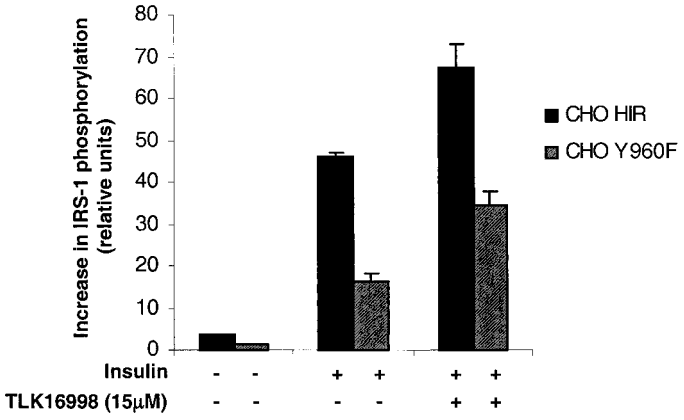

FIG. 6. Effect of TLK16998 on IRS-1 phosphorylation in CHO HIR and CHO Y960F mutant cell lines. The cell lysates were immunoprecipitated with anti–IRS-1 antibody followed by immunoblotting with either antiphosphotyrosine antibody or anti–IRS-1 antibody as indicated. The cells were incubated either with insulin (2.5 nmol/l), TLK16998 (15 μmol/l), vehicle (–), or combinations thereof for 15 min, as indicated.

a small effect on this pattern. In contrast, stimulation of the cells with 100 nmol/l insulin resulted in GLUT4 immunofluorescence being localized primarily on the cell surface. This change in distribution is consistent with insulin-dependent translocation of GLUT4 from intracellular storage sites to the membrane (10). TLK16998 (20 μmol/l) plus 5.6 nmol/l insulin resulted in a distribution of GLUT4 immunofluorescence that was indistinguishable from that produced by 100 nmol/l insulin. The effect of TLK16998 on GLUT4 translocation was inhibited by wortmannin and cytochalasin B (2,9) (data not shown).

Effect of TLK16998 in vivo. The in vivo effectiveness of TLK16998 was examined in two animal models of type 2 diabetes. The first model was the 8-week-old *db/db* mouse (13). These mice were moderately hyperglycemic, with blood glucose levels between 300 and 400 mg/dl. TLK16998 (10 mg/kg i.p.) decreased blood glucose levels by 21% compared with controls (Fig. 8). The peak effect occurred between 15 min and 2 h after administration of the compound, but the decrease was sustained for at least 4 h.

The ability of TLK16998 to lower blood glucose levels was also examined in (C57BL/6J) mice fed an HFD followed by treatment with a low dose of streptozotocin (STZ/HFD). This animal model incorporates both the insulin resistance and the hyperglycemia seen in human type 2 diabetes (16). The animals used in this study had moderate hyperglycemia, with blood glucose levels ranging from 300 to 400 mg/dl. TLK16998, at 10 mg/kg, produced an average decrease of 28% in blood glucose levels 2–6 h after administration (Fig. 9). The small elevation of blood glucose seen in the first hour after administration was due to the injection procedure itself, because a similar trend was observed in the vehicle-injected group.

DISCUSSION

In the present studies, we observed that a nonpeptide molecule, TLK16998, acts as an IR sensitizer in cultured cells. TLK16998 acted directly on the IR β-subunit to increase insulin-stimulated IR autophosphorylation with subsequent enhancement of downstream signaling events, including IRS-1 phosphorylation, PI 3-kinase recruitment, GLUT4 translocation, and glucose uptake in cells. TLK16998 by itself did not activate either IR signaling or glucose uptake in cells, making it a sensitizer of the IR rather than an agonist. TLK16998 increased the sensitivity of cells to insulin but did not increase maximal response. Thus, TLK16998 did not produce supraphysiological levels of IR stimulation, again consistent with its function as an insulin sensitizer. In contrast to its effect on IR signaling, the compound had no effect on the EGF receptor autophosphory-

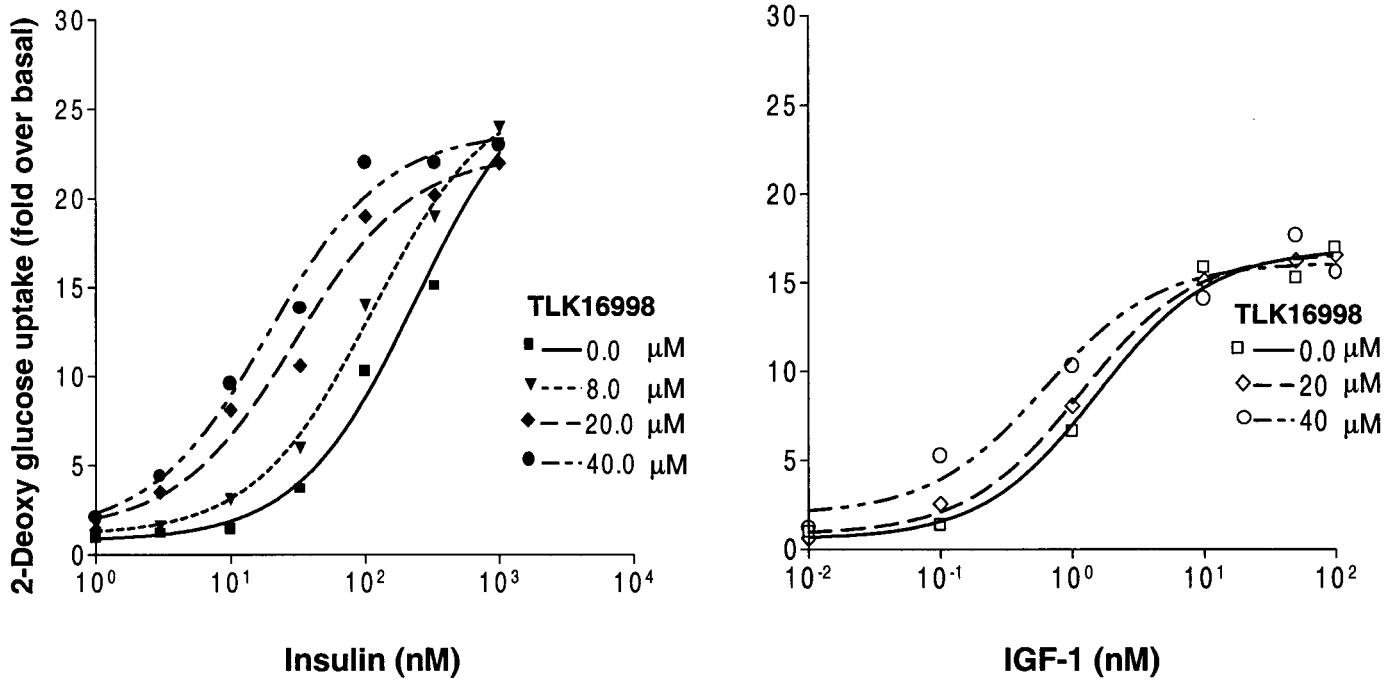

FIG. 7. Differential effect of TLK16998 on insulin or IGF-1–stimulated 2-deoxy-D-glucose uptake in 3T3-L1 adipocytes. The cells were incubated with insulin and IGF-1 plus various concentrations of TLK16998 for 30 min at 37°C, and 2-deoxy-D-glucose uptake was measured. Values are the means of four to five separate experiments.

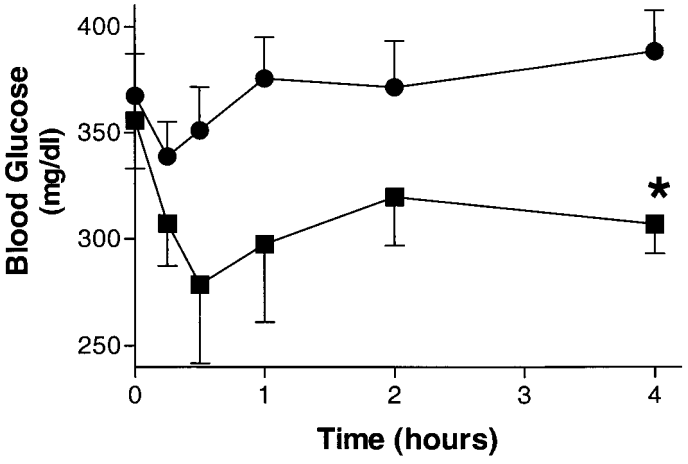

FIG. 8. Decreasing of blood glucose levels in diabetic *db/db* mice by TLK16998. Mice were injected (intraperitoneally) with either vehicle (PBS) (●) or TLK16998 (■) (30 mg/kg). Values are the mean ± SE for five control and five TLK16998-treated animals. TLK16998 produced a significant decrease in blood glucose levels at 4 h after administration compared with the PBS control (Student's *t* test, *$P < 0.01$).

lation and only small effects on the IGF-1–induced glucose uptake. Therefore, TLK16998 is not a general sensitizer of receptor tyrosine kinase domains. In diabetic mice, TLK16998 lowered blood glucose levels. TLK16998 represents, therefore, the first small nonpeptide molecule that specifically and directly sensitizes cells to insulin.

It has recently been reported that L-783,281, a fungal metabolite, activated the IR both in vitro and in vivo (18). Like TLK16998, this compound acts directly on the IR β-subunit. Unlike TLK16998, however, L-783,281 was a direct IR agonist, increasing IR autophosphorylation and downstream signaling events in the absence of insulin. L-783,281 also differs structurally from TLK16998, having a quinone backbone. Nevertheless, the results with L-783,281, in addition to our results with TLK16998, indicate that the IR β-subunit is an important molecular target for the treatment of type 2 diabetes.

TLK16998 stimulated autophosphorylation of the isolated CKD, whereas it served as an insulin sensitizer and not as an independent stimulator of autophosphorylation of the full-length IR. The sites of autophosphorylation in the IR suggest a mechanistic basis for this difference. ATP favors phosphorylation of the activation loop and COOH-terminal sites and decreases phosphorylation of the juxtamembrane region in the native IR (19). Recent studies with the CKD (20) indicated that ATP binding promotes a conformational change in the activation loop to a "gate-open" state that is essential for its autophosphorylation. These findings suggest that the kinase domain of the receptor can be primed independently for autophosphorylation, but insulin binding is still the event that triggers the reaction in cells (R. Kohanski, unpublished data). A similar priming effect of TLK16998 is likely to stimulate CKD autophosphorylation in vitro and to potentiate insulin-dependent IR autophosphorylation in cells.

The decrease in phosphorylation of the 960 tyrosine in the juxtamembrane domain of the IR in cells treated with TLK16998 and insulin, compared with cells treated with insulin alone, was surprising because insulin-induced IRS-1 phosphorylation was stimulated by the compound. Other studies have shown that phosphorylation of the juxtamembrane subdomain is important for IRS-1 phosphorylation, possibly to promote interaction of IRS-1 with the IR CKD (15,21). However, Yenush et al. (22), while demonstrating the critical role played by the plekstrin homology domain in IRS-1 recruitment, also showed that juxtamembrane phosphorylation of the IR is not absolutely required, although it does enhance insulin-stimulated IRS-1 phosphorylation through interaction with the phosphotyrosine binding–domain of IRS-1. In the present work, increased phosphorylation of the activation loop in the presence of the compound should increase kinase activity, which may compensate for diminished juxtamembrane phosphorylation. Alternatively, the effect of the compound on the IR CKD, although decreasing juxtamembrane autophosphorylation, may itself promote IRS-1 recruitment.

TLK16998 demonstrates efficacy in two animal models of type 2 diabetes. The first, the *db/db* mouse, has a truncation of the leptin receptor, which leads to development of insulin resistance, hyperinsulinemia, and hyperglycemia. Eventually, insulin levels begin to decrease, producing severe hyperglycemia and death. In the present report, the animals were used at 8 weeks of age, a time when increasing insulin resistance and decreasing insulin levels mimic the situation in human type 2 diabetes (13). At a dose of 10 mg/kg i.p., TLK16998 produced a 21% decrease in blood glucose levels.

TLK16998 demonstrated efficacy in a second animal model of type 2 diabetes, the STZ/HFD mouse. In this model, insulin resistance was induced by feeding the animal a diet high in fat, and hyperglycemia was then produced by a low dose of streptozotocin. Animals treated in this way have been reported to exhibit moderate hyperglycemia in the presence of relatively normal circulating insulin levels, a syndrome that models human type 2 diabetes (16). TLK16998 produced a 28% decrease in blood glucose levels in this model when given at a dose of 10 mg/kg i.p.

Insulin-dependent activation of the IR by a small molecule is a potential new paradigm for the treatment of type 2 diabetes and may offer unique advantages over other therapies (23,24). Acting directly at the level of the IR

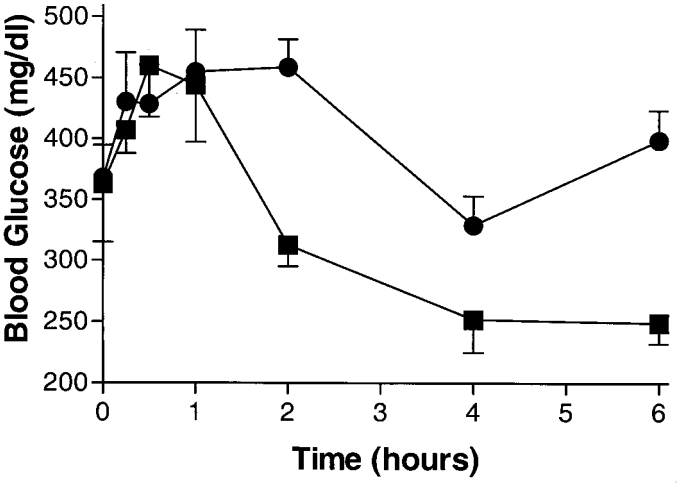

FIG. 9. Decreasing of blood glucose levels in diabetic STZ/HFD mice by TLK16998. Mice were injected (intraperitoneally) with either vehicle (PBS) (●) or TLK16998 (■) (30 mg/kg). Values are the mean ± SE for nine control and three TLK16998-treated animals. TLK16998 produced a significant decrease in blood glucose levels compared with the PBS control (two-way analysis of variance, $F = 13.16$, $P = 0.0005$).

should allow induction of the entire range of actions of insulin while minimizing unwanted side effects. At the same time, avoiding IR agonism should reduce the risk of functional hypoglycemia. Perhaps more importantly, because the effect of an IR-sensitizing drug would be proportional to the level of endogenous insulin, more precise control of glucose levels might be achieved. Thus, when blood glucose levels increase, leading to an elevation of insulin levels, such a drug would have a greater effect. The in vitro and in vivo profiles of TLK16998 reported here provide a rationale for developing drugs that sensitize the IR as a potential therapy for type 2 diabetes. In addition, TLK16998 provides a chemical starting point for the development of IR-sensitizing drugs.

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REFERENCES

1. Harris MI: *Diabetes in America*, 2nd ed., 1–13 National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, 1995 (Pub. No. 95–1468)
2. Davis SN, Granner DK: Insulin, oral hypoglycemic agents and the pharmacology of the endocrine pancreas. In *Goodman & Gilman's the Pharmacological Basis of Therapeutics*. 9th ed. Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG, Eds. New York, McGraw Hill, 1996, p. 1487–1517
3. Goldfine ID: Unravelling the riddle of insulin resistance. *J Clin Lab Med* 134:100–102, 1999
4. Taylor SI: Deconstructing type 2 diabetes. *Cell* 97:9–12, 1999
5. Thies RS, Molina JM, Ciaraldi TP, Freidenberg GR Olefsky JM: Insulin-receptor autophosphorylation and endogenous substrate phosphorylation in human adipocytes from control, obese, and NIDDM subjects. *Diabetes* 39:250–259, 1990
6. White MF, Kahn CR: The insulin signaling system. *J Biol Chem* 269:1–4, 1994
7. Di Guglielmo GM, Drake PG, Baass PC, Authier F, Posner BI, Bergeron JJ: Insulin receptor internalization and signalling. *Mol Cell Biochem* 182:59–63, 1998
8. Virkamaki A, Ueki K, Kahn CR: Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest* 103:931–943, 1999
9. Czech MP, Corvera S: Signaling mechanisms that regulate glucose transport. *J Biol Chem* 274:1865–1868, 1999
10. Shepherd PR, Kahn BB: Glucose transporters and insulin action, implications for insulin resistance and diabetes mellitus. *N Engl J Med* 341:248–257, 1999
11. Sportsman JR, Villar HO, Kauvar LM, Spevak WR: Nonpeptide insulin receptor agonists. U.S. patent 5,851,988, 1998
12. Cann AD, Kohanski RA: Cis-autophosphorylation of juxtamembrane tyrosines in the insulin receptor kinase domain. *Biochemistry* 36:7681–7689, 1997
13. Kodama H, Fujita M, Yamaguchi I: Development of hyperglycemia and insulin resistance in conscious genetically diabetic (C57BL/KsJ-db/db) mice. *Diabetologia* 37:739–744, 1994
14. Kauvar LM, Higgins DL, Villar HO, Sportsman JR, Engqvist-Goldstein A, Bukar R, Bauer KE, Dilley H, Rocke DM: Predicting ligand binding to proteins by affinity fingerprinting. *Chem Biol* 2:107–118, 1995
15. Villalba M, Wente SR, Russell DS, Ahn JC, Reichelderfer CF, Rosen OM: Another version of the human insulin receptor kinase domain: expression, purification, and characterization. *Proc Natl Acad Sci U S A* 86:7848–7852, 1989
16. Luo J, Quan J, Tsai J, Hobensack CK, Sullivan C, Hector R, Reaven GM: Nongenetic mouse models of non-insulin-dependent diabetes mellitus. *Metabolism* 47:663–668, 1998
17. Kaburagi Y, Momomura K, Yamamoto-Honda R, Tobe K, Tamori Y, Sakura H, Akanuma Y, Yazaki Y, Kadowaki T: Site-directed mutagenesis of the juxtamembrane domain of the human insulin receptor. *J Biol Chem* 268:16610–16622, 1993
18. Zhang B, Salituro G, Szalkowski D, Li Z, Zhang Y, Royo I, Vilella D, Diez MT, Pelaez F, Ruby C, Kendall RL, Mao X, Griffin P, Calaycay J, Zierath JR, Heck JV, Smith RG, Moller DE: Discovery of a small molecule insulin mimetic with antidiabetic activity in mice. *Science* 284: 974–977,1999
19. Kohanski RA: Insulin receptor autophosphorylation. 1. Autophosphorylation kinetics of the native receptor and its cytoplasmic kinase domain. *Biochemistry* 32:5766–5772, 1993
20. Frankel M, Bishop SM, Ablooglu AJ, Han Y-P, Kohanski RA: Conformational changes in the activation loop of the insulin receptor's kinase domain. *Protein Sci* 8:2158–2165, 1999
21. Keren P, Voliovitch H, Hadari YR, Roberts CT Jr, LeRoith D, Zick Y: Interaction between the insulin receptor and its downstream effectors. *J Biol Chem* 271:6998–7003,1996
22. Yenush L, Makati KJ, Smith-Hall J, Ishibashi O, Myers MG Jr, White MF: The pleckstrin homology domain is the principal link between the insulin receptor and IRS-1. *J Biol Chem* 271:24300–24306, 1996
23. DeFronzo RA: Pharmacologic therapy for type 2 diabetes mellitus. *Ann Intern Med* 131:281–303, 1999
24. Saltiel AR, Olefsky JM: Thiazolidinediones in the treatment of insulin resistance and type 2 diabetes. *Diabetes* 45:1661–1669, 1996