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

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
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 1x phosphoric acid to remove background salts. The plates were then soaked in water and 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.

**Whole-cell IR autophosphorylation.** Either differentiated 3T3-L1 adipocytes or CHO cells expressing wild type or 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% fetal bovine serum (FBS), 25 mMol/l dexamethasone (DEX), and 0.5% isobutylmethylxanthine (IBMX). The cells were shifted to a medium containing 1.7% FBS using DEX or IBMX for 48 h. After a 2-h washout period, the cells were returned to normal serum-supplemented medium for 4 days. The cells were serum-starved (Dubelcco’s modified Eagle’s medium supplemented with 0.1% BSA) for 16 h and then stimulated with TLK16998 with or without insulin (5.6 mMol/l for 3T3-L1 adipocytes and 2.5 mMol/l for CHO HIR and CHO Y960F mutant) for 15 min at 37°C. After stimulation with cold PBS, lysed, and centrifuged, the supernatant from 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. 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.

**IR phosphorylation mapping.** Partially purified native IR was autophosphorylated on 50 mMol/l Tris HCl, pH 7.0, 5 mMol/l magnesium acetate, 10 mMol/l [32P]ATP. The incubations contained 0.1% insulin with or without 20 mMol/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 [32P]-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 plates were incubated for 2 h at room temperature, washed, incubated with an additional hour with horseradish peroxidase–conjugated anti-phosphoseryl-phosphotyrosine antibody PT20 (Zymed Laboratories, South San Francisco, CA), washed, and incubated with 100 μl tetramethyl benzidine (TMB) substrate (Kerkwegard and Perry Laboratories, Gaithersburg, MD). The plate was read kinetically on a Vmax reader (Molecular Devices, Sunnyvale, CA) at 650 nm.

**In vivo diabetogenic activity**

**db/db mice.** Male 8-week-old db/db mice (C57BL/L6j 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 C57BL6j 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 μM or less and reached a maximum at 5.6 μM. Increasing the concentration of TLK16998 to 10 μM diminished this activation. Second, the effect of TLK16998 on insulin binding was measured. TLK16998, at concentrations up to 100 μM, had little or no effect on binding (Fig. 3). Insulin, in contrast, produced a progressive decrease in binding at concentrations between 1 and 100 μM. These data indicate that TLK16998 acted on the IR β-subunit.
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. 4A). 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).

Effect of TLK16998 on autophosphorylation sites. To study in more detail the effect of TLK16998 on IR autophosphorylation, [32P]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...
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. 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.
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 autophosphorylation.
studies have shown that phosphorylation of the juxtamembrane domain of the IR in 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 of IRS-1 inactivation, 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.

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).

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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