Activation of Insulin Signal Transduction Pathway and Anti-diabetic Activity of Small Molecule Insulin Receptor Activators*

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We recently described the identification of a non-peptidyl fungal metabolite (1-783,281, compound 1), which induced activation of human insulin receptor (IR) tyrosine kinase and mediated insulin-like effects in cells, as well as decreased blood glucose levels in murine models of Type 2 diabetes (Zhang, B., Salituro, G., Szalkowski, D., Li, Z., Zhang, Y., Royo, I., Vilella, D., Diez, M. T., Pealez, F., Ruby, C., Kendall, R. L., Mao, X., Griffin, P., Calaycay, J., Zierath, J. R., Heck, J. V., Smith, R. G. & Moller, D. E. (1999) Science 284, 974–977). Here we report the characterization of an active analog (compound 2) with enhanced IR kinase activation potency and selectivity over related receptors (insulin-like growth factor I receptor, epidermal growth factor receptor, and platelet-derived growth factor receptor). The IR activators stimulated tyrosine kinase activity of partially purified native IR and recombinant IR tyrosine kinase domain. Administration of the IR activators to mice was associated with increased IR tyrosine kinase activity in liver. In vivo oral treatment with compound 2 resulted in significant glucose lowering in several rodent models of diabetes. In db/db mice, oral administration of compound 2 elicited significant correction of hyperglycemia. In a streptozotocin-induced diabetic mouse model, compound 2 potentiated the glucose-lowering effect of insulin. In normal rats, compound 2 improved oral glucose tolerance with significant reduction in insulin release following glucose challenge. A structurally related inactive analog (compound 3) was not effective on insulin receptor activation or glucose lowering in db/db mice. Thus, small molecule IR activators exert insulin mimetic and sensitizing effects in cells and in animal models of diabetes. These results have implications for the future development of new therapies for diabetes mellitus.

Insulin elicits a diverse array of biological responses by binding to its specific receptor (1). The insulin receptor (IR)1 is a heterotetrameric protein consisting of two extracellular α subunits and two transmembrane β subunits. The binding of the ligand to the α subunit of IR not only concentrates insulin at its site of action, but also induces conformational changes in the receptor, which in turn stimulates the tyrosine kinase activity intrinsic to the β subunit of the IR. Extensive studies have indicated that the ability of the receptor to autophosphorylate and phosphorylate intracellular substrates is essential for its mediation of the complex cellular responses of insulin (2–5). Insulin receptors trans-phosphorylate several immediate substrates (on Tyr residues), including insulin receptor substrate (IRS) proteins 1–4, Shc, and Gab 1, each of which provide specific docking sites for other signaling proteins containing Src homology 2 domains (6). These events lead to the activation of downstream signaling molecules, including phosphatidylinositol 3-kinase (PI 3-kinase). Numerous studies have adduced that PI 3-kinase is required for the metabolic effects of insulin. Although the discrete pathways that couple PI 3-kinase to glucose regulation remain poorly defined, Akt (or PKB), a Ser/Thr kinase known to be PI 3-kinase-dependent, appears to be involved in insulin-mediated activation of glucose transport (7) and glycogen synthesis (8).

Insulin is essential for maintaining glucose homeostasis and regulating carbohydrate, lipid, and protein metabolism (9). The central role of the insulin receptor in metabolic and growth control has been validated in insulin receptor null mouse models (10, 11). Decreased cellular responses to insulin or perturbation of the insulin signaling pathways are associated with a number of pathological states. Mutations in insulin receptor gene that lead to alterations of receptor synthesis, degradation, and function have been described in patients with several uncommon syndromes associated with severe insulin resistance (12). The molecular basis for insulin resistance that proceeds, or is associated with, common forms of Type 2 diabetes remains poorly understood. However, several studies have shown modest decreases in insulin receptor number attributed to down-regulation in response to hyperinsulinemia in tissues or cells from Type 2 diabetic patients (13, 14). Substantial decreases in insulin-stimulated receptor tyrosine kinase activity and an even more substantial defect in receptor-mediated IRS phosphorylation or PI 3-kinase activation have been described using samples of tissue (e.g. muscle or fat) from rodents or human subjects with Type 2 diabetes (15–17). Although controversial, diminished insulin-stimulated Akt activation was documented in skeletal muscle from Type 2 diabetic patients (18, 19). Thus, in humans with Type 2 diabetes there are...
clear defects involving the insulin receptor and proximal steps in insulin signaling. Pharmaceutical intervention aimed at augmenting insulin receptor function may ultimately prove beneficial in patients with diabetes.

We have previously reported the discovery of a small molecule fungal metabolite (L-783,281) with insulin-like activities in cells and in animal models of diabetes (20). Here we describe a new active analog of L-783,281 with improved potency for activation of insulin receptor in cells as well as improved selectivity toward insulin receptor versus other homologous receptor tyrosine kinases. Moreover, a structurally related inactive analog was also synthesized and used, along with the active compound, to establish the correlation between insulin receptor activation in vitro and glucose lowering in animals. The results of these studies further validate approaches designed to identify new small molecule insulin receptor activators as potential novel anti-diabetic agents.

**Experimental Procedures**

**Materials**—All biological reagents used here were obtained from commercial sources. Radiochemicals were purchased from PerkinElmer Life Sciences. Compound 1 (L-783,281) was prepared as described previously (20). Compounds 2 and 3 were prepared synthetically as described previously (21). Compounds stock solutions were prepared in 100% dimethyl sulfoxide (Me2SO) and diluted in appropriate media just prior to use.

**Animals**—Male db/db and db/+ mice were from Jackson Laboratories. Male Harlan Sprague-Dawley rats were from Charles River Breeding Laboratories, Inc. Animals were allowed access to standard rodent chow and water ad libitum. Animals were treated orally (by gavage) with vehicle or test compounds. Blood glucose level was measured using One TouchTM Glucometer (LifeScan) or Accu-ChekTM blood glucose monitoring system (Roche Molecular Biochemicals). Plasma glucose concentration was measured using a glucose oxidase kit (Sigma). Plasma insulin concentration was measured with a radioimmunooassay kit (Linco). Animal care was in accordance with institutional guidelines.

**Cell Culture and Treatment**—Chinese hamster ovary cells expressing human insulin receptor (CHO.IR, a gift from Dr. Richard Roth, Stanford University, Stanford, CA) were maintained as described previously (20). For experiments, cells were treated in the appropriate serum-free media containing compounds dissolved in Me2SO. Control cells received equivalent amounts of Me2SO, and the final concentration of Me2SO was always kept below 0.1%.

**Tyrosine Kinase Assays**—The IRTK activity in CHO.IR cells and mouse liver extracts was determined using a previously described procedure (20). To determine IRTK in a cell-free assay, insulin receptor was partially purified from CHO.IR cells using WGA-agarose columns (22). For the in vitro kinase assay, 2 μg of WGA-purified IR was incubated with a buffer (final volume 50 μl) containing 50 mM MgCl2, 50 mM HEPES (pH 7.5), 0.1% Triton X-100, insulin or test compounds at 25 °C for 20 min. ATP (25 μM, 0.25 μCi/μl) was added and incubation continued for 20 min. The mixture was then incubated for 5 min at 25 °C with 100 μM concentration of a peptide substrate based on insulin receptor autophosphorylation sites (TRIDYETDYYRK) (23). The reaction was terminated by addition of 10 μl of 1% bovine serum albumin followed by 20 μl of 20% trichloroacetic acid. The mixtures were centrifuged, and 20 μl of the supernatant was applied to phosphocellulose filter strip. The filters were washed several times with 20% trichloroacetic acid, and radioactivity was determined in a scintillation counter.

To determine the activity of recombinant IRTK, a GST fusion protein containing the 48-kDa intracellular domain of IR (5 μg) (24) was incubated in a buffer containing 50 mM HEPES (pH 7.5), 10 mM MgCl2, and 0.1% Triton X-100, test compounds, and ATP (0–200 μM) at 25 °C for 30 min. Biotinylated IR peptide substrate (above) was added and the reaction continued for 30 min. The reaction was then terminated, and IRTK activity was determined by measuring tyrosine phosphorylation of the IR peptide substrate using anti-phosphotyrosine antibody in a coupled fluorescence resonance energy transfer reaction according to standard methodology (25).

**SDS-PAGE and Western Blot**—CHO.IR cells were incubated with serum-free medium for 2 h and then stimulated with insulin or test compounds. The cells were lysed (20), and clarified cell lysates were fractionated on 4–12% or 4–20% SDS-PAGE. The proteins were then transferred to nitrocellulose filters which were subsequently incubated with antibodies directed against phosphotyrosine or Akt protein. After incubation with secondary antibodies conjugated to horseradish peroxidase, the signals were detected with Renaissance® ECL kit (PerkinElmer Life Sciences).

**Measurement of Liver Insulin Receptor Kinase Activity**—Male db/+ mice (7–8 weeks old) were orally treated with vehicle (0.5% methylcellulose) or single doses of test compounds followed by immediate removal of food (with free access to water). Blood glucose concentrations were monitored prior to dosing and post dosing at indicated intervals. For chronic studies, mice were treated with a single oral dose of test compounds per day, and blood glucose was measured at 24 h post last dose on the indicated days. For generation of the streptozotocin-mouse model, male lean (db+) mice (7 weeks old) were administered streptozotocin (Sigma) (180 mg/kg intraperitoneal, 0.2 ml/25 g) in distilled water. The efficacy of the streptozotocin on blood glucose levels was assessed after 5 days. Compound (10 mg/kg) or vehicle was administered by oral gavage. Insulin (Humulin R, Lilly) at the appropriate dose was administered intraperitoneal in a volume of 200 μl/mouse. Blood glucose level was monitored at indicated time points.

**Oral Glucose Tolerance Test**—Male Harlan Sprague-Dawley rats (350 g) were orally dosed with vehicle (0.5% methylcellulose) or test compound for 3 days. At days 1 and 2, blood samples were collected prior to and 6 h post-dosing via exteriorized cannula in femoral vein. At day 3, food was withheld following dosing, and blood samples were collected at 4 h post-dosing. Oral glucose challenge (1 g/kg) was then administered, and blood samples were collected at indicated time point for measurement of plasma glucose and insulin levels.

**Other Procedures**—Protein concentrations were determined using Bradford reagent (Bio-Rad) following the manufacturer's instruction. Data are expressed as means ± S.E. Statistical analysis was conducted using Student’s t test or analysis of variance.

**Results**

**Activation of Insulin Receptor Tyrosine Kinase and Signal Transduction in CHO Cells**—To identify a more potent and selective analog of L-783,281 (compound 1) (Table I) derivatives of this compound were synthesized and tested in a cell-based assay that monitors activation of IR tyrosine kinase activity in CHO cells expressing human insulin receptor (CHO.IR) (20). One of these derivatives (compound 2) increased the insulin receptor tyrosine kinase activity in these cells with an EC50 of 300 nM, reflecting a greater than 10-fold improvement in the potency compared with compound 1 (Table I). In contrast, a closely related analog, compound 3, was not effective in activating IRTK in the same assay at concentrations up to 100 μM.

**Table I**

| Compound | EC50 (μM) |
|----------|-----------|
| Compound 1 | ≤ 5 μM |
| Compound 2 | 0.3 μM |
| Compound 3 | > 100 μM |
we tested the ability of compound to activate IR in cell-free assays. Insulin receptor was partially purified from CHO(IR) cells using WGA affinity chromatography. When the partially purified IR was incubated in the presence [γ-32P]ATP and a 12-mer IR peptide substrate, insulin stimulated IR kinase activity in a dose-dependent manner as measured by increased incorporation of radiolabel into the substrate peptide. Under similar conditions compound 2, but not compound 3, produced a dose-dependent increase in IR tyrosine kinase activity (Fig. 2A). These results suggest that the activation of IR by compound 2 is due to a direct effect of the compound on the insulin receptor. To further delineate the site of interaction of this compound on the IR, we examined the effect of compound 2 on a recombinant GST fusion protein containing the cytoplasmic domain of the human IR (Gln983 to the COOH-terminal stop codon) (GST-IRK) expressed in baculovirus. Compound 2 stimulated tyrosine kinase activity of this protein as well (Fig. 2B). As predicted, compound 3 had no measurable effect in this assay.

**Correction of Hyperglycemia in db/db Mice**—The results of in vitro experiments demonstrated that compound 2 is a potent and selective insulin receptor activator, thus it was initially tested in the db/db mouse model to determine whether an in vivo glucose lowering effect might occur. Single dose oral administration of compound 2 (5 mg/kg) resulted in significant lowering of blood glucose (over 2–4 h; food withheld), achieving ~50% transient correction of hyperglycemia (percent correction of mean db/db glucose levels relative to mean glucose levels in db/+ mice) (Fig. 3). However, treatment of db/db mice with compound 3 (at 30 mg/kg) did not alter the elevated blood glucose levels. This finding is consistent with the inability of this compound to activate IR in the in vitro assays.

Having established that compound 2 can lower glucose upon acute treatment, we next determined the ability of compound 2 to correct hyperglycemia in db/db mice after long term treatment. Mice were treated with a daily dose of compound 2 or 3 for 8 days. At day 8, the extent to which compound 2 treatment resulted in correction of hyperglycemia was 35% at 1 mg/kg and 76% at 10 mg/kg, respectively. Under similar conditions compound 3 (at 10 mg/kg) was not efficacious in lowering glucose in these animals. Furthermore, similar treatment with compound 2 at 1 or 10 mg/kg had no significant effect on blood glucose levels in normal lean mice.

**Insulin-dependent Effect of Compound 2 on Hyperglycemia in Streptozotocin-induced Diabetic Mouse Model**—The results of studies using db/db mice described above indicated that compound 2 was an effective agent for reducing hyperglycemia. However, since db/db mice are obese and have sustained endogenous insulin levels, it was difficult to differentiate the insulin mimetic versus sensitizing effects of the compound in
these animals. To address this issue, we tested compound 2 in a streptozotocin-induced insulin-deficient diabetic mouse model (28). As expected, 1 week following streptozotocin treatment, the mice had blood glucose levels that were elevated from ∼100 to 300–400 mg/dl and plasma insulin levels reduced by ∼70%. We titrated insulin in streptozotocin-treated mice and estimated that the dose of insulin needed for partial correction of hyperglycemia (∼50% reduction in blood glucose at 4 h post-intraperitoneal injection) was ∼2.5 units/kg. Having established the efficacy of insulin we then used these animals to establish whether compound 2 would have an effect on blood glucose levels in the absence and presence of exogenous insulin. To do so, these animals were given two daily oral doses of vehicle or compound 2. Immediately following the second daily dose, mice were injected with saline or insulin at 2.5 units/kg. Blood glucose levels were measured prior to injection of saline or insulin (zero time) and 4 h later. The results showed that while treatment with either insulin or compound alone had no significant effect on blood glucose levels in these animals, a combination of the two agents produced a significant decrease in blood glucose (∼34%) (Fig. 4). These results suggest that compound 2 had insulin sensitizing effects in this animal model.

Improvement of Glucose Tolerance in Rat—An earlier manifestation of the onset of diabetes in man is the reduced ability to dispose of glucose after a meal. To establish if treatment with compound 2 will result in a more efficient glucose disposal from plasma, we investigated the effects of the compound on glucose tolerance in Harlan Sprague-Dawley rats. Rats were treated with single daily oral dose of vehicle or compound 2 (at 1 or 10 mg/kg) for 3 days. This treatment did not significantly change glucose levels in these animals during the first 2 days. On day 3, an oral glucose tolerance test was administered at 4 h post-dose. As shown in Fig. 5, treatment of these animals with compound 2 at 10 mg/kg (but not at 1 mg/kg) resulted in improved glucose tolerance. Moreover, treatment with the compound 2 was associated with a dose-dependent decrease in elevation of plasma insulin level following the oral glucose challenge. Taken together, these data suggest that compound 2 is able to maintain or improve glucose disposal in the presence of reduced insulin levels in this non-diabetic animal model without causing hypoglycemia.

Activation of IRTK Tyrosine Kinase Activity in Mouse Liver—To determine whether the compounds are capable of modulating activation of insulin receptors in vivo, we examined the IRTK activity in liver extracts prepared from mice. Lean normal mice (db/+ ) were treated with compound 1 (at 50 and 150 mg/kg) or vehicle for 2 h and then given an injection of saline or insulin via the tail vein prior to preparation of liver extracts. In vehicle-treated groups, a high dose of insulin (2 units/kg) induced an ∼6-fold increase in the hepatic IRTK activity. Treatment with compound 1 resulted in significant increase in basal IRTK activity (Fig. 6) to a level that was comparable with ∼30% of that stimulated by injection of moderate dose of insulin (0.4 unit/kg). Furthermore, compound 1 potentiated insulin activation of IRTK in the liver (not shown). Similar insulin sensitizing effect was also observed in mice treated with compound 2 (at 10 mg/kg). In studies with compound 2, insulin-stimulated
INSULIN RECEPTOR ACTIVATORS

This class of compounds.

In vivo IRTK means.

Results shown are means ± S.E. (n = 8–10 mice in each group). *p < 0.01 comparing vehicle- or compound 1-treated groups. Cpd, compound.

IRTK activities in treated groups were 177% and 143% of those in the vehicle groups for db/+ and db/db mice, respectively (p < 0.05, n = 8–10 in each group). In contrast, treatment with compound 3 (at 10 mg/kg) had no effect on insulin-stimulated IRTK activity. These data demonstrate the ability of compounds 1 and 2 to activate and enhance insulin activation of IRTK in vivo, which may account for the anti-diabetic effects of this class of compounds.

DISCUSSION

The pathogenesis of Type 2 diabetes is complex, involving the progressive development of insulin resistance and a relative deficiency in insulin secretion, leading to overt hyperglycemia. The molecular basis for insulin resistance in Type II diabetes is not fully understood. However, abnormalities in insulin receptor expression, structure (rarely), and function are present in association with chronic insulin resistance and diabetes. Thus, it has been described that patients with obesity and diabetes have impaired insulin receptor binding in liver, muscle, and adipose tissue (13–15, 29). The defect is exacerbated by additive defect in insulin receptor kinase that is also present in tissues of patients with overt diabetes (14, 29, 30). More recently, defects in the insulin receptor-mediated signal transduction pathway, including IRS-1 phosphorylation and PI 3-kinase activation, have been found in tissues from Type 2 diabetic patients or rodent models (16, 31, 32). Thus, augmenting insulin signaling by targeting insulin receptor activation may represent a potential approach to alleviate insulin resistance and improve glucose homeostasis in Type 2 diabetes.

We previously reported on the discovery of a small molecule fungal metabolite (compound 1) that functions as an insulin mimetic in cells and in animal models of diabetes (20). In this study, we have described active and inactive analogs of compound 1. We have shown that compound 2 is a potent and selective activator of insulin receptor. In contrast, a closely related analog (compound 3) is completely inactive, suggesting that the effect of the active compound is likely to be specific on the target receptor. Thus, compound 1 was not simply an isolated example, but it represents a lead from which the directed synthesis of new compounds with improved features can be derived.

Insulin receptor belongs to a large family of receptor tyrosine kinases. Binding of insulin to the extracellular domain of the dimeric IR leads to autophosphorylation of key tyrosine residues in the intrinsic tyrosine kinase that resides in the cytoplasmic domain. This event triggers a cascade of signal transduction steps. The mechanism for activation of insulin receptor tyrosine kinase has been a subject of intensive investigation. High resolution structural information has been obtained through crystallographic studies of IR kinase domain (33, 34). Based on crystal structures of the unphosphorylated low activity form, as well as the phosphorylated, active form of the IR, a model of cis-inhibition and trans-activation of the receptor was proposed. The unliganded receptors exist in the autoinhibitory conformation that prevents access of ATP and substrate to the active site. Upon autophosphorylation of Tyr1156, Tyr1162, and Tyr1163 in the activation loop, the IR kinase undergoes a major conformational change resulting in unrestricted access of ATP and substrate to the active site and full activation of the kinase.

More recently, the three-dimensional structure of insulin receptor bound to insulin was determined by electron cryomicroscopy (35). The three-dimensional reconstruction of the quaternary structure reveals that the both α subunits are involved in insulin binding and that the two β subunits are poised for trans-autophosphorylation. These structural studies have provided molecular basis of activation of IR.

In the current study, we demonstrated that insulin receptor activator compound 2 stimulated activation of recombinant insulin receptor kinase (Fig. 2). It is possible that interaction of the compound with the inactive receptor kinase domain alters conformation of the protein, thereby partially relieving the autoinhibition and increasing accessibility of ATP to the active site. This hypothesis is being investigated. Further structural studies will be necessary to further define the mechanism of action of the insulin receptor activators at molecular level.

Compounds 1 and 2 were capable of potentiating activation of IRTK in liver of normal mice following in vivo treatment at doses that were efficacious for hyperglycemia (in diabetic mice). Consistent with the lack of activity in cell-based and cell-free insulin receptor activation assays, compound 3 was without effect on insulin stimulation of IRTK in liver. When tested in the db/db mouse model, compound 2 elicited a glucose lowering effect at 2–4 h following single oral dose (5 mg/kg) (food withdrawn) or following chronic treatment, whereas compound 3 was not effective at an equivalent or higher dose. These results demonstrated a correlation between modulation of insulin receptor activation and glucose-lowering efficacy in the animal models and further validated the role of insulin receptor in the regulation of glucose homeostasis.

It is of interest to point out that although compound 2 (10 mg/kg) was able to potentiate activation of hepatic IR by exogenous insulin in lean euglycemia mice, it did not cause hypoglycemia in this mouse model at the dose tested. In the murine model with insulinopenic diabetes induced by streptozotocin, oral administration of compound 2 alone (10 mg/kg) was without effect on blood glucose levels. Streptozotocin-induced diabetic rodent model is characterized with in vivo insulin resistance (36, 37) and altered regulation of insulin signal transduction pathway (32, 38–40). In this study, co-administration of the compound with another subeffective dose of insulin resulted in a significant degree of glucose lowering (Fig. 4), suggesting that the compound could function as an insulin sensitizer in this animal model of diabetes. In addition, we observed that treatment with compound 2 was able to improve glucose tolerance without causing hypoglycemia in Harlan Sprague-Dawley rats.

The above results, along with the apparent lack of hypoglycemic effects of the compounds in lean non-diabetic mice, suggest that compounds 1 and 2 could potentially function as insulin sensitizers in vivo in the animal models. This notion contrasts with the ability of the compounds to function as full...
insulin-mimetics in CHO.IR cells. However, relative to the effects of exogenous insulin, the extent of in vivo IR activation by compounds administered alone was modest. Thus, we speculate that the molecular mechanism of IR activation by compounds is sufficient, only in part, to mimic that of insulin, this effect may be exaggerated in CHO.IR cells, an artificial system where >500,000 IRs per cell are expressed. In vivo, it is possible that direct interactions of compound with IRs may allow for further insulin-mediated activation (in all or selected subsets) of cell-surface receptors.

In summary, we have characterized small molecule insulin receptor activator compounds that are capable of activating the receptor kinase in a variety of in vitro assays and in tissue following in vivo treatment. The availability of the structurally related inactive analog provided an invaluable tool to establish the correlation between insulin receptor activation and glucose lowering in the animal model of diabetes. Exploratory mechanistic studies indicate that the active compounds interacted with and activated insulin receptor. In rodent models of diabetes, the active compounds had both insulin-like and insulin-sensitizing effects. These studies further validated the approach of targeting the insulin receptor for potential novel anti-diabetic agents.

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