Original Article

Oral Insulin-Mimetic Compounds That Act Independently of Insulin

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The hallmarks of insulin action are the stimulation and suppression of anabolic and catabolic responses, respectively. These responses are orchestrated by the insulin pathway and are initiated by the binding of insulin to the insulin receptor, which leads to activation of the receptor's intrinsic tyrosine kinase. Severe defects in the insulin pathway, such as in types A and B and advanced type 1 and 2 diabetes lead to severe insulin resistance, resulting in a partial or complete absence of response to exogenous insulin and other known classes of antidiabetes therapies. We have characterized a novel class of arylalkylamine vanadium salts that exert potent insulin-mimetic effects downstream of the insulin receptor in adipocytes. These compounds trigger insulin signaling, which is characterized by rapid activation of insulin receptor substrate-1, Akt, and glycogen synthase kinase-3 independent of insulin receptor phosphorylation. Administration of these compounds to animal models of diabetes lowered glycemia and normalized the plasma lipid profile. Arylalkylamine vanadium compounds also showed antidiabetic effects in severely diabetic rats with undetectable circulating insulin. These results demonstrate the feasibility of insulin-like regulation in the complete absence of insulin and downstream of the insulin receptor. This represents a novel therapeutic approach for diabetic patients with severe insulin resistance. *Diabetes* 56:486–493, 2007

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B4V10, tetraquis(benzylammonium) decavanadate; B5V10, pentaquis(benzylammonium) decavanadate; B6V10, hexaquis(benzylammonium) decavanadate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; GH, growth hormone; GSK, glycogen synthase kinase; IRS, insulin receptor substrate; SCZ, semicarbazide; SSAO, SCZ-sensitive amine oxidase; V10, sodium decavanadate salt; VAP-1, vascular adhesion protein-1.

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Activation of insulin receptors phosphorylate several immediate substrates, including insulin receptor substrate (IRS) proteins (1–5). IRS proteins, in turn, trigger the activation of downstream signaling molecules such as phosphatidylinositol 3-kinase, protein kinase B, or atypical forms of protein kinase C. A number of defects in the insulin signaling pathway have been reported in type 2 diabetic patients (6–10). Current antidiabetes therapies are generally safe and effective but suffer from certain limitations. For instance, insulin requires the action of functional insulin receptor, and noninsulin oral antidiabetes therapies additionally require minimum endogenous levels of insulin. As a result of these limitations, patients with severe insulin resistance, which includes a subset of severely advanced type 1 and 2 diabetic patients, currently lack effective treatment. This is a growing concern because the number of patients with severe insulin resistance is increasing in direct relation to the current global diabetes epidemic (11,12). The development of insulin-mimetic compounds that act independently of insulin and the insulin receptor may thus represent a novel therapeutic strategy for severe insulin-resistant patients and type 1 and 2 diabetic patients.

We have previously reported that the combination of semicarbazide (SCZ)-sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1) substrates and low doses of vanadate show antidiabetic effects in streptozotocin-induced diabetic and Goto-Kakizaki diabetic rats (13,14) and significantly enhance glucose transport in adipocytes (15,16) and in skeletal muscle (17). In addition, the combination of SSAO substrates and vanadate ameliorates insulin secretion in Goto-Kakizaki rats (13). During these studies, we noted the possibility of generating vanadium salts containing arylalkylamines that are also substrates of SSAO/VAP-1 such as benzylamine. We reasoned that administration of arylalkylamine vanadium salts may exhibit more potent effects when compared with the administration of vanadate and SSAO/VAP-1 substrates separately. This was an important consideration because the known antidiabetic effects of vanadium are detected in vivo using relatively high doses that may exhibit some degree of toxicity in humans (18–22) and in rodents (23–27). In this regard, and supporting a possible therapeutic use of vanadium, it has been recently shown that low, nontoxic doses of vanadium improve the recovery from lipopoly-

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saccharide-induced sickness in mice (28). Here, we describe a salt composed by a series of arylalkylamines combined with vanadate that permit the effective antidiabetic dose of vanadium at nontoxic levels to be lowered. This compound is orally active and efficacious under in vivo experimental conditions in which insulin is absent.

RESEARCH DESIGN AND METHODS

2-[1,2-^{3}H]-D-deoxyglucose (26 Ci/mmol) was from NEN Life Science Products, and [^{14}C]mannitol (59 Ci/mmol) was from Amersham Pharmacia Biotech. Purified porcine insulin and growth hormone (GH) were a gift from Eli Lilly (Indianapolis, IN). SCZ hydrochloride, benzylamine hydrochloride, sodium orthovanadate, wortmannin, epidermal growth factor (EGF), and other chemicals were purchased from Sigma Aldrich (St. Louis, MO). LY294002 was purchased from Calbiochem (San Diego, CA). Ketamine was obtained from Mérieux (Rhône; Mérieux, Lyon, France). Collagenase type I was obtained from Worthington (Lakewood, NJ) and collagenase from Innogenetics (Ghent, Belgium). The osmotic minipumps used in chronic studies were from Alza. All electrophoresis reagents and molecular weight markers were obtained from Bio-Rad. Enhanced chemiluminescence reagents (super signal substrate) were from Amersham (Arlington Heights, IL). Anti–phospho-tyrosine monoclonal antibody and anti–insulin receptor β-chain polyclonal antibodies were purchased from BD Biosciences. Anti-PKB, phospho-Thr308–PKB, anti–phosho-Ser473–PKB, anti–IGF-I receptor, anti-EGF receptor, anti–glycogen synthase kinase (GSK)3β, and anti–phospho-Ser9–GSK3β polyclonal antibodies were purchased from Cell Signaling (Beverly, MA). Anti-JAK2 polyclonal antibodies were from Millipore, and Amplex Red and IGF-I were from Invitrogen.

Male Wistar rats weighing 180–220 g were purchased from Harlan Interfauna Ibèrica (Barcelona, Spain). Diabetes was induced by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (in some studies the dose was 45 mg/kg body wt, and in others 100 mg/kg body wt was dissolved in 50 mmol/l citrate buffer, pH 4.5). Only diabetic animals with glycemia >300 mg/dl were used. The animals were housed in animal quarters at 22°C with a 12-h light/12-h dark cycle and were fed ad libitum. All procedures used were approved by the animal ethical committee of the University of Barcelona. Male mice C57BL/Ks bearing the *db/db* mutation (The Jackson Laboratories) were purchased from Harlan France (Gannat, France). C57BL/6J male mice were assigned for 16 weeks to very high–fat diet containing (in kcal): 72% from fat, 28% from proteins, and <1% from carbohydrates (29).

Chronic treatments of diabetic animals. Osmotic minipumps delivering hexaquis(benzylammonium) decavanadate (B6V10; 2.5 μmol · kg body wt^{-1} · day^{-1}) or decavanadate (2.5 μmol · kg body wt^{-1} · day^{-1}) were implanted subcutaneously in diabetic rats anesthetized by ketamine hydrochloride (95 mg/kg) and xylasine (10 mg/kg). Animals that did not receive B6V10 or decavanadate were sham operated. Glycemia was measured on arterio-venous blood collected from the tail vessels at 9:00 A.M., before the administration of vanadate for 2 weeks. Insulin concentrations were determined before and after treatment. In another set of experiments, B6V10 was orally administered at a single dose of 5 μmol · kg^{-1} · day^{-1} during the 1st week and 10 μmol · kg^{-1} · day^{-1} during 2 additional weeks by gastric gavage. A control group received the corresponding decavanadate salt in the absence of benzylamine. At the end of the treatment, animals were killed and tissues were kept at −80°C and the plasma at −20°C.

Metabolic measurements in isolated rat or mouse adipocytes. Adipocytes were isolated from epididymal and perirenal fat pads from Wistar rats (180–220 g) or from internal adipose tissue of male FVB mice (25–30 g). At the end of the incubation with the indicated drugs and/or hormones, 2-D-deoxyglucose uptake measurements were performed as reported (14,30). Lipolytic activity was determined by using glycerol release as an index as previously reported (31).

Cell culture. 3T3-L1 or 3T3 F442A cells were seeded at a density of 4,000 cells/cm^2 and grown at 37°C in a 7% CO_2 atmosphere. Cells were kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% donor calf serum until confluence. Confluent 3T3 F442A cells were induced to differentiate in DMEM in the presence of 5% FCS and 50 nmol/l insulin for 8 days. When differentiation was induced by insulin or other chronic treatments, fresh medium containing the tested compounds was changed daily. Postconfluent cells kept for the same period in DMEM containing 5% FCS served as control cells. Cell number was determined using a cell counter (Coulter Electronics). Differentiation of adipose cells was assessed by evaluating both cell morphology, using phase contrast microscopy, and biochemical parameters, such as triacylglycerol and protein content. For these procedures, 3T3 F442A cells were first washed twice with PBS, then scraped, resuspended in 50 mmol/l Tris/HCl (pH 7.5)/1 mmol/l EDTA buffer, and homogenized. Triacylglycerol content was quantified by a colorimetric reaction using the Triglyceride Enzymatic Trinder kit (Biotrol Diagnostic, Chennevieres, France). Protein content was determined using the Bio-Rad DC Protein Assay kit.

Measurements in incubated soleus muscle. Isolated strips of soleus muscles were incubated, and 2-D-deoxyglucose uptake measurements were performed as reported previously (13). Muscle homogenates were obtained from strips of rat soleus. Muscles were frozen, collected into an Eppendorf, placed on ice containing 200 μl fresh lysis buffer, and then homogenized with an Eppendorf homogenizer. Homogenates were taken to a final volume of 1 ml/strip by adding lysis buffer and then centrifuged at 15,000 rpm for 10 min at 4°C. The pellet was discarded, and supernatant was collected and stored at −80°C until use. Protein was measured by the Bradford method.

Measurements of insulin secretion. The experiments were performed with islets isolated by collagenase digestion followed by hand picking of the pancreas from C57BL/6J male mice (fed a very high–fat diet for 16 weeks) killed by decapitation. The pancreata were removed and distended with bicarbonate-buffered physiological salt solution. The medium used for islet isolation was a bicarbonate-buffered solution containing 120 mmol/l NaCl, 4.8 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 5 mmol/l HEPES, and 24 mmol/l $NaHCO_3$. It was gassed with O_2-CO_2 (94:6) to maintain a pH of 7.4 and supplemented with 1 mg/ml BSA (Boehringer Mannhein, Germany) and 10 mmol/l glucose. After isolation, the islets were preincubated at 37°C for 90 min in a medium containing 15 mmol/l glucose before being distributed into batches of three. Each batch of islets was then incubated for 60 min in 1 ml medium containing 5.5 mmol/l or 16.7 mmol/l glucose and appropriate concentrations of the test substances. At the end of the incubation, a portion of the medium was withdrawn and diluted before insulin assay measurement was performed by double-antibody radioimmunoassay (Schering España, Madrid, Spain).

Amine oxidase activity assays. The continuous fluorimetric detection of SSAO-dependent H_2O_2 production based on a peroxidase-coupled reaction was performed as previously described (32) and following the procedure described by Salmi et al. (33).

Analytical methods. In glucose tolerance tests and in chronic treatments, the circulating glucose concentration was determined by a rapid glucose analyzer (Accutrend Sensor Comfort; Roche). Plasma insulin concentration was determined by enzyme-linked immunosorbent assay method (Crystal Chem, Downers Grove, IL). Plasma triglycerides and nonesterified fatty acids were determined with standard colorimetric methods (Biosystems, Barcelona, Spain, and Wako Chemicals, Neuss, Germany, respectively).

Analysis of intracellular signaling. Isolated fat cells were disrupted for total membrane preparation by hypo-osmotic lysis in a 20 mmol/l HES buffer and an antiprotease and antiphosphatase cocktail as reported previously (13). Protein concentrations were determined by the BCA method (34) with BSA as a standard. Immunoprecipitation and immunoblot assays were performed as previously described (17) with the use of a monoclonal antiphosphotyrosine antibody for the immunoprecipitation and an anti-insulin receptor antibody for immunoblotting, respectively. SDS-PAGE was performed on membrane proteins following the method of Laemmli. Proteins were transferred to Immobilon, and immunoblotting was performed as reported (35).

Oxidative stress analysis. Antioxidant defense status and oxidative stress markers were analyzed in tissue homogenates: antioxidant enzyme activities (catalase, superoxide dismutase, glutathione peroxidase, and reductase) by spectrophotometric procedures (36); antioxidant vitamins and substrates (coenzyme Q9, coenzyme Q10, tocopherol, and total glutathione) and free radical damage markers (malondialdehide evaluated as thiobarbituric reaction and percentage of oxidized coenzyme Q9 and Q10) by high-performance liquid chromatography with electrochemical detection and spectrophotometry (36) or electrochemical detection (37).

RESULTS

We have previously reported that the combination of SSAO/VAP-1 substrates and low doses of vanadate show antidiabetic effects in streptozotocin-induced diabetic and Goto-Kakizaki diabetic rats (13,14). During these studies, we noted the possibility of generating vanadium salts containing arylalkylamines that are also substrates of SSAO/VAP-1, which may offer some pharmacological advantages compared with the administration of vanadate and SSAO/VAP-1 substrates separately. Based on this, we optimized the synthesis of salts composed by a series of arylalkylamines combined with vanadate. The preparations of arylalkylamine vanadium salts and compounds

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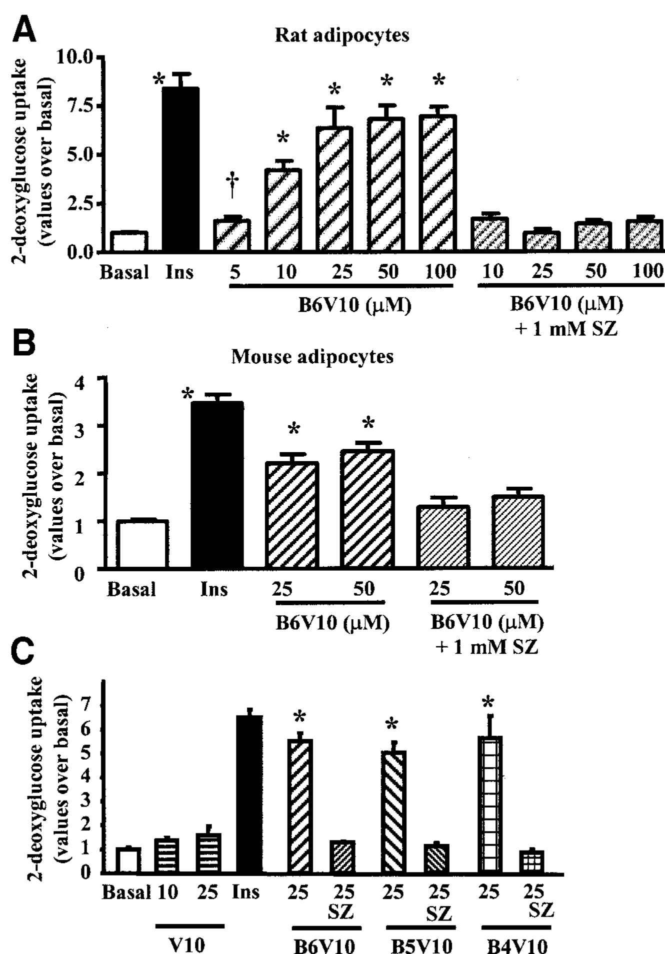

FIG. 1. Stimulatory effect of B6V10, B5V10, and B4V10 on glucose transport in adipose cells. *A*: Adipose cells from Wistar rats were incubated for 45 min in basal conditions (Basal) or in the presence of 100 nmol/l insulin (Ins) and different concentrations of B6V10 in the absence or in the presence of 1 mmol/l SCZ. Subsequently, 2-DG transport was measured over 5 min. Values are means ± SE of four to five observations per group. †A significant stimulation of 2-DG uptake compared with basal transport value at $P < 0.05$. *A significant stimulation of 2-DG uptake compared with basal transport value at $P < 0.001$. *B*: Adipose cells from FVB mice were incubated for 45 min in basal conditions (Basal) or with 100 nmol/l insulin (Ins) and different concentrations of B6V10 in the absence or in the presence of 1 mmol/l SCZ. Values are means ± SE of four to five observations per group. *A significant stimulation of 2-DG uptake compared with basal transport value at $P < 0.001$. *C*: Adipose cells from Wistar rats were incubated for 45 min in basal conditions (Basal) or with 100 nmol/l insulin (Ins) and different concentrations (μmol/l) of decavanadate (V10), B6V10, B5V10, or B4V10 in the absence or in the presence of 1 mmol/l SCZ. Values are means ± SE of four to five observations per group. *A significant stimulation of 2-DG uptake compared with basal transport value at $P < 0.001$.

were extensively characterized in solution and in the solid state by infrared spectrum, ^{51}V-NMR, ^{1}H/^{13}C-NMR, elemental analysis, and X-ray diffraction analysis (F.Y., L.M., S.G.-V., F.A., A.Z., M.R., unpublished data). Three different salts were prepared starting from benzylamine and vanadate: B6V10 [$(C_7H_{10}N)_6V_{10}O_{28}0.2H_2O$], pentaquis (benzylammonium) decavanadate [$(C_7H_{10}N)_5HV_{10}O_{28}$; B5V10], and tetraquis(benzylammonium) decavanadate [$(C_7H_{10}N)_4H_2V_{10}O_{28}$; B4V10]. B6V10 stimulated glucose transport in rat adipocytes in a concentration-dependent manner (Fig. 1*A*) with maximal effect at 85% relative to maximal stimulation caused by insulin. Interestingly, 25 μmol/l B6V10 showed a greater stimulation of glucose transport than the combination of 150 μmol/l benzylamine and 250 μmol/l vanadate (data not shown). The stimulatory effect of B6V10 was completely blocked by SCZ, which indicates that SSAO activity is required (Fig. 1*A*). In contrast, sodium decavanadate salt (V10) alone at concentrations ranging from 5 to 50 μmol/l did not stimulate glucose transport (data not shown; Fig. 1*C*). Similar stimulatory effects of B6V10 were detected in isolated mouse adipocytes (Fig. 1*B*). B6V10, B5V10, and B4V10 showed a similar potency as activators of glucose transport activity in isolated rat adipocytes (Fig. 1*C*), indicating that a lower benzylamine/vanadium ratio does not alter the insulin-mimicking abilities of these compounds. B6V10 also induced adipogenesis in 3T3-F442A cells cultured for 8 days in the absence of insulin as assessed by an increase in triglyceride concentration (1 μmol/l B6V10 induced an increase in cellular triglyceride, which was 43% of values obtained when cells were differentiated with 50 nmol/l insulin) (data not shown).

In silico modeling studies (32) of SSAO binding allowed us to determine the basic structural requirements of potential substrates. Commercial compounds that met these basic structural requirements and novel compounds obtained through combinatorial chemistry were included in a library that was subsequently screened for attractive SSAO substrates. Screening yielded four novel and high-affinity SSAO substrates: 2-(4-fluorophenyl)ethylamine (compound A), 3-phenylpropylamine (compound B), 4-fluorobenzylamine (compound C), and 4-phenylbutylamine (compound D) (Fig. 2*A*). Some of these compounds, in fact, showed high V_{max} values and high affinity for rat SSAO compared with benzylamine (Fig. 2*B*). Salts of these compounds and vanadium were prepared and characterized, and their insulin-mimicking activity was assayed in isolated rat adipocytes (Fig. 2*C*). All four compounds markedly stimulated glucose transport of rat adipocytes (Fig. 2*C*), and the maximal stimulatory effect was similar for the vanadium salts generated with compounds C and D. These studies indicate that arylalkylamine vanadium salts represent a novel class of insulin-mimetic agents, which can be generated from different amines compounds and vanadate.

We have also investigated the mechanism of action of B6V10 in isolated rat adipocytes. To this end, we have analyzed the tyrosine phosphorylation of insulin receptors, IRS-1, and the phosphorylation of protein kinase B and GSK-3. The results indicate that B6V10 rapidly stimulated (at 5 min of its addition) the phosphorylation of IRS-1 (19% of the effect of insulin), protein kinase B (33% of the effect of insulin), and GSK-3 (49% of the effect of insulin) in the absence of insulin receptor phosphorylation (Fig. 3). The phosphorylation of IRS-1, protein kinase B, and GSK-3, induced by B6V10, was parallel to activation of glucose transport (Fig. 3). At later times of incubation with B6V10 (15 min), the phosphorylation of IRS-1, Akt, and GSK-3 was maintained, and this was concomitant to a transient phosphorylation of insulin receptors (9% of the effect of insulin). The results indicate that B6V10 initially stimulates insulin signaling downstream of insulin receptor in isolated rat adipocytes, and this is followed by a transient stimulation of insulin receptors. The activation of IRS-1, protein kinase B, or GSK-3 induced by B6V10 was blocked by SCZ, and it was not observed with decavanadate alone (Fig. 3). In addition, inhibitors of phosphatidylinositol 3-kinase, wortmannin, and LY294002 blocked B6V10-induced glucose transport (data not shown). We have analyzed the specificity of the effects of arylalky-

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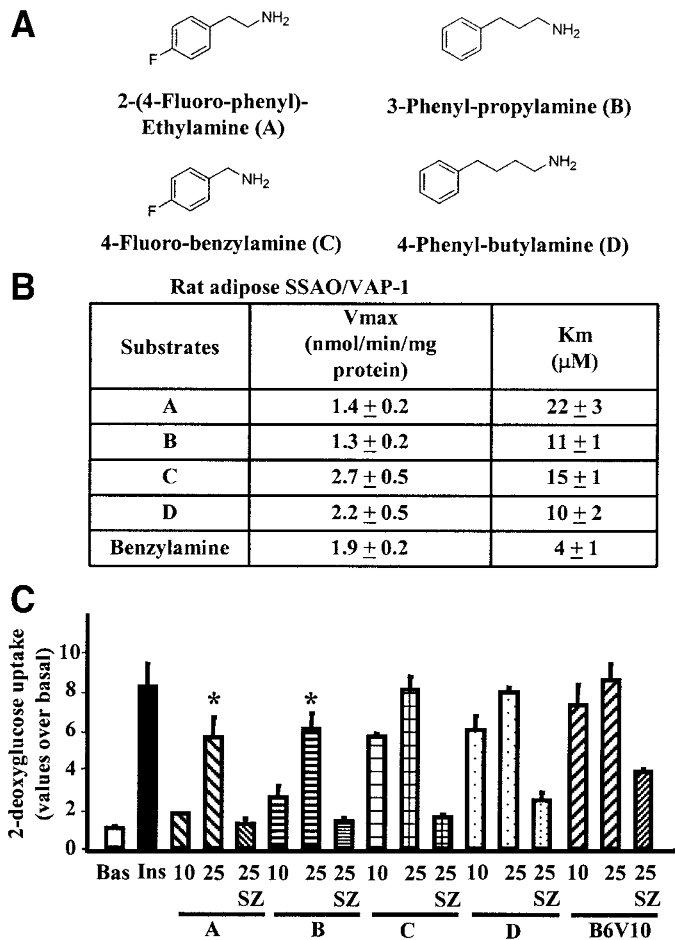

Substrates	Vmax (nmol/min/mg protein)	Km (μM)
A	1.4 ± 0.2	22 ± 3
B	1.3 ± 0.2	11 ± 1
C	2.7 ± 0.5	15 ± 1
D	2.2 ± 0.5	10 ± 2
Benzylamine	1.9 ± 0.2	4 ± 1

FIG. 2. Structures (*A*) and kinetic properties (*B*) of novel SSAO substrates and effects of their vanadium salts on glucose transport (*C*) by isolated rat adipocytes. Adipose cells from Wistar rats were incubated for 45 min in basal conditions (Basal) or with 100 nmol/l insulin (Ins) and different concentrations (μmol/l) of vanadium salts of 2-(4-fluoro-phenyl)-ethylamine (compound A), 3-phenyl-propylamine (compound B), 4-fluoro-benzylamine (compound C), and 4-phenyl-butylamine (compound D) in the absence or in the presence of 1 mmol/l SCZ (SZ). Values are means ± SE of four to five observations per group. *A significant 2-DG uptake in groups incubated in the presence of 25 μmol/l compounds compared with 25 μmol/l B6V10-stimulated transport values at $P < 0.05$.

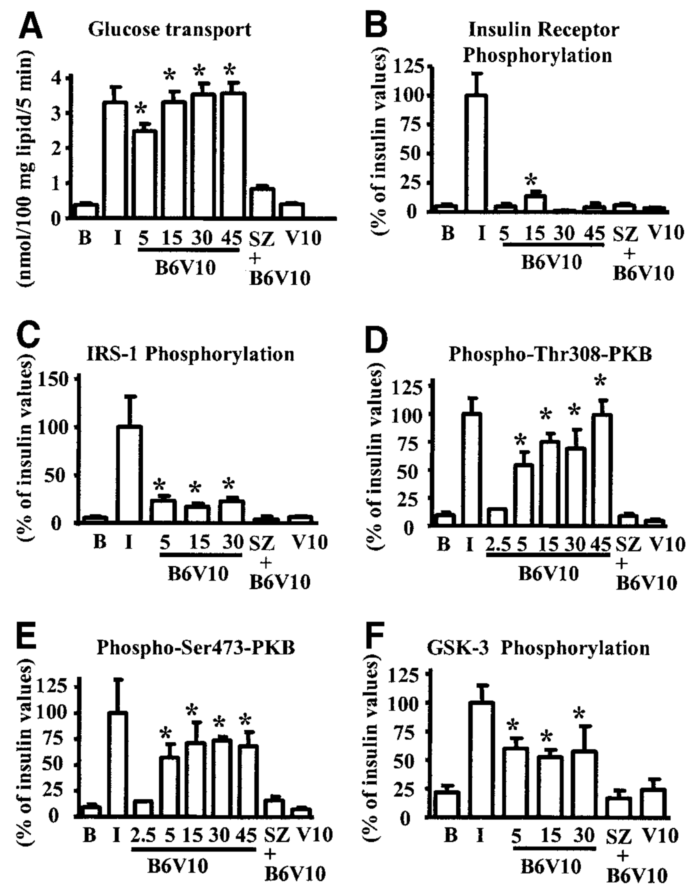

FIG. 3. Intracellular signaling pathway activated by B6V10 in adipose cells. *A–D*: Adipose cells from Wistar rats were incubated for different times in the presence of 25 μmol/l B6V10. Cells were also incubated in the presence of insulin (100 nmol/l, 45 min), decavanadate (25 μmol/l, 45 min), or SCZ (1 mmol/l, 45 min). Subsequently, 2-deoxyglucose uptake (*A*), tyrosine phosphorylation of insulin receptor (*B*), tyrosine phosphorylation of IRS-1 (*C*), phosho-Thr308–protein kinase B (*D*), phospho-Ser473–protein kinase B (*E*), and phosphorylated GSK-2 (*F*) were measured. Values are means ± SE of four to five observations per group. *A significant stimulation by B6V10 compared with basal values at $P < 0.05$.

lamine vanadium salts in 3T3-L1 cells by studying whether these compounds affect the GH signaling pathway (phosphorylation of JAK2) or whether they phosphorylate IGF-I or EGF receptors. Incubation in the presence of B6V10 for 5 or 15 min did not cause any stimulatory effect on the phosphorylation of JAK2, IGF-I receptor, or EGF receptor under conditions in which GH, IGF-I, or EGF caused stimulation of their respective signaling pathways (data not shown). Under these conditions, B6V10 for 5 min caused stimulation of Akt and GSK-3 (data not shown).

Next, we analyzed the effects of B6V10 in skeletal muscle glucose transport and insulin signaling in preparations of incubated soleus muscles. To this end, soleus muscles were incubated with or without insulin (100 nmol/l), decavanadate, or B6V10 in the absence or in the presence of membranes from rat adipocytes as a source of SSAO. The results indicate that B6V10 but not decavanadate stimulated glucose transport by muscle only in the presence of adipocyte membranes (Fig. 4). The stimulation was blocked by the inhibitor SCZ. Under these conditions, B6V10 caused a limited stimulation of insulin receptor (<25% of the effect of insulin) and more substantial stimulation of IRS-1 phosphorylation (40% of the effect of insulin) or Akt phosphorylation (80% of the effect of insulin) (Fig. 4). These stimulatory effects depended upon the presence of adipocyte membranes, they were blocked by SCZ, and they were not shown by decavanadate (Fig. 4).

To determine whether B6V10 shows in vivo metabolic effects, we examined whether its acute administration improved glucose tolerance in conscious mice. An intraperitoneal injection of B6V10 15 min before a glucose load reduced the hyperglycemic response in normoglycemic C57BL/6J mice (data not shown). Similar glucose tolerance tests were performed 15 min after B6V10 injection in mice made glucose intolerant by a very high–fat diet (29) (Fig. 5). Again, the acute B6V10 administration enhanced glucose tolerance in these mildly obese and diabetic mice (Fig. 5*A*). Prior administration of B6V10 ameliorated the profile of plasma insulin during the hyperglycemic response, which was characterized by reduced basal values and increased insulin after a glucose load (Fig. 5*B*). Glucose-stimulated insulin secretion in isolated pancreatic islets from very high–fat diet C57BL/6J mice was not

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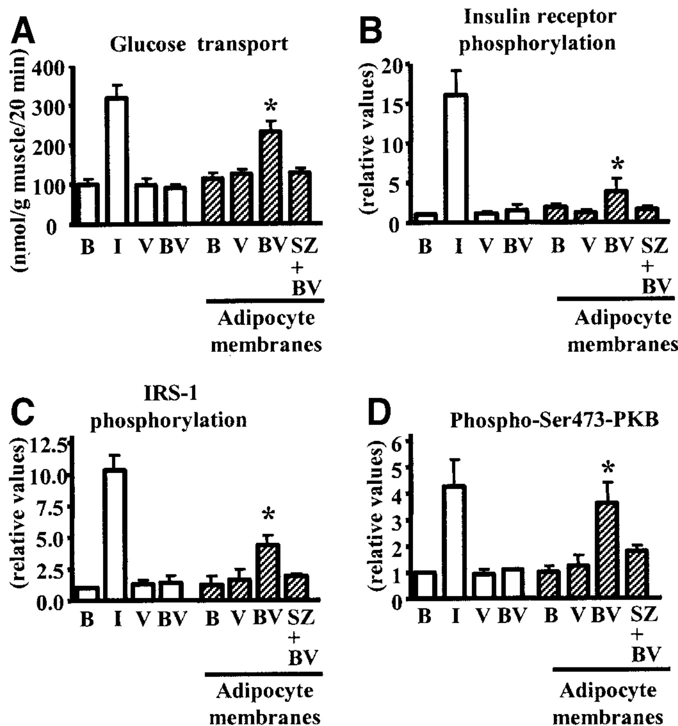

FIG. 4. Glucose transport and insulin signaling pathway activated by B6V10 in soleus muscle. Soleus muscles were incubated for 30 min in the absence (*B*) or in the presence of different combinations of 100 nmol/l insulin (I), 10 μmol/l B6V10 (BV), 100 mmol/l decavanadate (V10), or 10 μmol/l B6V10 and 1 mmol/l (BV + SCZ) and 100 μg of total membranes obtained from adipose tissue. After 2-deoxyglucose uptake, muscles were digested, and radioactivity was measured (*A*). Homogenates were also obtained, and 200 μg of total homogenate was loaded on SDS-PAGE for Western blot assays using anti-PKB, anti–phospho-Ser^{473}–PKB (*E*), anti–phospho-Thr^{308}–PKB (*D*), and anti–phospho-GSK-3 (*F*) antibodies. Homogenates were also immunoprecipitated with anti-phosphotyrosine antibodies to detect phosphorylated insulin receptors (*B*) or IRS-1 (*C*). Autoradiograms were densitometrically quantified, and results are shown as the means ± SE obtained from five different samples. *$P < 0.001$ vs. basal group.

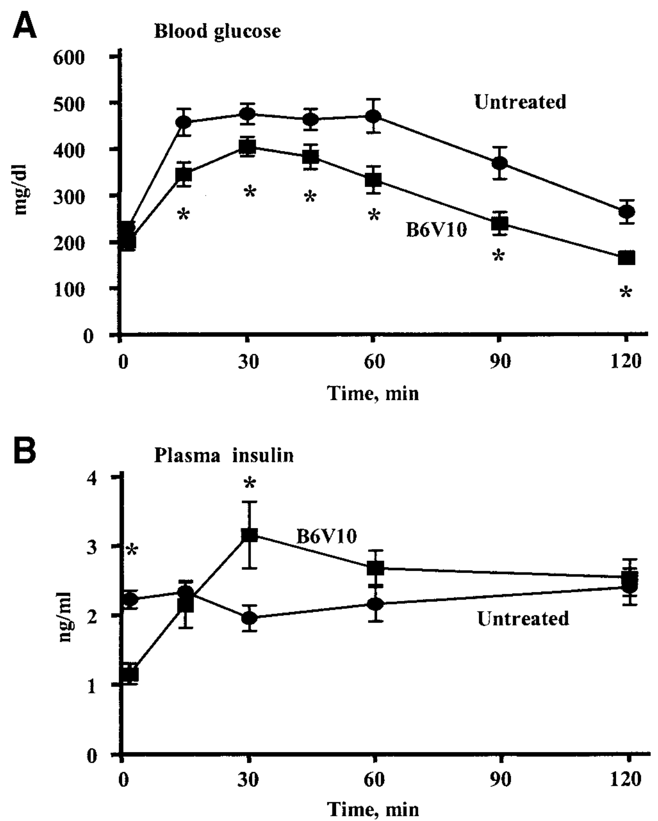

FIG. 5. Single injection of B6V10 ameliorates glucose tolerance and plasma insulin in high-fat–fed mice. Blood glucose (*A*) and plasma insulin (*B*) concentrations after an intraperitoneal glucose load (1 g/kg) starting 15 min after intraperitoneal injection of vehicle (solid circles, untreated) or 7 μmol/kg of B6V10 (solid squares) in C57BL/6J high-fat–fed mice. Mice were fasted for 5 h before the start of the test. Values are means ± SE of seven mice per group. *A significant difference compared with the vehicle-treated group, at $P < 0.05$.

stimulated by the presence of 15 μmol/l B6V10 in the incubation medium (data not shown).

We also examined the chronic in vivo efficacy of B6V10 in streptozotocin-induced diabetic rats and in *db/db* mice. Chronic subcutaneous administration of B6V10 for 12 days resulted in significant correction of hyperglycemia in streptozotocin-induced diabetic rats (Fig. 6*A*). Daily oral administration of B6V10 for 17 days also resulted in significant correction of hyperglycemia in diabetic rats (Fig. 6*B*). Treatment with identical doses of decavanadate did not alter glycemia in streptozotocin-induced diabetic rats (Fig. 6*A* and *B*). Intraperitoneal treatment with B6V10 also reduced glycemia in *db/db* mice (Fig. 6*C*), indicating that this compound was also effective in type 2 diabetic animal models.

We have also analyzed whether B6V10 treatment may induce oxidative stress in diabetic rats. Chronic subcutaneous administration of B6V10 did not alter concentrations of glutathione, malondialdehyde, vitamin E, coenzyme Q9, and coenzyme Q10 or activities of glutathione peroxidase, glutathione reductase, or superoxide dismutase in liver, lung, or heart (Table 1; data not shown); under these conditions, only hepatic catalase activity was moderately reduced by B6V10 treatment (32%). Oral administration of B6V10 caused no changes in hepatic levels of antioxidant metabolites and enzymes or free radical damage markers (data not shown) or in plasma amylase, alanine aminotransferase, or urea (online appendix supplementary table 1 [available at http://dx.doi.org/10.2337/db06-0269]), suggesting the absence of hepatic, pancreatic, or renal toxicity.

We next tested whether B6V10 has antidiabetic effects in the complete absence of insulin. To this end, rats were made diabetic by the injection of a large dose of streptozotocin (100 mg/kg) that eliminates pancreatic β-cells (38). These rats showed undetectable levels of insulin in plasma (Fig. 7*B*). Diabetic rats responded to subcutaneous treatment with B6V10 by reducing glycemia (Fig. 7*A*). Treatment with decavanadate did not show any change in circulating glucose (data not shown). Chronic treatment with therapeutic doses of B6V10 did not affect body weight and spared epididymal and perirenal adipose tissue weight (data not shown). Under these conditions, we also measured the concentration of circulating lipids. Plasma free fatty acid and triglyceride concentrations were normalized in diabetic rats treated with B6V10 but not with decavanadate alone (Fig. 7*B*). In keeping with these observations, B6V10 inhibited the lipolytic effect of isoprenaline in isolated adipocytes (data not shown).

DISCUSSION

Arylalkylamine vanadium salts remarkably mimic the in vivo function of insulin. Neither administration of the

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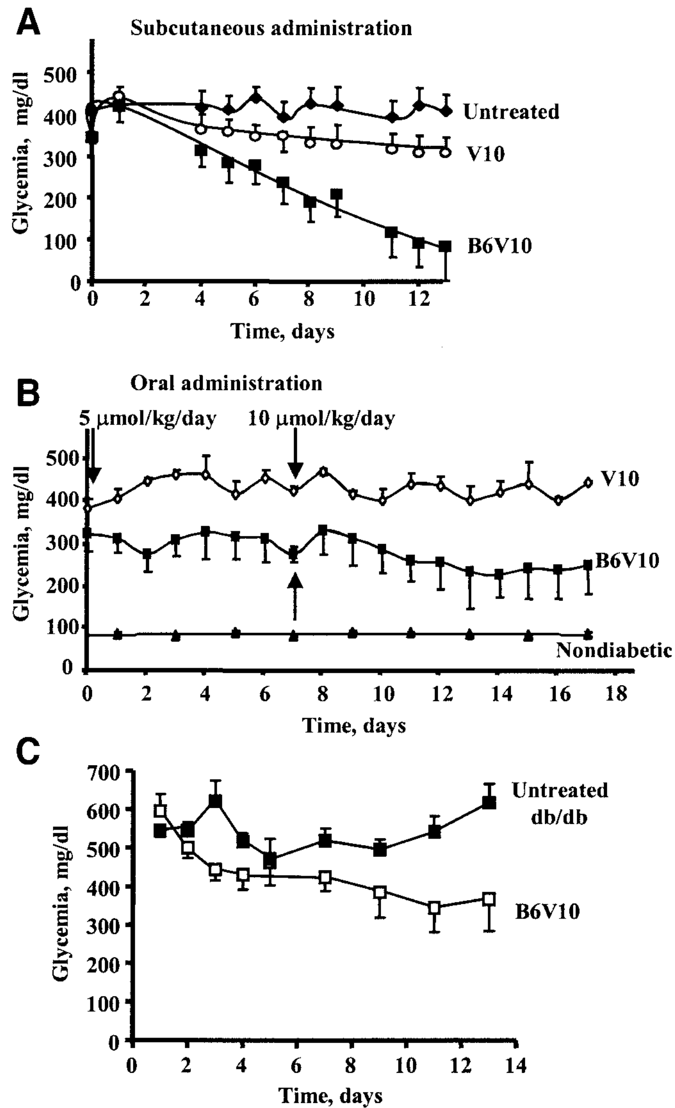

FIG. 6. Antidiabetic efficacy of administered B6V10 in rat or mouse models of diabetes. *A*: Streptozotocin-induced (45 mg/kg) diabetic rats were subcutaneously treated with B6V10 (2.5 μmol/kg) (B6V10, solid squares) or with decavanadate (2.5 μmol/kg) (V10, open circles) delivered subcutaneously by osmotic minipumps. Diabetic rats were also sham operated (untreated, solid diamonds). Values are means ± SE of six to seven observations. Two-way ANOVA indicated the existence of significant differences between the B6V10 and the untreated or V10 groups, at $P < 0.01$. Post hoc tests indicated significant differences in the B6V10 group compared with the untreated group after day 8 of treatment, at $P < 0.01$. *B*: Streptozotocin-induced (45 mg/kg) diabetic rats were orally treated with B6V10 (5 μmol/kg from day 0 to 7 and 10 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ from day 7 to 17) (B6V10, solid squares) or received decavanadate (10 μmol/kg) (V10, open circles). Nondiabetic rats were also untreated (solid triangles). Values are means ± SE of six to seven observations per group. Two-way ANOVA indicated the existence of significant differences between the B6V10 and the V10 groups, at $P < 0.01$. *C*: Thirteen-week-old diabetic *db/db* mice were intraperitoneally injected with B6V10 (7 $\mu mol \cdot kg^{-1} \cdot day^{-1}$) (B6V10, open squares) or with saline (Untreated, solid squares). Values are means ± SE of six to seven observations per group. Two-way ANOVA indicated the existence of significant differences between B6V10 and untreated groups at $P < 0.006$. Post hoc tests indicated significant differences between the two groups at days 11 and 13 at $P < 0.05$.

vanadium or SSAO substrate part of the salt alone produced notable effects. These compounds are likely to act as pro-drugs that require the generation of hydrogen peroxide from the arylalkylamine moiety (substrates of SSAO/VAP-1), and in this regard, we have determined that

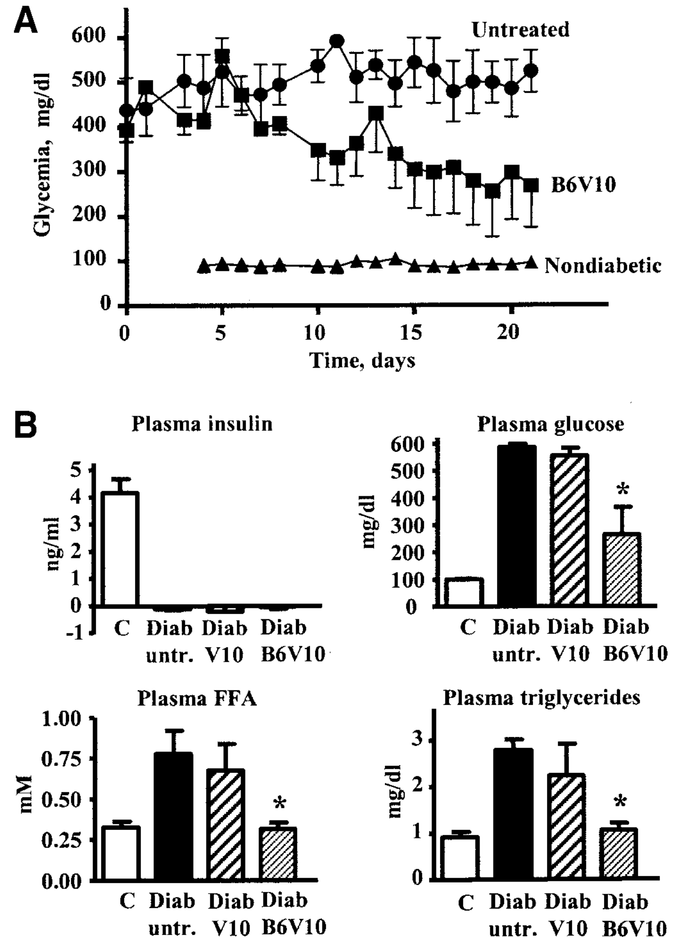

FIG. 7. Antidiabetic and antidyslipidemic efficacy of administered B6V10 in streptozotocin-induced diabetic rat with undetectable circulating insulin. *A*: Streptozotocin-induced (100 mg/kg) diabetic rats were subcutaneously treated with B6V10 (2.5 μmol/kg) (solid squares) delivered by osmotic minipumps or left untreated (solid circles). Sham-operated nondiabetic rats were also untreated (solid triangles). Values are means ± SE of six to seven observations. Two-way ANOVA indicated the existence of significant differences between the B6V10 and the untreated groups, at $P < 0.01$. *B*: After 28 days of treatment of diabetic rats with B6V10 (2.5 μmol/kg) (wide striped bars), with decavanadate (close striped bars) delivered by osmotic minipumps, in untreated diabetic (solid bars) or nondiabetic rats (open bars), plasma insulin, glucose, free fatty acids, and triglycerides were measured. Values are means ± SE of six to seven observations. *Significantly different from untreated diabetic group, at $P < 0.05$.

inhibition of SSAO activity blocks the signaling events and all the biological effects in adipose cells. Previously, we reported that the combination of benzylamine and vanadate generates peroxovanadium compounds (13), which inhibits protein tyrosine phosphatase in adipose cells (17,39). Here, we have found that arylalkylamine vanadium salts activate the intracellular insulin signaling pathway downstream of the insulin receptor in adipose cells. Thus, the intracellular activation followed by these compounds in adipocytes was characterized by rapid phosphorylation of IRS-1, protein kinase B (in both Thr308 and Ser473 residues), and GSK-3, which occurred in the absence of activation of insulin receptors. At later times, the activation of IRS-1, protein kinase B, and GSK-3 was maintained and a certain activation of the insulin receptor was detectable. Based on these observations, we propose that arylalkylamine vanadium salts trigger a number of signaling events secondary to peroxovanadate formation. These events probably include initial inhibition of protein

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TABLE 1
Hepatic concentrations of parameters related to oxidative stress in diabetic rats treated subcutaneously with B6V10

	Nondiabetic	Untreated diabetic	Vanadate-treated diabetic	B6V10-treated diabetic
Glutathione (nmol/mg protein)	39.7 ± 3.0	34.1 ± 2.8	44.8 ± 3.8	42.6 ± 4.8 (NS)
Superoxide dismutase (mU/mg protein)	7,729 ± 236	7,936 ± 494	7,697 ± 333	7,649 ± 412 (NS)
Catalase (mU/mg protein)	3,622 ± 213	2,784 ± 100	2,944 ± 181	1,897 ± 205*†
Glutathione peroxidase (mU/mg protein)	453 ± 25	534 ± 33	559 ± 32	593 ± 51 (NS)
Glutathione reductase	103 ± 2	100 ± 3	97 ± 2	88 ± 4
(mU/mg protein)	295 ± 27	314 ± 20	320 ± 20	393 ± 38 (NS)
Malondialdehyde (nmol/mg protein)				
Vitamin E (μmol/mg protein)	46 ± 5	62 ± 4	54 ± 6	49 ± 2 (NS)
CoQ9 (nmol/mg protein)	0.63 ± 0.04	0.66 ± 0.05	0.65 ± 0.06	0.71 ± 0.04 (NS)
Reduced CoQ9 (%)	60 ± 2	56 ± 4	53 ± 4	49 ± 2 (NS)
CoQ10 (nmol/mg protein)	0.19 ± 0.02	0.25 ± 0.03	0.22 ± 0.02	0.30 ± 0.03 (NS)
Reduced CoQ10 (%)	88 ± 1	89 ± 2	90 ± 2	91 ± 2 (NS)

Data are means ± SE of six to seven observations. Streptozotocin-induced (45 mg/kg) diabetic rats were subcutaneously treated with B6V10 (2.5 μmol/kg), with V10 (2.5 μmol/kg) delivered by osmotic minipumps, or left untreated. Sham-operated nondiabetic rats were also untreated. After 14 days of treatment, livers were collected and processed for antioxidant status measurements. The data from B6V10-treated groups were analyzed statistically as follows. *Significantly different from untreated diabetic rats ($P < 0.05$). †Significantly different from vanadate-treated diabetic rats.

tyrosine phosphatase activity mediated by peroxovanadium compounds, followed by activation of IRS-1, phosphatidylinositol 3-kinase, protein kinase B/Akt, and GSK-3, which occurs in the absence of activation of insulin receptor tyrosine kinase. Our data also indicate that arylalkylamine vanadium salts stimulate glucose transport and insulin signaling in skeletal muscle, which requires an exogenous SSAO activity.

Based on previous reports in which animals were treated with the combination of benzylamine and vanadate, we propose that arylalkylamine vanadium salts generate locally peroxovanadate in adipose tissue and perhaps in other sites characterized by high SSAO/VAP-1 activity such as endothelial cells. As a consequence, arylalkylamine vanadium salts have a profound activity in different tissues of relevance for metabolic homeostasis. Thus, arylalkylamine vanadium salts have a marked insulin-mimetic activity in adipose tissue where they activate glucose transport and inhibit lipolysis, and they also have the capacity to promote adipogenesis. Skeletal muscle also responds to arylalkylamine vanadium salts by acutely enhancing glucose uptake and insulin signaling, which requires an intact SSAO activity. As a consequence of the effects in adipose tissue and in muscle, the in vivo administration of arylalkylamine vanadium salts causes a potent insulin-mimicking activity in animal models of diabetes. The components of arylalkylamine vanadium salts also enhance insulin secretion in pancreatic islets obtained from Goto-Kakizaki diabetic rats, indicating the potential capacity of peroxovanadate generated by arylalkylamine vanadium salts to enhance insulin secretion by pancreatic β-cells (13). In this regard, we have found in this report that acute administration of B6V10 ameliorated the pattern of plasma insulin levels in mice made glucose intolerant by a high-fat diet. The precise mechanisms responsible for the improved response to glucose are unclear, and insulin secretion studies performed in isolated islets did not show any effects of arylalkylamine vanadium salts.

In vivo treatment with arylalkylamine vanadium salts also cause a marked insulin-mimetic action in animal models of diabetes. Thus, chronic oral or subcutaneous treatment with B6V10 ameliorated hyperglycemia in rats made diabetic by streptozotocin administration, in obese diabetic *db/db* mice, or in diabetic rats with undetectable circulating insulin after administration of a very large dose of streptozotocin. B6V10 did not cause hypoglycemia in animals, and at the doses used, it did not show any short-term toxicological side effects. It must be mentioned that the effective dose of B6V10 as an antidiabetes agent represents a very low dose of vanadium, which is below the "no-observed-adverse-effects level" dose of vanadium in rodents (40). This is in contrast to previous reports using high doses of vanadium as an antidiabetes agent, which is thought to work through a different set of mechanisms than described in this report. Because, arylalkylamine vanadium salts stimulate insulin signaling at a step downstream of the insulin receptor, we propose that development of these compounds may be appropriate for not only type 1 and 2 diabetes but particularly for the treatment of conditions characterized by the lack of insulin receptor activity such as in patients affected by type A or type B insulin-resistance syndromes or in other types of severe insulin resistance that are refractory to treatment with insulin sensitizers and in which the only effective therapy is the use of large doses of insulin (12,41).

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