

Semicarbazide-Sensitive Amine Oxidase/Vascular Adhesion Protein-1 Activity Exerts an Antidiabetic Action in Goto-Kakizaki Rats

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In this study we have explored whether the bifunctional protein semicarbazide-sensitive amine oxidase (SSAO)/vascular adhesion protein-1 (VAP-1) represents a novel target for type 2 diabetes. To this end, Goto-Kakizaki (GK) diabetic rats were treated with the SSAO substrate benzylamine and with low ineffective doses of vanadate previously shown to have antidiabetic effects in streptozotocin-induced diabetic rats. The administration of benzylamine in combination with vanadate in type 2 diabetic rats acutely stimulated glucose tolerance, and the chronic treatment normalized hyperglycemia, stimulated glucose transport in adipocytes, and reversed muscle insulin resistance. Acute *in vivo* administration of benzylamine and vanadate stimulated skeletal muscle glucose transport, an effect that was also observed in incubated muscle preparations coincubated with adipose tissue explants or with human recombinant SSAO. Acute administration of benzylamine/vanadate also ameliorated insulin secretion in diabetic GK rats, and this effect was also observed in incubated pancreatic islets. In keeping with these observations, we also demonstrate that pancreatic islets express SSAO/VAP-1. As far as mechanisms of action, we have found that benzylamine/vanadate causes enhanced tyrosine phosphorylation of proteins and reduced protein tyrosine phosphatase activity in adipocytes. In addition, incubation of human recombinant SSAO, benzylamine, and vanadate generates peroxovanadium compounds *in vitro*. Based on these data, we propose that benzylamine/vanadate administration generates peroxovanadium locally in pancreatic islets, which stimulates insulin secretion and also produces peroxovanadium in adipose tissue, activating glucose metabolism in adipocytes and in neighboring muscle. This opens the possibility of using the SSAO/VAP-1 activity as a local

generator of protein tyrosine phosphatase inhibitors in antidiabetic therapy. *Diabetes* 52:1004–1013, 2003

The semicarbazide-sensitive amine oxidase (SSAO)/vascular adhesion protein-1 (VAP-1) is a bifunctional membrane protein. On one hand, SSAO/VAP-1 is a copper-containing ectoenzyme with amine oxidase activity that is inhibited by carbonyl-reactive compounds like semicarbazide (1). On the other hand, SSAO/VAP-1 is an inflammation-inducible endothelial molecule involved in leukocyte subtype-specific rolling under physiological shear (2–4).

SSAO/VAP-1 is expressed in a variety of tissues, and under normal conditions expression is high in adipose cells. In isolated rat adipocytes, SSAO/VAP-1 is mainly at the plasma membrane, and nearly 17×10^6 copies of this protein are present at the cell surface in a single adipocyte (5,6). In contrast, SSAO activity is very low or absent in other insulin-responsive tissues such as skeletal muscle or heart (7). In fact, it has been demonstrated that SSAO/VAP-1 is not expressed in 3T3-L1 fibroblasts and that SSAO/VAP-1 gene expression is induced during adipogenesis (8). This finding is in complete agreement with the previous observation of an increase in the SSAO activity of stroma-vascular preadipocytes from rat adipose tissue during their conversion into adipocytes when cultured *in vitro* (9). This suggests that SSAO/VAP-1 is a member of the adipogenic gene program and, in addition, that SSAO/VAP-1 may contribute to the acquisition of some final characteristics of fully differentiated adipose cells. Most of the SSAO/VAP-1 expressed in rat adipocytes is found in plasma membrane (5,6). Thus, subcellular fractionation of membranes from 3T3-L1 adipocytes or isolated rat adipocytes has demonstrated that SSAO/VAP-1 protein is far more abundant in the plasma membrane than in the light microsomes (5,6). In addition, the distribution of SSAO/VAP-1 in adipose cells does not seem to be regulated hormonally. Thus, the incubation of isolated rat adipocytes or 3T3-L1 adipose cells with insulin does not alter SSAO/VAP-1 protein abundance or SSAO activity in intracellular membranes or in plasma membrane preparations (5,6,10).

We have previously determined that substrates of SSAO strongly stimulate glucose transport and recruitment of GLUT4 to the cell surface in isolated rat adipocytes or 3T3-L1 adipocytes in the presence of low vanadate con-

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IAPP, islet amyloid polypeptide; IRS, insulin receptor substrate; LAR, leukocyte antigen-related phosphatase; NMR, nuclear magnetic resonance; PTP1B, protein tyrosine phosphatase-1B; SHP2, src-homology phosphatase-2; SSAO, semicarbazide-sensitive amine oxidase; VAP-1, vascular adhesion protein-1.

centrations (6,10–12). Stimulation of glucose transport by SSAO substrates has also been demonstrated in isolated human adipocytes (13). As to the mechanisms involved, we have demonstrated that the combination of SSAO substrates and vanadate stimulates tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and -3 proteins and activates phosphatidylinositol 3-kinase (10), i.e., crucial components of insulin signal transduction. In addition, chronic incubation of 3T3 adipocytes with SSAO substrates caused an enhanced insulin sensitivity (14). In keeping with these *in vitro* insulinomimetic effects, chronic treatment with benzylamine and vanadate lower hyperglycemia in streptozotocin-induced diabetic rats (12).

In this study, we have studied whether the utilization of SSAO substrates exerts insulin-like effects in the diabetic Goto-Kakizaki (GK) rat, an animal model of type 2 diabetes. We demonstrate that the administration of benzylamine (SSAO substrate) in combination with low ineffective doses of vanadate acutely stimulates glucose tolerance, and the chronic treatment normalizes hyperglycemia and reverses muscle insulin resistance in diabetic rats. The mechanism of action involves the generation of peroxovanadium, inhibition of protein tyrosine phosphatase activity, stimulation of adipocyte and muscle glucose transport, and improvement of insulin secretion.

RESEARCH DESIGN AND METHODS

Materials. The 2-[1,2-³H]-D-deoxyglucose (26 Ci/mmol) was from NEN Life Science Products (Boston, MA) and the [¹⁴C]Benzylamine (59 Ci/mmol) was from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). Purified porcine insulin was a kind gift from Eli Lilly (Indianapolis, IN). Semicarbazide hydrochloride, benzylamine hydrochloride, sodium orthovanadate, and other chemicals were purchased from Sigma Aldrich (St. Louis, MO). Ketamine was obtained from Mérieux (Imalgene, Mérieux, France). Collagenase type I was obtained from Worthington (Lakewood, NJ) and collagenase P from Roche Diagnostics (Basel, Switzerland). The osmotic minipumps used in chronic studies were from Alza (Mountainview, CA). All electrophoresis reagents and molecular weight markers were obtained from Bio-Rad (Hercules, CA). Enhanced chemiluminescence reagents (super signal substrate) were from Amersham (Arlington Heights, IL). Anti-GLUT4 antibody (OSCRX) was produced from rabbit as previously reported (15). Rabbit polyclonal antibodies against rat β_1 -integrin were kindly given by Dr. C. Enrich (Universitat de Barcelona, Barcelona, Spain). Anti-SSAO/VAP-1 was a kind gift from Dr. S. Jalkanen (University of Turku, Turku, Finland), and monoclonal antiphosphotyrosine antibody 4G10 was from Upstate Biotechnology. Human recombinant SSAO was provided by BioTie (Turku, Finland). Antibody against amylose was from Sigma Aldrich. Antibody against islet amyloid polypeptide (IAPP) was kindly given by Dr. A. Clark (Radcliffe Infirmary, Oxford, U.K.).

Animals. Male Wistar rats weighting 180–220 g were purchased from Harlan (Interfauna Ibérica S.A., Sant Felin de Codines, Spain). GK weight-matched diabetic rats were purchased from M&B Animal Models (Ry, Denmark). The animals were housed in animal quarters at 22°C with a 12-h light/dark cycle and were fed *ad libitum*. All procedures used were approved by the animal ethical committee of the University of Barcelona.

Glucose tolerance tests. Unanesthetized Wistar or GK diabetic rats were injected intravenously into the tail vein with vehicle (PBS, pH 7.4) or with a dose of 7 μ mol/kg body wt benzylamine and/or 20 μ mol/kg sodium orthovanadate. An oral glucose tolerance test (2 g/kg body wt) was carried out 15 min after benzylamine and vanadate injection. Blood samples were collected before (0 min) and 30, 45, 60, 90, and 120 min after glucose administration. Plasma was obtained for the determination of glucose and insulin concentrations. Food was withdrawn at 8:00 A.M. to ensure a 3-h fast before the beginning of the study.

Chronic treatments of diabetic animals. Osmotic minipumps containing benzylamine (84 μ mol \cdot kg body wt⁻¹ \cdot day⁻¹) were implanted subcutaneously in GK diabetic rats anesthetized by ketamine hydrochloride (95 mg/kg) and xylazine (10 mg/kg). Animals that did not receive benzylamine were sham operated. A single intraperitoneal injection of vanadate (25 μ mol/kg body wt) or the vehicle (PBS) was administered daily at 0900. Glycemia was measured

on arterio-venous blood collected from the tail vessels at 0900, before the administration of vanadate for 2 weeks. Insulin concentrations were determined before and after treatment.

Glucose transport measurements in isolated rat adipocytes and in incubated muscle preparations. Adipocytes were isolated from epididymal and perirenal fat pads in nondiabetic and GK diabetic male rats (180–220 g), and 2-D-deoxyglucose uptake measurements were performed as reported (10,12). Isolated fat cells from control or treated rats were disrupted for total membrane preparation by hypo-osmotic lysis in a 20 mmol/l HES buffer and an antiprotease cocktail as reported (10). Protein concentrations were determined by the Bradford method (16) with γ -globulin as a standard. Isolated strips of soleus muscles were incubated, and 2-D-deoxyglucose uptake measurements were performed as reported (17). Adipose tissue explants used for coinubation experiments with soleus muscle were prepared by dissecting and cutting epididymal and perirenal fat pads into small pieces that were further rinsed with Krebs-Ringer buffer before use.

Determination of insulin secretion by isolated pancreatic islets and preparation of extracts. Pancreata from Wistar and GK diabetic rats were enzymatically digested with collagenase (Roche Diagnostics) using the procedure of pancreatic duct cannulation and density gradient purification (Histopaque; Sigma Diagnostics) as described (18). Islets were separated from the remaining exocrine tissue by handpicking under a stereomicroscope.

Batches of six freshly isolated islets were incubated in a shaking water bath for 90 min at 37°C in 1.0 ml bicarbonate buffered medium (pH 7.4) containing 0.5% BSA, D-glucose (at 5.5 or 16.7 mmol/l), and test substances as appropriate: sodium orthovanadate (100 μ mol/l), benzylamine hydrochloride (100 μ mol/l), or semicarbazide hydrochloride (1 mmol/l). For the first 10 min of incubation, the vials containing the medium with the islets were gassed with 95% O₂ 5% CO₂. At the end of the incubation period the supernatants were collected and stored at -20°C until insulin determination by radioimmunoassay (CIS Biointernational, Gyf-Sur-Yvette, France).

Amine oxidase activity assays. The radiochemical determination of amine oxidase activity was performed basically as described by Fowler and Tipton (19), with slight modifications (11).

Analytical methods and immunoblot analysis. The glucose concentration in plasma was determined by the glucose oxidase method (MPR-three glucose/GOD-PAP Method; Boehringer-Mannheim) in glucose tolerance experiments and with a rapid glucose analyser (Accutrend Sensor Comfort; Roche) in chronic treatments. Plasma immunoreactive insulin concentration was determined with a Sensitive Rat insulin RIA kit (Linco Research, St. Charles, MO). SDS polyacrylamide gel electrophoresis was performed on membrane proteins following Laemmli (20). Proteins were transferred to Immobilon, and immunoblotting was performed as reported (15).

⁵¹V nuclear magnetic resonance spectroscopy analysis. The ⁵¹V nuclear magnetic resonance (NMR) spectra for vanadate and peroxovanadate detection were recorded on a Varian Unity 300-MHz spectrometer. Field frequency stabilization was achieved by dissolving samples with D₂O. Spectral widths of 380 ppm (78.84 MHz), a 90° pulse angle, and an accumulation time of 0.5 s were used. The chemical shifts are reported relative to the external reference standard VOCl₃ (assigned to 0 ppm, where ppm represents the chemical shift of an atom in a defined chemical environment and is directly proportional to the resonance frequency of the atom in that environment). Exponential line-broadening (10 Hz) was applied before Fourier transformation.

Determination of protein tyrosine phosphatase activity. Rat adipocyte suspension was incubated at 37°C with the indicated compounds at 100 μ mol/l during 45 min. At the end of the incubation adipocytes were homogenized in 10 mmol/l Tris buffer containing protease inhibitors (pH 7.4). After a 15,000g centrifugation at 4°C for 5 min, the fat cake was discarded. The homogenates, after desalting step by Sepharose-G25 column, were conserved at -80°C until use for phosphatase activity determination. Phosphatase activity measurement was estimated by the potency of the homogenates (20 μ g proteins) to hydrolyze *p*-nitrophenyl phosphate (5 mmol/l pNPP) in 10 mmol/l Tris-HCl, pH 7.5, in the presence of 2 mmol/l dithiothreitol. The extent of hydrolysis was determined by spectroscopy at 405 nm after 1 h of incubation at 37°C, as described by Kremerskothen and Barnekow (21).

Calculations and statistical analysis. Insulin and glucose responses during the glucose tolerance test were calculated as the incremental plasma values integrated over a period of 120 min after the injection of glucose. Areas under curve of insulin and glucose responses were calculated using the Graph Prism program. Data were presented as mean \pm SE, and unpaired Student's *t* test was used to compare two groups. When experimental series involved more than two groups, statistical analysis was done by one-way ANOVA and further post hoc Dunnett's or Tukey's *t* tests.

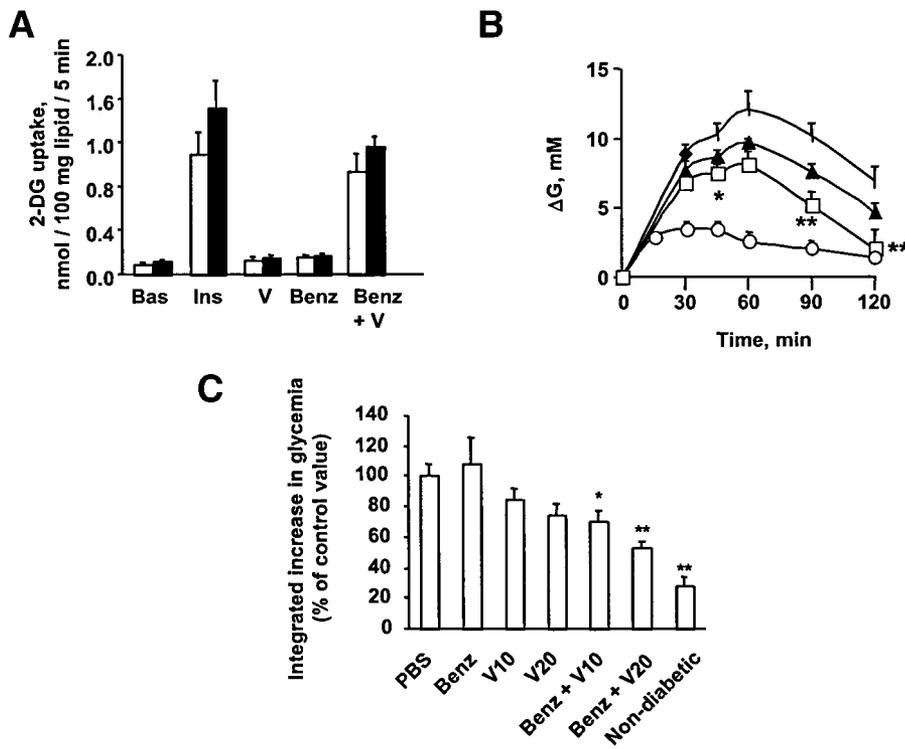


FIG. 1. Benzylamine and vanadate enhance glucose tolerance and stimulate glucose transport in adipose cells from GK diabetic rats. **A:** Adipose cells from GK diabetic rats (□) or nondiabetic Wistar rats (■) were incubated for 45 min in basal conditions (Bas) or in the presence of 100 nmol/l insulin (Ins), 100 μmol/l vanadate (V), 100 μmol/l benzylamine (Benz), or both vanadate and benzylamine (Benz + V). Subsequently, 2-deoxyglucose (2-DG) transport was measured over 5 min. Values are mean ± SE of four to five observations per group. *Significant stimulation of 2-DG uptake compared with basal transport value at $P < 0.001$. **B:** Relative changes in glycemia with an oral glucose load (2 g/kg) starting 15 min after intravenous injection of vehicle (PBS) (◇), 20 μmol/kg vanadate (▲), or 20 μmol/kg vanadate and 7 μmol/kg benzylamine (□) in diabetic rats. Data obtained after oral glucose load in nondiabetic rats are also shown (○). Results are expressed as increment in plasma glucose. * $P < 0.05$ and ** $P < 0.001$ vs. the PBS diabetic group. **C:** Integrated increase in glycemia. Concentrations of plasma glucose at zero time were 11.5 ± 0.4 , 11.1 ± 0.4 , 11.8 ± 0.5 , 11.9 ± 0.5 , 13.1 ± 0.4 , 12.4 ± 0.4 , and 5.6 ± 0.3 mmol/l in untreated (PBS), benzylamine (Benz), 10 μmol/kg vanadate (V10), 20 μmol/kg vanadate (V20), benzylamine/10 μmol/kg vanadate (Benz + V10), benzylamine/20 μmol/kg vanadate (Benz + V20), and nondiabetic groups, respectively. Values are mean ± SE of 4–10 observations expressed as a percentage, 100% corresponding to the PBS group. * $P < 0.05$ and ** $P < 0.01$ vs. the PBS group.

RESULTS

Acute administration of benzylamine and vanadate enhances glucose transport in GK rats. To determine whether SSAO/VAP-1 is a drug target for type 2 diabetes, we selected the GK diabetic rats. These animals are hyperinsulinemic, nonobese, and hyperglycemic, and they show defective insulin secretion and peripheral insulin resistance but normal insulin-stimulated glucose transport in adipocytes (22–25). Adipose tissue from GK diabetic rats showed normal SSAO activity (162 ± 12 and 173 ± 19 nmol benzylamine \cdot min $^{-1}$ \cdot mg protein $^{-1}$ in control and GK rats), and the combination of 0.1 mmol/l benzylamine and 0.1 mmol/l vanadate markedly stimulated glucose transport in isolated adipocytes from GK rats (Fig. 1A). In fact, benzylamine/vanadate-stimulated and maximally insulin-induced glucose transport was similar in control Wistar and in GK rats (Fig. 1A), the latter in agreement with previous data (25). Next, GK diabetic rats were injected via tail vein with vehicle or a dose of 7 μmol/kg body wt benzylamine and/or 10 or 20 μmol/kg sodium orthovanadate. An oral glucose tolerance test (2 g/kg body wt) was carried out 15 min after drug injection (Fig. 1B). The acute intravenous administration of benzylamine and vanadate enhanced glucose tolerance in GK rats (30 and 47% increase after benzylamine/vanadate 10 μmol/kg and benzylamine/vanadate 20 μmol/kg, respectively) (Fig. 1B and C). In fact, administration of benzylamine and 20 μmol/kg vanadate ameliorated a glucose tolerance test of GK diabetic rats to values approaching those of nondiabetic rats (Fig. 1C). In these conditions, administration of benzylamine or vanadate alone did not alter glucose tolerance (Fig. 1B and C). The improvement in glucose tolerance induced by benzylamine and vanadate was also detected in nondiabetic Wistar rats (10 and data not shown).

Chronic treatment with benzylamine and vanadate normalizes glycemia and ameliorates muscle insulin

responsiveness. Next, we tested the effects of the chronic administration of these compounds in GK diabetic rats. To this end, GK diabetic rats were implanted subcutaneously with osmotic minipumps releasing benzylamine ($84 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) or were sham operated. Benzylamine is stable for 2 weeks in implanted osmotic minipumps as based on its capacity to further stimulate glucose transport in isolated adipocytes after this period (data not shown). A group of animals was also subjected to daily intraperitoneal injection of vanadate (25 μmol/kg) for 2 weeks. While treatment with vanadate caused a moderate and insignificant reduction of glycemia, the combination of benzylamine and vanadate during 1 week significantly reduced the hyperglycemia to levels close to those of the nondiabetic group (Fig. 2A). Administration of benzylamine plus vanadate for 2 weeks did not alter SSAO/VAP-1 or GLUT4 protein expression in adipose cells (Fig. 2B). The normalization of glycemia caused by chronic treatment with both benzylamine and vanadate took place in the absence of changes in plasma insulin concentrations (data not shown).

To determine whether the antidiabetic effects caused by the chronic combined treatment with benzylamine and vanadate were a consequence of stimulation of peripheral glucose utilization, we analyzed glucose transport in adipose cells obtained from rats subjected to chronic treatment. A 2-week treatment with benzylamine and vanadate stimulated basal glucose transport and GLUT4 abundance at the plasma membrane in isolated adipocytes (Fig. 2C). We also detected a statistically significant correlation between the abundance of GLUT4 at the cell surface and basal glucose transport in adipocytes from untreated and benzylamine/vanadate-treated diabetic rats (Fig. 2C). These effects on glucose transport were not detected in adipose cells obtained from rats treated with benzylamine or vanadate alone (data not shown).

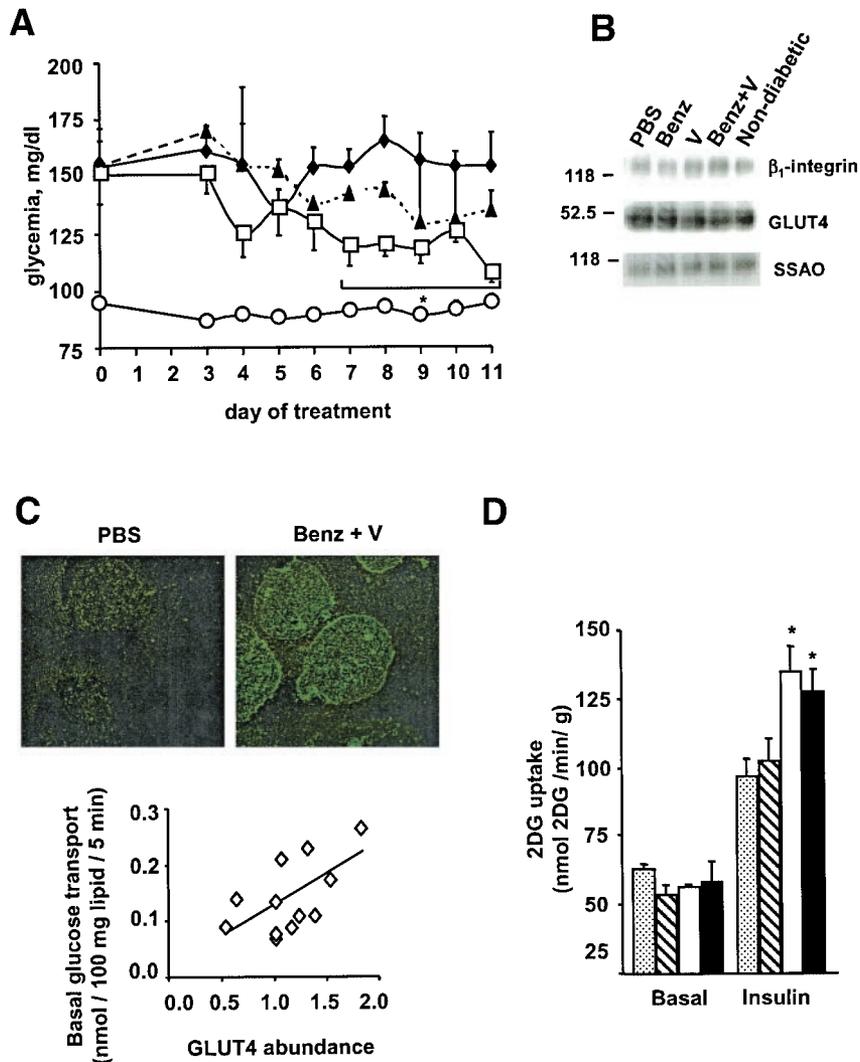


FIG. 2. Chronic treatment with benzylamine and vanadate ameliorates glycemia and enhance glucose transport in muscle and adipose cells. **A:** GK diabetic rats were treated by a daily intraperitoneal injection of vanadate (25 $\mu\text{mol/kg}$) alone (\blacktriangle) or in combination with benzylamine delivered by osmotic minipumps (84 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) (\square) or received a daily injection of the vehicle (PBS) (\blacklozenge). Nondiabetic rats treated with PBS were also studied (\circ). Values are mean \pm SE of 4–12 observations. * $P < 0.05$ vs. the PBS diabetic group from day 6. **B:** After 2 weeks of treatment, isolated adipocytes from untreated diabetic rats (PBS), benzylamine-treated diabetic rats (Benz), vanadate-treated diabetic rats (V), benzylamine and vanadate treated diabetic rats (Benz + V), or nondiabetic rats were obtained from epididymal adipose tissue. SSAO, GLUT4, and β_1 -integrin protein expression in total membranes from the different groups was assayed by Western blot. A representative autoradiogram from five separate experiments is shown. **C:** After 2 weeks of treatment, isolated adipocytes from untreated GK diabetic rats and benzylamine and vanadate treated diabetic rats were obtained from epididymal adipose tissue. Adipose cells were incubated for 30 min in basal conditions, and GLUT4 present at the cell surface and glucose transport were measured. 2-Deoxyglucose transport was measured for 5 min. Plasma membrane lawns were obtained, and immunofluorescence assays were performed using specific GLUT4 antibodies. Correlation between basal glucose transport and GLUT4 abundance at the plasma membranes in isolated rat adipocytes was statistically significant ($r = 0.59$, $P < 0.01$). **D:** After 2 weeks of treatment, soleus muscles from untreated GK diabetic rats (\square), vanadate-treated diabetic rats (\blacksquare), benzylamine- and vanadate-treated diabetic rats (\blacksquare), and nondiabetic rats (\blacksquare) were incubated in the absence (basal) or in the presence of 100 nmol/l insulin. Following 2-deoxyglucose uptake, muscles were digested and radioactivity was measured. * $P < 0.05$ vs. the untreated diabetic group.

Chronic treatment with benzylamine and vanadate also had an impact on muscle insulin responsiveness. Indeed, incubation of soleus muscle preparations from GK rats showed normal basal glucose transport and insulin resistance (Fig. 2D), which is in keeping with previous observations (21,26). Chronic treatment with benzylamine/vanadate reversed the insulin response to normal levels (Fig. 2D). No effects were observed on muscle glucose transport after chronic administration with vanadate alone (Fig. 2D). Chronic administration of benzylamine/vanadate did not alter adipose mRNA levels of leptin, tumor necrosis factor- α , adiponectin, or resistin or plasma concentrations of free fatty acids (data not shown). These data indicate that the effects of chronic benzylamine/vanadate on glycemia and muscle insulin responsiveness are unrelated to changes on adipocytokines gene expression or on circulating free fatty acids.

SSAO/VAP-1 activity stimulates muscle glucose transport under in vivo or in vitro conditions. To determine the basis for the improvement in glucose tolerance detected in nondiabetic and diabetic rats after acute treatment with benzylamine/vanadate, nondiabetic rats were acutely treated with benzylamine (7 $\mu\text{mol/kg}$ body wt) and/or vanadate (20 $\mu\text{mol/kg}$) or with insulin (10 units/kg), and 30 min later, soleus muscles were incubated and

glucose transport measured. Previous in vivo insulin administration caused a 75% stimulation of muscle glucose transport, and previous benzylamine/vanadate treatment also caused a marked enhancement of glucose transport (40% increase) (Fig. 3A). Next, soleus muscles were incubated in the presence of different combinations of 0.1 mmol/l benzylamine and/or 0.1 mmol/l vanadate in the absence or presence of adipose tissue explants (Fig. 3B). In the absence of adipose tissue, muscle glucose transport remained unaffected by benzylamine/vanadate, which is in keeping with the fact that SSAO activity is very low in rat skeletal muscle; however, in the presence of adipose tissue explants, muscles underwent a marked stimulation of glucose transport by benzylamine/vanadate (Fig. 3B), and this effect was inhibited by the SSAO inhibitor semicarbazide. The presence of adipose tissue explants did not alter basal or insulin-stimulated glucose transport in the incubated soleus muscle (data not shown).

In a further set of experiments, soleus muscles were incubated in the presence of benzylamine/vanadate and human recombinant SSAO/VAP-1 (Fig. 3C). As mentioned before, benzylamine/vanadate caused no effect on muscle glucose transport; however, the presence of SSAO/VAP-1 caused the stimulation of muscle glucose transport that was comparable with the effect of a supramaximal con-

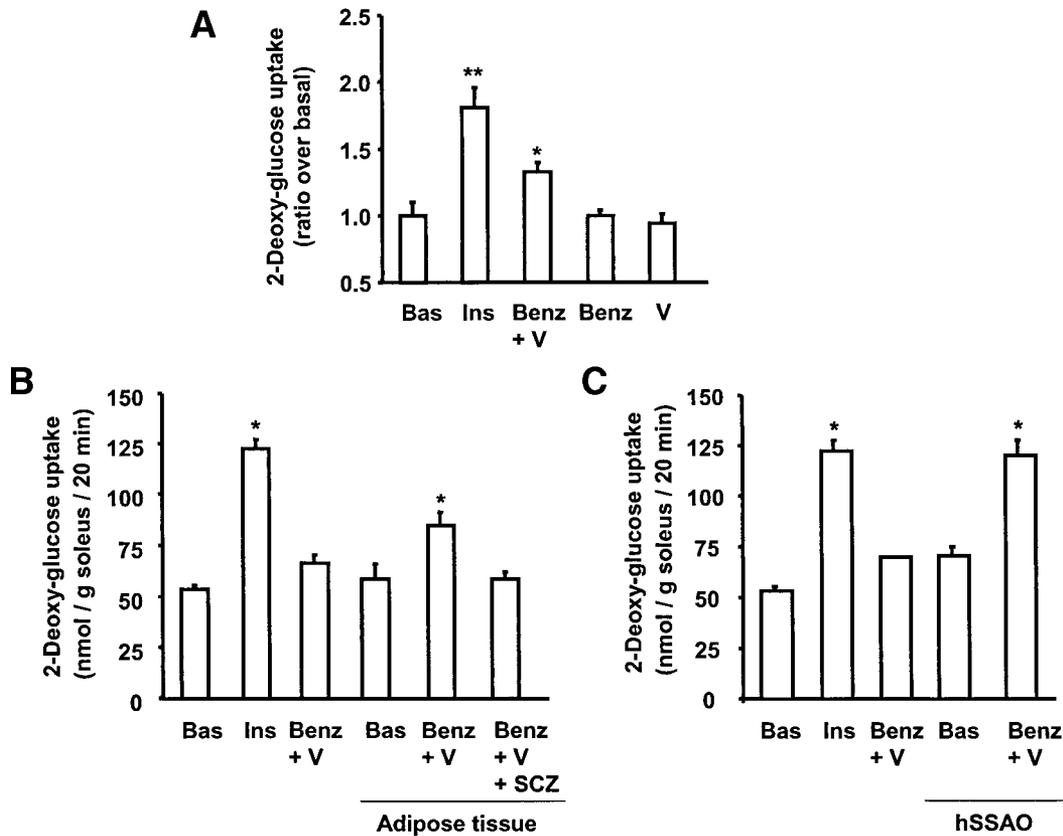


FIG. 3. Muscle glucose transport is stimulated by acute exposure to benzylamine, vanadate, and SSAO. *A*: Nondiabetic rats were intravenously injected with vehicle (Bas), 10 units/kg insulin (Ins), 7 $\mu\text{mol/kg}$ benzylamine (Benz), 20 $\mu\text{mol/kg}$ vanadate (V), or with benzylamine and vanadate (Benz + V); 30 min later, soleus muscles were incubated and glucose transport measured. Values are mean \pm SE of 6–12 observations per group. * $P < 0.05$ and ** $P < 0.01$ vs. the basal group. *B*: Soleus muscles were incubated for 30 min in the absence (Bas) or in the presence of different combinations of 100 nmol/l insulin, 0.1 mmol/l benzylamine, 0.1 mmol/l vanadate, 1 mmol/l semicarbazide (SCZ), and 300 mg epididymal adipose tissue explants. Following 2-deoxyglucose uptake, muscles were digested and radioactivity was measured. Values are mean \pm SE of 9–12 observations per group. * $P < 0.05$ vs. the basal group. *C*: Soleus muscles were incubated for 30 min in the absence (Bas) or in the presence of different combinations of 100 nmol/l insulin, 0.1 mmol/l benzylamine, 0.1 mmol/l vanadate, 1 mmol/l semicarbazide (SCZ), and human recombinant SSAO/VAP-1 (hSSAO). Following 2-deoxyglucose uptake, muscles were digested and radioactivity was measured. Values are mean \pm SE of seven observations per group. * $P < 0.05$ vs. the basal group.

centration of insulin and was also inhibited by semicarbazide (Fig. 3C). In all, these data indicate that the *in vivo* treatment with the combination of benzylamine/vanadate stimulates muscle glucose transport, and this is a consequence of signals that depend on SSAO activity and that may originate in adipose tissue.

Benzylamine and vanadate enhance insulin secretion.

In a further step, we tested the possibility that insulin secretion was responsible for some of the effects of the acute treatment with benzylamine/vanadate on glucose tolerance detected in GK diabetic rats. Thus, we measured plasma insulin concentrations at different times after the glucose tolerance test in nondiabetic and GK diabetic rats injected with PBS or in GK rats administered with benzylamine/vanadate. GK diabetic rats showed a marked deficiency in insulin secretion (Fig. 4A); in contrast, previous administration of benzylamine/vanadate showed a normalization of the profile of insulin concentrations after a glucose challenge (Fig. 4A and B). The acute treatment with vanadate alone showed no effect on plasma insulin concentrations (Fig. 4B). Also, no effect of benzylamine/vanadate on plasma insulin concentrations was detected in nondiabetic rats (12). To test whether benzylamine/vanadate acted directly on pancreatic islets, isolated islets from nondiabetic Wistar and GK diabetic rats were incu-

bated in the presence of benzylamine and/or vanadate, and the response to glucose was evaluated. Insulin secretion measured at 16.7 and 5.5 mmol/l glucose was defective in pancreatic islets from GK diabetic rats compared with those from controls (values of insulin secretion at 16.7 mmol/l glucose were 81 ± 12 and 50 ± 5 μU islet/90 min, and at 5.5 mmol/l glucose was 51 ± 8 and 34 ± 4 μU islet/90 min in control and GK rats, respectively); in keeping with the *in vivo* observations, benzylamine/vanadate markedly enhanced insulin secretion in pancreatic islets from GK diabetic rats but not from nondiabetic rats (Fig. 4C and D). In addition, the effect of benzylamine/vanadate on insulin secretion was blocked by semicarbazide (Fig. 4D). In keeping with these observations, we detected a similar abundance of SSAO/VAP-1 protein in extracts from isolated pancreatic islets compared with extracts from total pancreas; under these conditions, IAPP (a marker of the pancreatic islets) was highly enriched in pancreatic islets compared with total pancreas, whereas amylase abundance (a marker of exocrine pancreas) was very low in pancreatic islets (Fig. 4E). The abundance of SSAO/VAP-1 protein was much higher in adipose tissue than in pancreatic islets (Fig. 4E). In addition, SSAO activity was nearly 300-fold greater in extracts from adipocytes than in pancreatic islets (data not shown). In all,

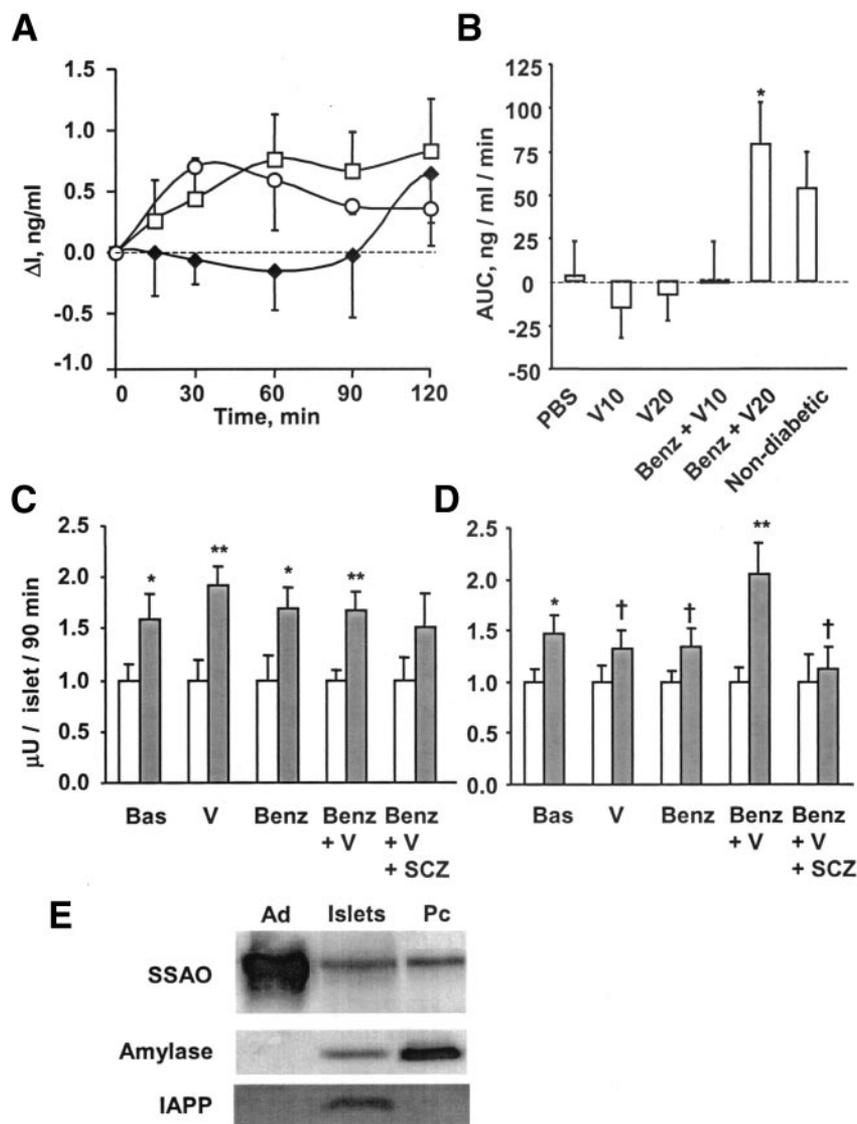


FIG. 4. Benzylamine and vanadate stimulate insulin secretion in GK diabetic rats. **A:** Plasma insulin concentrations after an oral glucose load (2 g/kg) starting 15 min after intravenous injection of vehicle (◆), 20 $\mu\text{mol/kg}$ vanadate, and 7 $\mu\text{mol/kg}$ benzylamine (□) in GK diabetic rats and vehicle in nondiabetic Wistar rats (○). Values are mean \pm SE of three to six rats per group and are given as nanograms of insulin per milliliter of plasma and as area under the curve. * $P < 0.05$ vs. the vehicle-treated (PBS) GK group. **B:** Integrated increase in plasma insulin. Concentrations of plasma insulin at zero time were 1.15 ± 0.36 , 1.19 ± 0.20 , 0.98 ± 0.46 , 0.80 ± 0.09 , 1.41 ± 0.17 , and 0.19 ± 0.05 ng/ml in untreated (PBS), 10 $\mu\text{mol/kg}$ vanadate (V10), 20 $\mu\text{mol/kg}$ vanadate (V20), benzylamine/10 $\mu\text{mol/kg}$ vanadate (Benz + V10), and benzylamine/20 $\mu\text{mol/kg}$ vanadate (Benz + V20) in GK diabetic rats and in nondiabetic groups, respectively. Values are mean \pm SE of four to six observations expressed area under the curve. * $P < 0.05$ vs. the PBS group. **C and D:** Insulin secretion in pancreatic islets from Wistar (**C**) or GK diabetic rats (**D**). Six pancreatic islets from nondiabetic or diabetic rats were incubated for 90 min in the absence or in the presence of 0.1 mmol/l vanadate, 0.1 mmol/l benzylamine, benzylamine, and vanadate or 1 mmol/l semicarbazide (SCZ), and insulin released into the incubation medium was measured by radioimmunoassay. Values are mean \pm SE of 5–14 observations per group and are relative to the values obtained at 5.5 mmol/l glucose. * $P < 0.05$ and ** $P < 0.01$ for 16.7 vs. 5.5 mmol/l glucose. † $P < 0.05$ vs. the Benz + V group. **E:** Extracts from isolated rat adipocytes (Ad), from isolated rat pancreatic islets (Islets), or from rat pancreas (Pc) were obtained. Expression of SSAO/VAP-1, IAPP, and amylase were assayed by Western blot using specific antibodies. A representative autoradiogram from three separate experiments is shown.

our results show that endocrine islets express SSAO/VAP-1 protein but at lower levels than in adipose cells.

Exposure of adipocytes to SSAO substrates generates peroxovanadium compounds and inhibits protein tyrosine phosphatase activity. As to the nature of the molecules that promotes the effects on adipose cells, skeletal muscle, and pancreatic islets in response to benzylamine/vanadate, we have ruled out the involvement of changes in gene expression of adipocytokines and, at least, for skeletal muscle they only require the presence of benzylamine, vanadate, and the enzymatic activity of human recombinant SSAO. Next, we tested the possible formation of peroxovanadium compounds, based on the fact that SSAO catalysis generates hydrogen peroxide, and this is known to react with vanadate, generating peroxovanadium. Vanadate was detected by NMR assays as a number of peaks at approximately -560 and -580 ppm (Fig. 5A), which was independent of the presence of human recombinant SSAO (data not shown). Under our assay conditions, the presence of benzylamine caused no NMR signals (data not shown). Incubation of vanadate and hydrogen peroxide generated monoperoxovanadium (identified as a peak at -630 ppm) and triperoxovanadium

(identified as a peak at -730 ppm) compounds detected by NMR (Fig. 5A) (27). Furthermore, the incubation of vanadate, benzylamine, and human recombinant SSAO also caused a substantial formation of monoperoxovanadium and triperoxovanadium compounds in NMR assays (Fig. 5A). Further support for the in vivo generation of peroxovanadium complexes was the observation that the combination of benzylamine and vanadate caused a very potent stimulation of protein tyrosine phosphorylation in extracts from isolated rat adipocytes. The effect of benzylamine/vanadate was much greater than that caused by insulin; it was not observed in the presence of benzylamine or vanadate alone, and it was blocked by semicarbazide (Fig. 5B). The effect of benzylamine/vanadate was similar to that caused by peroxovanadate (Fig. 5B). This is consistent with the powerful inhibitory effect of peroxovanadium on protein tyrosine phosphatases (28, 29). Moreover, we have found that the incubation of adipocytes with benzylamine and vanadate caused a marked inhibition of protein tyrosine phosphatase activity, whereas incubation with benzylamine or vanadate alone had no effect on protein tyrosine phosphatase (Fig. 5C). The effect of benzylamine/vanadate on protein tyrosine

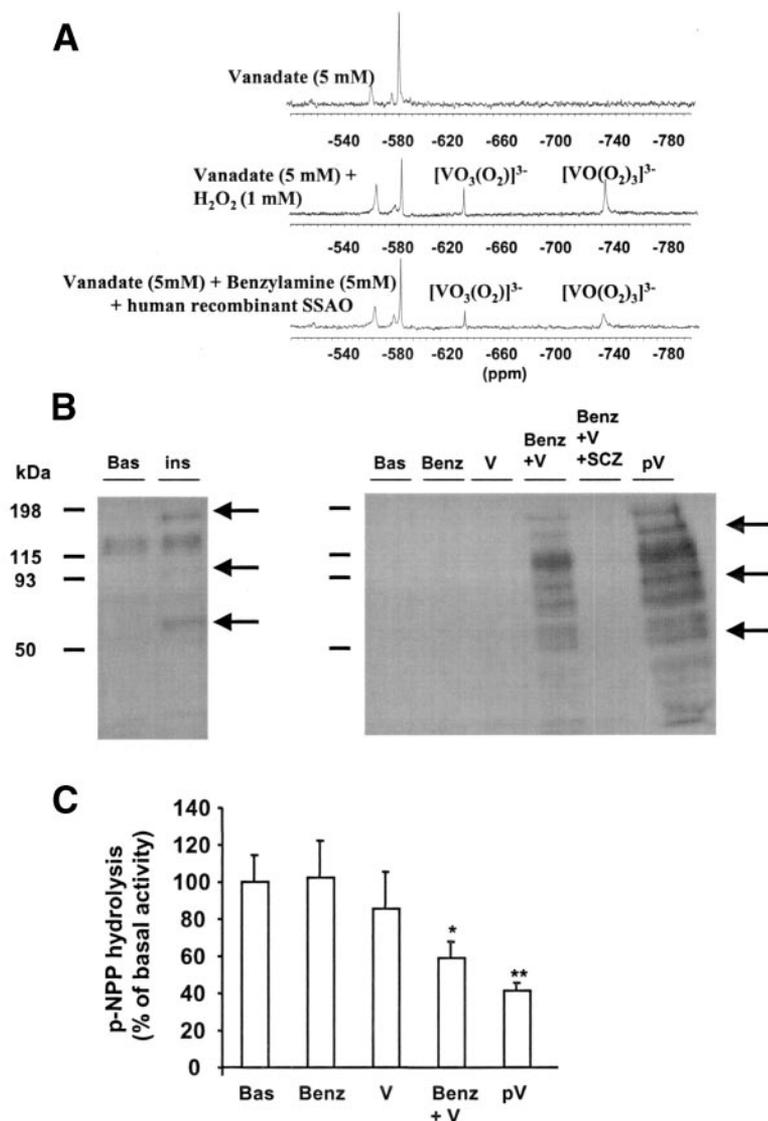


FIG. 5. Benzylamine, vanadate, and SSAO generate peroxovanadium and inhibit protein tyrosine phosphatase activity. **A:** Identification of vanadate and peroxovanadium compounds by resonance of V51 after incubation of 5 mmol/l sodium vanadate alone, 5 mmol/l sodium vanadate and 1 mmol/l hydrogen peroxide, and 5 mmol/l sodium vanadate, 5 mmol/l benzylamine, and human recombinant SSAO/VAP-1. **B:** Incubation in the presence of benzylamine and vanadate enhances the level of protein tyrosine phosphorylation in isolated rat adipocytes. Cells were incubated for 15 min in the absence (bas) or presence of insulin (100 nmol/l) (ins), benzylamine (0.1 mmol/l) (Benz), vanadate (0.1 mmol/l) (V), semicarbazide (1 mmol/l) (SCZ), or freshly prepared peroxovanadate (pV, 0.1 mmol/l). Thereafter, total cell extracts were obtained and the level of protein tyrosine phosphorylation was assayed by Western blot with specific antiphosphotyrosine antibodies. Arrows point out the major proteins phosphorylated on tyrosine residues by insulin showing apparent molecular weights of 170, 120, and 67 kDa. The two autoradiograms were exposed for different times. **C:** Incubation in the presence of benzylamine and vanadate inhibits protein tyrosine phosphatase activity in isolated rat adipocytes. Cells were incubated for 45 min in the absence or presence of benzylamine (0.1 mmol/l), vanadate (0.1 mmol/l), the combination of both, or with 0.1 mmol/l peroxovanadate (pV). Thereafter, total cell extracts were obtained and the protein tyrosine phosphatase activity was determined for each condition by measuring pNPP hydrolysis. The results are expressed as a percentage of basal values. Values are means \pm SE of three to five determinations. * $P < 0.05$ and ** $P < 0.01$ vs. the basal group.

phosphatase was similar to the effect caused by 100 μ mol/l peroxovanadate (Fig. 5C).

DISCUSSION

In this study, we have demonstrated that the utilization of the SSAO/VAP-1 substrate benzylamine in combination with ineffective doses of vanadate has very remarkable pharmacological properties in the treatment of type 2 diabetes. Thus, both acute and chronic treatment with benzylamine and vanadate stimulates glucose utilization in adipose cells and in skeletal muscle and reverses muscle insulin resistance in GK diabetic rats. In addition, benzylamine and vanadate also acutely ameliorate insulin secretion in GK diabetic rats. Therefore, treatment with benzylamine and vanadate shows two types of antidiabetic effects: 1) metabolic effects in adipose and muscle cells that lead to enhanced glucose utilization and 2) stimulatory effects on insulin secretion. This type of double action is ideal in the treatment of type 2 diabetes, which is characterized by insulin resistance affecting peripheral tissues and defective insulin secretion.

The biological effects of benzylamine/vanadate require an intact SSAO activity. This is an important consideration

because benzylamine may also be metabolized via mono-amino oxidase activity (14). The experimental evidence in support of the view that SSAO activity is essential for the effects of benzylamine/vanadate is as follows: 1) the effects of benzylamine/vanadate on glucose transport in isolated rat adipocytes are blocked with semicarbazide but not with pargyline (5,10); 2) the acute effects of benzylamine/vanadate on glucose disposal in nondiabetic and streptozotocin-induced diabetic rats are blocked in rats previously treated with semicarbazide (12); 3) the effects of benzylamine/vanadate on skeletal muscle glucose transport (in coinubation studies with adipose tissue explants) is prevented in the presence of semicarbazide (this study); 4) the effect of benzylamine/vanadate on insulin secretion in isolated rat pancreatic islets is also prevented in the presence of semicarbazide (this study); and 5) the effect of benzylamine/vanadate on protein phosphorylation on tyrosine residues and activation of IRS-1 and -3 in adipocytes is prevented in the presence of semicarbazide (10 and this study).

In this study we only used benzylamine as a SSAO/VAP-1 substrate. However, current available evidence indicates that other SSAO substrates also show similar biological

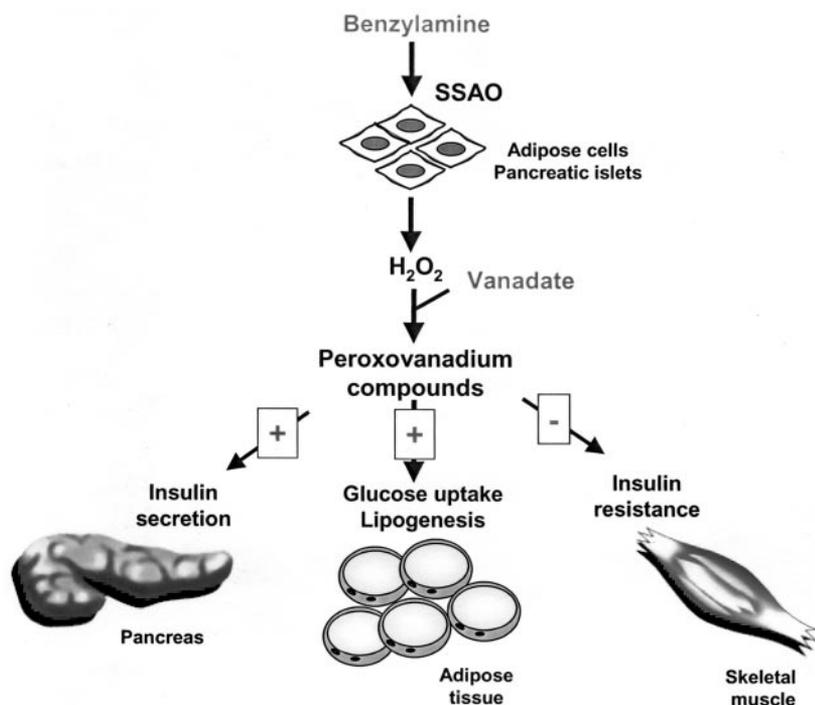


FIG. 6. Model on the cellular effects of SSAO/VAP-1 effects on glucose homeostasis.

effects, which correlates with their capacity of amines to be oxidized by SSAO activity (13). The evidence for this is as follows: other substrates of SSAO, such as tyramine, methylamine, *N*-decylamine, β -phenylethylamine, histamine, or *N*-acetylputrescine stimulate glucose transport in combination with vanadate in isolated rat adipocytes (10) or alone in isolated human adipocytes (13); 2) chronic incubation in the presence of benzylamine, tyramine, methylamine, or β -phenylethylamine stimulated adipose cell differentiation in 3T3 F442A preadipocytes (14,30); 3) the acute administration of tyramine (another SSAO substrate) enhances glucose utilization as assessed by glucose tolerance test in streptozotocin-induced diabetic rats, and this effect was blocked in rats chronically treated with semicarbazide, which shows completely inactive SSAO activity (31); and 4) the chronic administration for 2 weeks of tyramine together with vanadate enhanced glucose utilization as assessed by glucose tolerance test in streptozotocin-induced diabetic rats (31).

Diabetic GK rats show hyperglycemia, muscle insulin resistance, and defective insulin secretion despite normal circulating concentrations of insulin (22–25). Chronic treatment of diabetic GK rats with benzylamine/vanadate caused amelioration of plasma glucose, enhanced glucose transport in adipocytes, and improved insulin responsiveness in skeletal muscle, which occurred in the absence of alterations in circulating insulin. It is likely that amelioration of plasma glucose by benzylamine/vanadate is due to enhanced glucose transport in adipocytes and in skeletal muscle caused by benzylamine/vanadate together with the improvement in the capacity of muscle to respond to insulin. It is also likely that the amelioration in glucose-induced insulin secretion is involved in the effects of benzylamine/vanadate on glucose homeostasis. Benzylamine/vanadate did not alter plasma insulin concentrations in diabetic GK rats, which was not surprising because they were already normal under untreated conditions.

We have also demonstrated the nature of the mechanisms implicated in the metabolic effects associated with the utilization of SSAO/VAP-1 substrates, such as benzylamine, in combination with vanadate. Benzylamine metabolism catalyzed by SSAO generates hydrogen peroxide, and the combination of hydrogen peroxide and vanadate generates peroxovanadium compounds, a process that does not require enzymatic catalysis. This is the explanation by which the presence of benzylamine, human recombinant SSAO and vanadate produces peroxovanadium compounds. The generation of peroxovanadium compounds is relevant because they inhibit protein tyrosine phosphatase activity. In consequence, it is likely that the *in vivo* treatment with benzylamine and vanadate results in the production of peroxovanadium compounds. This type of compounds combination (SSAO substrate and vanadate) permits the generation of protein tyrosine phosphatase inhibitors in key sites such as adipose tissue or pancreatic islets, which can display a variety of local effects and which can also act in neighbor tissues such as skeletal muscle. The approach of generating inhibitors of protein tyrosine phosphatase activity is sensible, because protein tyrosine phosphatases are implicated in the dephosphorylation of signaling molecules that terminate insulin signaling. In addition, alteration in protein tyrosine phosphatase activity may contribute to insulin-resistant states. The expression of specific protein tyrosine phosphatases, including leukocyte antigen-related phosphatase (LAR), protein tyrosine phosphatase-1B (PTP1B), and src-homology phosphatase-2 (SHP2) are increased in muscle and adipose tissue from obese subjects or obese rodents (32–34). Enhanced PTP1B and SHP2 have also been detected in muscle from diabetic rats (32,35). Overexpression of the LAR in muscle causes insulin resistance characterized by hyperinsulinemia and reduced whole-body glucose disposal and muscle glucose uptake (36). Similarly, overexpression of PTP1B in adipose tissue im-

pairs insulin-stimulated glucose transport (37,38). This suggests that increased LAR or PTP1B activity may contribute to the pathogenesis of insulin resistance. Evidence indicating that mice lacking the PTP1B gene show increased insulin sensitivity and resistance to the development of obesity induced by a high-fat diet (39) has provided additional support to the view that inhibitors of protein tyrosine phosphatase activity may have beneficial properties.

Our data indicate that SSAO/VAP-1 is a drug target for type 2 diabetes and that the combination of SSAO substrates and low doses of vanadate efficiently normalizes glycemia and reverses muscle insulin resistance in GK diabetic rats. Moreover, our results are consistent with a model (Fig. 6) in which SSAO/VAP-1 activity, present in adipocyte cell surface or in pancreatic islets, generates hydrogen peroxide, and this promotes the formation of peroxovanadium complexes in the presence of vanadate. The peroxovanadium generated inhibits protein tyrosine phosphatase activity, which causes activation of protein tyrosine phosphorylation in adipose cells (Fig. 6). We propose that the peroxovanadium generated in the plasma membrane from adipose cells also reaches muscle fibers found in the neighborhood. This cascade of events is instrumental to acutely stimulate glucose transport and lipogenesis in adipose cells, glucose transport in skeletal muscle, and glucose-stimulated insulin secretion in pancreatic islets from GK rats (Fig. 6). This proposal is in keeping with the potent insulin-mimicking activity of peroxovanadate previously detected in adipocytes and in skeletal muscle (40–45). Our model also agrees with previous observations indicating that peroxovanadate regulates insulin secretion (46).

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