En aquest apartat s'inclou un article de revisió publicat després de la participació al 3er Congrés Internacional sobre Vitamina B6, PQQ, Catàlisi per grups Carbonil i Quinoproteïnes que tingué lloc l'abril de 2002 a la Universitat de Southampton, Anglaterra. En ell s'inclou un resum dels resultats obtinguts en el grup on s'ha desenvolupat aquesta tesi abans de la meva incorporació i un resum dels articles 1 i 2 presentats a l'apartat de resultats.

Semicarbazide-sensitive amine oxidase activity exerts insulin-like effects on glucose metabolism and insulin-signaling pathways in adipose cells

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Antonio Zorzano, **Anna Abella**, Luc Marti, Christian Carpéné, Manuel Palacín and Xavier Testar

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Substrates of semicarbazide-sensitive amine oxidase co-operate with vanadate to stimulate tyrosine phosphorylation of insulin-receptor-substrate proteins, phosphoinositide 3-kinase activity and GLUT4 translocation in adipose cells

Gemma ENRIQUE-TARANCÓN*, Isabelle CASTAN†, Nathalie MORIN‡, Luc MARTI*, Anna ABELLA*, Marta CAMPS*, Roser CASAMITJANA§, Manuel PALACÍN*, Xavier TESTAR*, Eva DEGERMAN†, Christian CARPÉNÉ‡ and Antonio ZORZANO*[1]

*Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Avenida Diagonal 645, 08028 Barcelona, Spain, †Department of Cell and Molecular Biology, Lund University, P.O. Box 94, S22100 Lund, Sweden, ‡Institut National de la Santé et de la Recherche Médicale (INSERM U317), Institut Louis Bugnard, Université Paul Sabatier, C. H. U. Rangueil, 31403 Toulouse Cedex 4, France, and §Laboratori Hormonal, Hospital Clínic i Provincial, C/ Villarroel 170, 08036 Barcelona, Spain

It has been shown that the combination of benzylamine or tyramine and low concentrations of vanadate markedly stimulates glucose transport in rat adipocytes by a mechanism that requires semicarbazide-sensitive amine oxidase (SSAO) activity and H_2O_2 formation. Here we have further analysed the insulin-like effects of the combination of SSAO substrates and vanadate and we have studied the signal-transduction pathway activated in rat adipocytes. We found that several SSAO substrates (benzylamine, tyramine, methylamine, n-decylamine, histamine, tryptamine or β-phenylethylamine), in combination with low concentrations of vanadate, stimulate glucose transport in isolated rat adipocytes. Furthermore, SSAO substrates together with vanadate stimulated the recruitment of GLUT4 to the cell surface in isolated rat adipocytes. Benzylamine plus vanadate also stimulated glucose transport and GLUT4 translocation in 3T3-L1 adipocytes. Benzylamine or tyramine in combination with vanadate potently stimulated the tyrosine phosphorylation of both insulin receptor substrate (IRS)-1 and IRS-3. In contrast, benzylamine and vanadate caused only a weak stimulation of insulin receptor kinase. Benzylamine or tyramine in combination with vanadate also stimulated phosphoinositide 3-kinase activity; wortmannin abolished the stimulatory effect of benzylamine and vanadate on glucose transport in adipose cells. Furthermore, the administration of benzylamine and vanadate *in vivo* caused a rapid lowering of plasma glucose levels, which took place in the absence of alterations in plasma insulin. On the basis of these results we propose that SSAO activity regulates glucose transport in adipocytes. SSAO oxidative activity stimulates glucose transport via the translocation of GLUT4 carriers to the cell surface, resulting from a potent tyrosine phosphorylation of IRS-1 and IRS-3 and phosphoinositide 3-kinase activation. Our results also indicate that substrates of SSAO might regulate glucose disposal *in vivo*.

Key words: benzylamine, glucose transport, H_2O_2, tyramine.

INTRODUCTION

Semicarbazide-sensitive amine oxidase (SSAO) constitutes a class of enzymes that catalyse the reaction in which a primary amine is converted into the corresponding aldehyde in the presence of oxygen, with the concomitant production of H_2O_2 and ammonia. The activity is inhibited by semicarbazide owing to the presence of a cofactor containing one or more carbonyl groups and is resistant to the acetylenic compounds clorgyline, pargyline and selegiline, inhibitors of mitochondrial monoamine oxidases. SSAOs are found in a wide variety of tissues such as in the vasculature, both in smooth-muscle cells and in endothelial cells [1–3], rat articular cartilage, bovine eye and pig dental pulp [4–9]. A high level of SSAO activity has also been found in rat adipocytes [10]. With regard to subcellular localization it has been recently reported that most of the SSAO expressed in rat adipocytes is found in plasma membrane [11,12], suggesting that the enzyme might metabolize extracellular amines. SSAO is an example of multifunctional proteins that, besides displaying amine oxidase activity, show vascular adhesion properties [13,14].

Several isoforms of SSAO have been cloned in human, rodent and bovine tissues. Thus a human placenta SSAO and a human retina-specific amine oxidase showing 65% similarity at the protein level have been cloned and sequenced [15–17]. Furthermore, two distinct bovine SSAO genes encoding a lung SSAO and a (presumably) serum SSAO have been identified [18,19]. On the basis of the fact that a partial clone corresponding to SSAO expressed in rat adipose cells shows 95% similarity to the mouse counterpart of human placenta SSAO [11,13,20], it is likely that adipocyte SSAO is the counterpart of human placental SSAO.

Recently we have demonstrated a stimulatory role of fat-cell SSAOs on glucose transport and GLUT4 translocation to the cell surface [12,21]. Benzylamine, a synthetic substrate of SSAO, and tyramine, an endogenous substrate of both SSAO and monoamine oxidase, stimulate markedly glucose uptake, and promote GLUT4 recruitment to the plasma membrane. The effects of benzylamine and tyramine are observed only in the presence of low concentrations of vanadate that are themselves unable to modify the basal glucose-transport rate in adipose cells [12,21]. Because the response to amines plus vanadate was sensitive to semicarbazide and also to catalase, we proposed that

Abbreviations used: FBS, fetal bovine serum; IRS, insulin receptor substrate; PI-3K, phosphoinositide 3-kinase; SSAO, semicarbazide-sensitive amine oxidase.

[1] To whom correspondence should be addressed (e-mail azorzano@porthos.bio.ub.es).

amines were able to stimulate glucose uptake through a mechanism dependent on H_2O_2 production, in synergism with vanadate [12,21].

All these results compelled us to characterize further the insulin-mimicking properties of SSAO substrates. In this regard, we have studied the signal pathway triggered by SSAO substrates in combination with vanadate and have explored the possible effect of these agents *in vivo*. Our results indicate that numerous SSAO substrates stimulate glucose transport in combination with vanadate in adipose cells. SSAO substrates cause GLUT4 recruitment to the cell surface. SSAO activity combined with vanadate triggers the activation of insulin receptor, induces the tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-3 and activates phosphoinositide 3-kinase (PI-3K). We propose that SSAO substrates and vanadate might be used to stimulate glucose transport in insulin-sensitive tissues under insulin-resistant conditions.

MATERIALS AND METHODS

Materials

^{125}I-labelled Protein A was purchased from ICN (Irvine, CA, U.S.A.). Enhanced chemiluminescence kit (ECL®) was from Amersham (Arlington Heights, IL, U.S.A.). 2-D-[1,2-^{3}H]Deoxyglucose (26 Ci/mmol) and D-[3-^{3}H]glucose(14 Ci/mmol) came from NEN (Les Ulis, France). [^{32}P]Orthophosphoric acid was from Amersham and [γ-^{32}P]ATP was synthesized as described [22]. Purified pig insulin was a gift from Eli Lilly (Indianapolis, IN, U.S.A.). γ-globulin, goat anti-mouse IgG, poly-(L-lysine), semicarbazide hydrochloride, pargyline, tyramine, benzylamine, sodium orthovanadate and most commonly used chemicals were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Wortmannin was kindly donated by Dr Trevor Payne (Sandoz, Basel, Switzerland). All electrophoresis reagents and molecular mass markers were obtained from Bio-Rad. Enhanced chemiluminescence reagents (super signal substrate) were from Pierce. Rabbit polyclonal antibodies against a C-terminal peptide of IRS-1 were kindly provided by Dr L. Mosthaf (Hagedorn Research Institute, Gentofte, Denmark). Mouse polyclonal phosphotyrosine antibodies (PY20) were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Monoclonal antibody against phosphotyrosine and polyclonal antibodies directed against the p85 subunit of PI-3K were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-GLUT4 antibody (OSCRX) was produced from rabbits after immunization with a peptide corresponding to the last 15 amino acid residues of the C-terminus [23]. A polyclonal antibody against the C-terminus of the rat insulin receptor was kindly donated by Dr Willy Stalmans (Katholieke Universiteit Leuven, Leuven, Belgium).

Preparation of adipocytes for hexose-transport measurements

Adipocytes were isolated from the epididymal fat pads of male Wistar rats (180–220 g) by digestion in KRBH [Krebs–Ringer buffer containing 15 mM sodium bicarbonate, 10 mM Hepes, 2 mM sodium pyruvate and 3.5% (w/v) BSA fraction V] containing 1.5 mg/ml collagenase. After digestion for 35–45 min at 37 °C, isolated fat cells were filtered and washed in KRBH buffer. After preincubation for 45 min at 37 °C, each vial (400 μl) containing the tested drugs received an isotopic dilution of 2-deoxy-D-[^{3}H]glucose, giving a final concentration of 0.1 mM (equivalent to approx. 1 300 000 d.p.m. per vial). Assays were incubated for a further 10 min and then stopped with 100 μl of 100 μM cytochalasin B. The radioactivity incorporated into the cells was counted as described by Olefsky [24], with dinonyl phthalate in order to separate intact fat cells from medium. The extracellular 2-deoxyglucose present in the cell fraction was determined as previously reported [25] and did not exceed 1% of the maximum 2-deoxyglucose transport in the presence of insulin.

Studies in 3T3-L1 cell cultures

3T3-L1 fibroblasts obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) were cultured in DMEM (Dulbecco's modified Eagle's medium) containing a high concentration of glucose and L-glutamine and supplemented with 10% (v/v) calf serum. Cells were maintained and passaged as preconfluent cultures at 37 °C in a humidified incubator under air/CO_2 (19:1). At 2 days after confluence (day 0), differentiation was induced with methylisobutylxanthine (0.5 mM), dexamethasone (0.25 μM) and insulin (5 μg/ml) in DMEM containing high glucose, L-glutamine and 10% (v/v) fetal bovine serum (FBS). After 2 days the methylisobutylxanthine and dexamethasone were removed and insulin was maintained for 2 further days. From day 4 onwards, DMEM and 10% (v/v) FBS were replaced every 2 days. Before each experiment, cell monolayers were incubated in serum-free DMEM for 2 h. Cells were used for experiments between days 8 and 14.

Plasma membrane lawn assays

The plasma membrane lawn assay is a technique previously described in [26,27] to generate highly purified plasma membrane fragments. Detection of the abundance of different proteins in plasma membrane fragments was achieved by using antibodies specific for cytoplasmic epitopes combined with immunofluorescence microscopy.

Isolated rat adipocytes were incubated in KRBH buffer containing 3.5% (w/v) BSA. At the end of the incubation, cells were washed in KRBH buffer without BSA to prevent BSA from sticking to the poly-(L-lysine)-coated coverslip. Washed cells were collected with a plastic Pasteur pipette and distributed in drops on a piece of Parafilm. By gently touching the drops (with the cells on top) with the poly-(L-lysine)-coated coverslips, cells were stuck immediately on it. Coverslips were rinsed once with KHMgE buffer [70 mM KCl/30 mM Hepes/5 mM $MgCl_2$/3 mM EGTA (pH 7.4)] and then were swollen by three rapid washes in hypotonic buffer (1/3 × KHMgE buffer); 1 ml of KHMgE buffer was added and the solution was aspirated up and down with a plastic Pasteur pipette to generate a lawn of plasma membrane fragments attached to the glass.

3T3-L1 adipocytes cultured on glass coverslips were treated as described in the figure legends. At the end of the incubation, cells were washed in PBS followed by treatment for 30 s in PBS containing 0.5 mg/ml poly-(L-lysine). The cells were swollen by three rapid washes in hypotonic buffer (1/3 × KHMgE buffer) and sonicated with a probe sonicator.

The membranes were fixed to the coverslips for 30 min with 3% (v/v) formaldehyde in KHMgE buffer. Cells were then washed three times in PBS and incubated for 30 min in PBS containing 20 mM glycine, followed by incubation for 1 h in PBS containing 10% (v/v) FBS. Coverslips were then incubated with primary antibody OSCRX (1:400) diluted in PBS containing 0.01% FBS for 1 h at room temperature. After being washed with PBS, coverslips were incubated with Oregon Green goat anti-rabbit IgG (Molecular Probes) for 1 h. Cells were washed three times with PBS before being mounted and viewed. Confocal images were obtained with a Leica TCS 4D laser confocal fluorescence microscope with a 63 × objective.

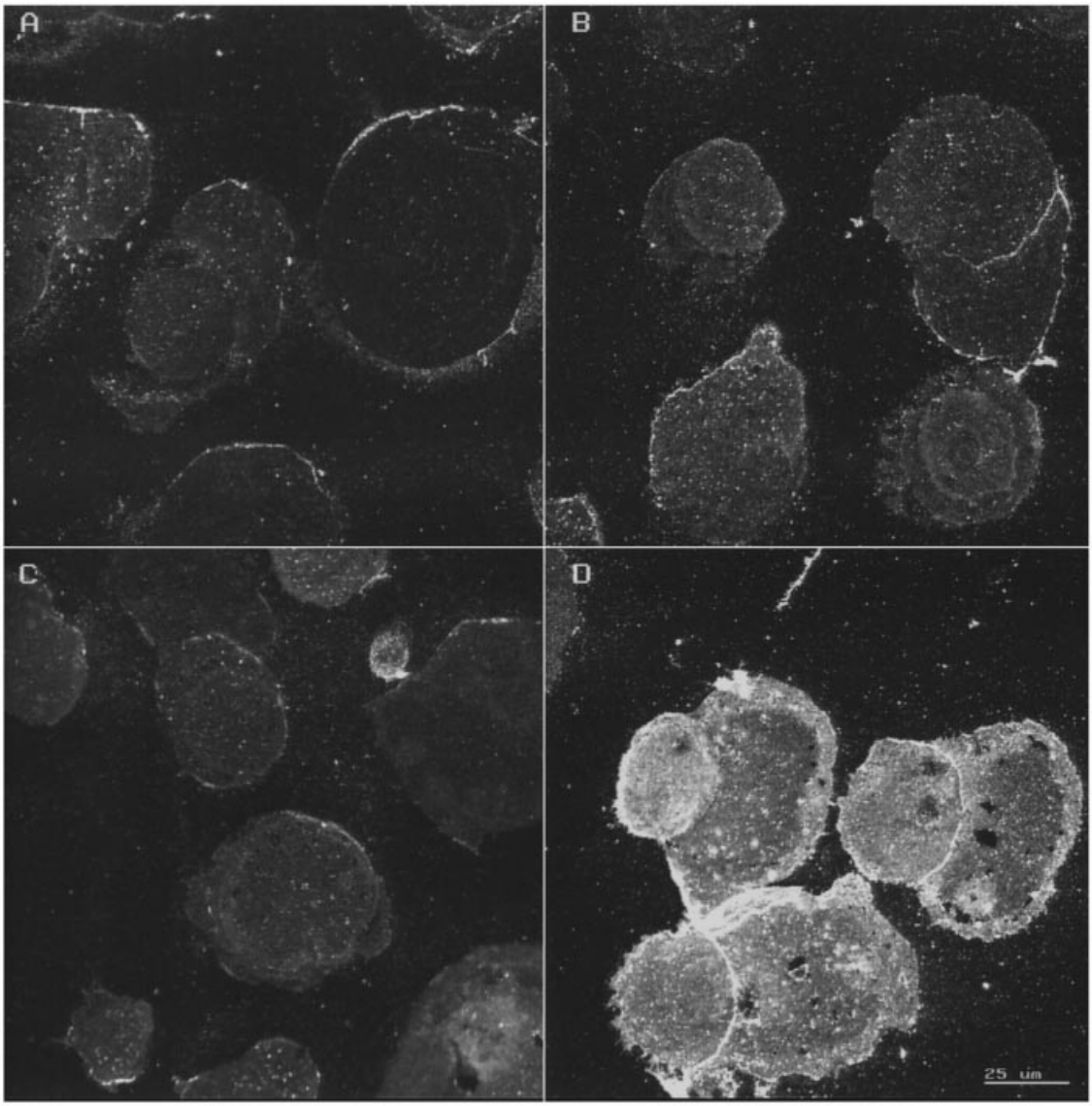

Figure 1 Benzylamine and vanadate recruit GLUT4 to the cell surface in isolated rat adipocytes

Adipocytes were incubated for 45 min in the absence (**A**) or in the presence (**B**) of 0.1 mM benzylamine alone, 0.1 mM vanadate alone (**C**) or a combination of both (**D**) before they were subjected to processing to obtain plasma membrane lawns. Immunofluorescence assays were performed on plasma membrane lawns by using specific anti-GLUT4 antibodies. Representative images are shown. Scale bar, 25 μm (for all panels).

Preparation of adipocytes for immunoprecipitation and immunoblotting

Isolated fat cells were washed with Krebs–Ringer medium, pH 7.4, containing 25 mM Hepes, 200 nM adenosine, 2 mM glucose and 1 % (v/v) BSA (KRH buffer). Adipocytes [2 ml of 10–12 % (v/v) cell suspension] were incubated in KRH buffer at 37 °C under shaking (120 cycles/min) with the drugs at the concentrations and times indicated.

Homogenization buffer (2 vol.) containing 10 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 100 mM Na_3VO_4, 0.1 mM diisopropyl fluorophosphate, 1 μg/ml pepstatin A, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 mM NaF and 20 mM sodium pyrophosphate was added to the adipocyte incubations to stop the reactions. Homogenates free from fat were supplemented with 1 % (v/v) Triton X-100 (final concentration) and solubilized for 1 h at 4 °C. Insoluble material was removed by centrifugation (14000 ***g***) for 10 min at 4 °C. Lysates were incubated with the anti-p85 antibody overnight at 4 °C and with 30 ml of Protein A–Sepharose 4B for 30–60 min at 4 °C. In some other experiments, homogenates were solubilized in the presence of 1 % Nonidet P40; solubilized lysates were immunoprecipitated with anti-phosphotyrosine antibodies bound to Protein G–agarose. The immunocomplexes were washed three times with PBS containing 0.1 % (v/v) *N*-laurylsarcosine, boiled in Laemmli sample buffer and subjected to SDS/PAGE [7 % (w/v) gel]. Proteins were transferred to PVDF membranes (Immobilon-P; Millipore). After being blocked with 0.5 % gelatin in TBST buffer [10 mM Tris/HCl (pH 7.6)/150 mM NaCl/0.1 % (v/v) Tween 20], the membranes were incubated with the appropriate antibodies at a 1:1000 dilution. Immunoblot analysis was performed with the enhanced chemiluminescence system and

horseradish peroxidase conjugated with anti-rabbit or anti-mouse IgG (Amersham). To reprobe the membrane with a second antibody, the membrane was stripped by incubation in 2% (w/v) SDS/62.5 mM Tris/HCl (pH 6.7)/100 mM 2-mercaptoethanol for 30 min at 50 °C. The autoradiograms were quantified by scanning densitometry. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range.

PI-3K assay

PI-3K activity was measured in immunoprecipitates with anti-phosphotyrosine antibody (PY20), as described previously [28]. In brief, immunoprecipitates prepared as mentioned above were washed twice with 25 mM Hepes buffer (pH 7.4)/1% (v/v) Nonidet P40, twice with 100 mM Tris/HCl (pH 7.4)/500 mM LiCl/100 mM Na_3VO_4 and twice with 10 mM Tris/HCl (pH 7.4)/100 mM NaCl/1 mM EDTA/100 mM Na_3VO_4. Finally, immunoprecipitates were resuspended in a final volume of 50 μl of assay buffer containing 40 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, a mixture of phosphatidylinositol and phosphatidylserine at final concentrations of 0.2 and 0.1 mg/ml respectively and 50 mM [γ-^{32}P]ATP (1 μCi). Wortmannin (100 nM) was added to some samples as indicated. After 15 min at 30 °C, the reactions were stopped by adding 40 μl of 4 M HCl and 160 μl of methanol/chloroform (1:1, v/v). The phospholipids in the organic phase were recovered and spotted on a Silica Gel TLC plate precoated with 1% (w/v) KOH/oxalate (Analtech). Migration was performed in methanol/chloroform/water/25% (v/v) NH_4OH (45:35:7:3, by vol.). Phosphatidylinositol 3-phosphate was used as a standard. ^{32}P incorporated into phosphatidylinositol was detected and quantified by a Fujix Bas 2000 system.

Animal studies

Male Wistar rats under post-absorptive conditions (approx. 4 h of fasting) were anaesthetized with sodium pentobarbital (5 mg/100 g body weight, administered intraperitoneal) and anaesthetized rats were administered intravenously with various doses of vanadate (10 or 20 μmol/kg) or benzylamine (7 or 70 μmol/kg) alone or in combination. Four to nine different rats were studied in each experimental group; rats from different groups were interspersed in a randomized manner to diminish the effect of small differences owing to time of sampling. At 30 min after the injection, blood samples were collected from the tail; plasma glucose and insulin concentrations were determined by standard methods.

RESULTS

Substrates of SSAO in combination with vanadate stimulate glucose transport and GLUT4 recruitment in isolated rat adipocytes and in 3T3-L1 adipocytes

We have recently reported that two different SSAO substrates, benzylamine and tyramine, when incubated in the presence of low and inefficient concentrations of vanadate, stimulate glucose transport in isolated rat adipocytes [12,21]. We have further investigated the effects of SSAO substrates in adipose cells. The effects of benzylamine, a selective SSAO substrate, on the rate of glucose transport were studied in adipose cells. Insulin (100 nM) caused a marked stimulation of glucose transport in conditions under which 0.1 mM benzylamine or 0.1 mM vanadate showed no effects (results not shown). However, combination of 0.1 mM benzylamine and 0.1 mM vanadate caused a large stimulation of glucose transport (12-fold increase) and semicarbazide (1 mM) blocked the effect of benzylamine and vanadate (results not shown). No additive effects of insulin and the combination of benzylamine and vanadate on glucose transport were found (results not shown). In parallel with these observations, we found that the combination of 0.1 mM benzylamine and 0.1 mM vanadate caused a marked enhancement in the GLUT4 present in the cell surface of rat adipocytes as assessed by the plasma membrane lawn assay (Figure 1). This effect was not detected in the presence of either 0.1 mM benzylamine or 0.1 mM vanadate alone (Figure 1). Benzylamine together with vanadate did not modify the abundance of GLUT1 on the cell surface (results not shown).

We also studied whether the effects promoted by benzylamine and tyramine were displayed by other SSAO substrates. With this aim we incubated isolated rat adipocytes in the presence of different concentrations (0.1 or 1 mM) of different SSAO substrates such as methylamine, n-decylamine, β-phenylethylamine,

Table 1 Effects of SSAO substrates and vanadate on glucose transport by rat adipocytes

Rat adipocytes were incubated for 45 min in the presence of different SSAO substrates at the indicated concentration without (no addition) or with 0.1 mM sodium vanadate; 2-deoxyglucose uptake was measured over a 10 min period. Results are expressed as a percentage of insulin-stimulated uptake of 2-deoxyglucose. Basal (0%) and 100 nM insulin-stimulated uptake (100%) were equivalent to 1.2 ± 0.1 and 10.6 ± 0.7 nmol/10 min per 100 mg of lipid respectively (n = 53). Glucose uptake by cells incubated in the presence of 0.1 mM vanadate alone or in combination with 100 nM insulin was equivalent to 5.1 ± 1.3% and 101.6 ± 2.5% of the insulin effect. Results are means ± S.E.M. for n (in parentheses) experiments. *Significant difference ($P < 0.05$) between the amine alone and the vanadate groups (analysis of variance followed by Scheffé F test).

Addition to the medium	Concentration	Uptake of 2-deoxyglucose (% of insulin effect)	
		No addition	Vanadate
Methylamine	1 mM	12.1 ± 1.6 (8)	88.0 ± 6.4 (11)*
Benzylamine	0.1 mM	9.3 ± 3.0 (10)	58.9 ± 3.5 (42)*
n-Decylamine	0.1 mM	10.3 ± 4.1 (7)	58.5 ± 9.2 (7)*
Tyramine	1 mM	4.4 ± 1.7 (22)	55.4 ± 3.7 (53)*
β-Phenylethylamine	1 mM	5.9 ± 0.7 (4)	47.5 ± 12.6 (4)*
Histamine	1 mM	7.9 ± 4.8 (4)	32.1 ± 8.2 (9)*
N-Acetylputrescine	1 mM	5.5 ± 1.3 (7)	28.2 ± 5.5 (9)*
Tryptamine	1 mM	13.0 ± 2.4 (7)	25.3 ± 4.1 (9)*

Table 2 Effect of benzylamine and vanadate on glucose transport by 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated for 3.5 h in incubation medium without or with 100 nM insulin, 1 mM benzylamine, 0.1 mM vanadate or a combination of them. Subsequently, 2-deoxyglucose uptake was measured over a 5 min period. Results are means ± S.E.M. for six separate experiments. *Significant difference ($P < 0.05$) between the benzylamine/vanadate and vanadate groups. †Significant difference ($P < 0.05$) between the basal and insulin groups (analysis of variance followed by Scheffé F test).

Group	Uptake of 2-deoxyglucose (nmol/5 min per mg of protein)
Basal	0.22 ± 0.03
Insulin	1.42 ± 0.03†
Vanadate	0.52 ± 0.04
Benzylamine	0.35 ± 0.03
Benzylamine + vanadate	1.12 ± 0.06*

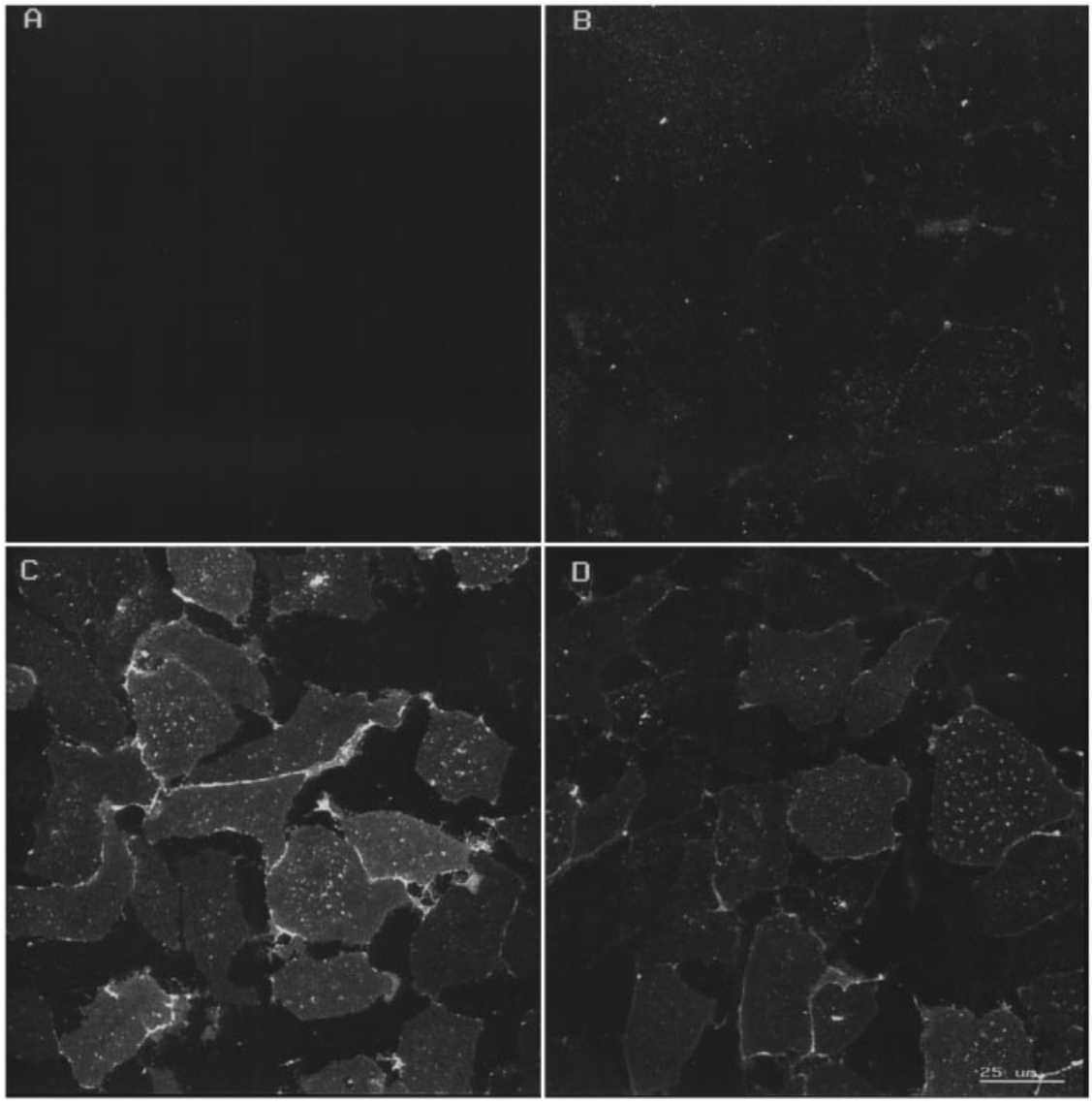

Figure 2 Benzylamine and vanadate recruit GLUT4 to the cell surface in 3T3-L1 adipocytes

Adipocytes were incubated for 3.5 h in the absence (**A**, **B**) or in the presence (**C**) of 100 nM insulin or a combination of 1 mM benzylamine and 0.1 mM vanadate (**D**). Cells were subjected to processing to obtain plasma membrane lawns. Immunofluorescence assays were performed on plasma membrane lawns by using specific anti-GLUT4 antibodies (**B**–**D**) or irrelevant antibodies (**A**). Representative images are shown. Scale bar, 25 μm (for all panels).

histamine, *N*-acetylputrescine or tryptamine [5–10,29–31]. Glucose transport remained unaltered after the incubation of adipose cells in the presence of 0.1 mM substrate (Table 1, and results not shown); the addition to the medium of 1 mM methylamine, n-decylamine or tryptamine caused a small increase in basal glucose transport (Table 1). Furthermore, the combination of SSAO substrates together with 0.1 mM vanadate led to a marked stimulation of glucose transport, ranging from 25 % to 88 % of the maximal stimulation caused by insulin (Table 1). In all, our results indicate that a variety of SSAO substrates markedly stimulate glucose transport when combined with low vanadate concentrations in isolated rat adipocytes, which is due to GLUT4 recruitment to the cell surface.

Next we determined whether the stimulatory effects of SSAO substrates and vanadate was specific to isolated rat adipocytes. With this aim we studied whether a similar pattern of effects occurred in 3T3-L1 adipocytes, an insulin-sensitive adipose cell line. In preliminary experiments we detected SSAO protein in crude membranes obtained from 3T3-L1 adipocytes. Furthermore, we found by subcellular fractionation of membranes from 3T3-L1 adipocytes that SSAO protein was more abundant in plasma membrane than in light microsomes (results not shown) in parallel with previous studies on isolated rat adipocytes [11,12]. Also in keeping with observations performed in isolated rat adipocytes [11,12], we found that the incubation of 3T3-L1 adipocytes for 30 min in the presence of 100 nM insulin did not alter SSAO protein abundance or SSAO activity in intracellular membranes or in plasma membrane preparations (results not shown).

Millimolar concentrations of vanadate stimulate glucose transport in 3T3-L1 adipocytes by a mechanism that requires long-term incubation [32]. In keeping with these observations,

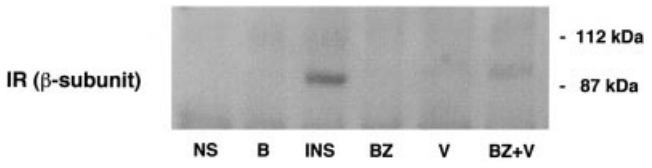

Figure 3 Combined addition of benzylamine and vanadate stimulates insulin receptor phosphorylation in isolated rat adipocytes

Rat adipocytes were incubated for 45 min in incubation medium without or with 100 nM insulin, 0.1 mM benzylamine, 0.1 mM vanadate or a combination of 0.1 mM benzylamine and 0.1 mM vanadate. Cell lysates (400 μg of protein) were incubated with 5 μg of anti-phosphotyrosine antibodies bound to Protein A or with an irrelevant antibody (NS). Immune complexes were subjected to SDS/PAGE and further immunoblotting with a specific antibody directed against the insulin receptor β-subunit. A representative autoradiogram is shown. The positions of molecular mass markers are indicated at the right.

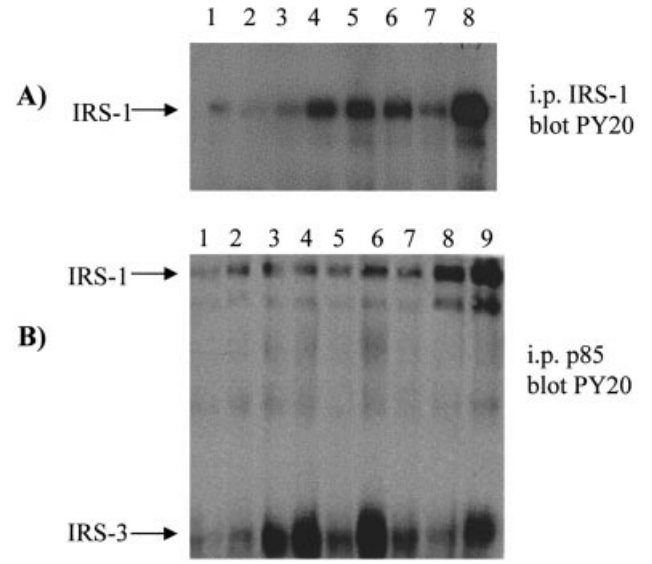

Figure 4 SSAO substrates and vanadate stimulate the tyrosine phosphorylation of IRS-1 and IRS-3

(**A**) Isolated rat adipocytes were treated for 15 min at 37 °C without vanadate (lane 1), with 0.1 mM vanadate (lane 2), with 0.1 mM vanadate plus 1 mM semicarbazide (lane 3), with 1 nM insulin (lane 4), with 1 nM insulin plus 1 mM semicarbazide (lane 5), with 100 nM insulin (lane 8), with 1 mM tyramine plus 0.1 mM vanadate (lane 6) and with tyramine plus vanadate plus semicarbazide (lane 7). After homogenization, proteins from whole-cell lysates were immunoprecipitated (i.p.) with anti-(IRS-1) antibody as described in the Materials and methods section, separated by SDS/PAGE [7% (w/v) gel], transferred to Immobilon-P membranes and probed with the anti-phosphotyrosine PY20 antibody. (**B**) Isolated adipocytes were treated for 15 min at 37 °C without vanadate (lane 1), with 0.1 mM vanadate (lane 2), with 5 mM vanadate (lane 3), with 1 mM tyramine plus 0.1 mM vanadate without (lane 4) or with (lane 5) 1 mM semicarbazide, with 0.1 mM benzylamine plus 0.1 mM vanadate without (lane 6) or with (lane 7) semicarbazide, 1 nM insulin (lane 8) and 100 nM insulin (lane 9). After homogenization, proteins from whole-cell lysates were immunoprecipitated with anti-p85 antibody as described in the Materials and methods section, separated by SDS/PAGE [7% (w/v) gel], transferred to Immobilon-P membranes and probed with the anti-phosphotyrosine PY20 antibody. Representative autoradiograms from five separate experiments are shown. Arrows labelled IRS-1 and IRS-3 indicate the expected electrophoretic mobility of the IRS proteins.

incubation of 3T3-L1 adipocytes in the presence of 0.1 mM vanadate for 30 min caused no alteration in glucose transport, whereas the addition of 1 mM benzylamine or a combination of 1 mM benzylamine and 0.1 mM vanadate caused 1.7-fold and 3-fold stimulations of glucose transport respectively (results not shown). The effect of incubation with the different compounds

Table 3 Effect of SSAO substrates and vanadate on PI-3K activity

Isolated adipocytes were treated for 15 min at 37 °C without or with 0.1 mM vanadate, 1 nM insulin, 1 mM tyramine plus 0.1 mM vanadate or 0.1 mM benzylamine plus 0.1 mM vanadate. After homogenization, whole-cell lysates were subjected to immunoprecipitation with PY20 antibody. A PI-3K assay was then performed in the absence or the presence of 100 nM wortmannin. Phospholipids were separated by TLC as described in the Materials and methods section. The incorporation of ^{32}P into phosphatidylinositol 3-phosphate was detected by digital imaging of ^{32}P. Quantification of labelled phosphatidylinositol 3-phosphate was performed with a Fujix Bas 2000 system. Results are expressed as percentages of the maximal activity induced by 1 nM insulin (equivalent to 1.1 ± 0.1 arbitrary units). Results are means ± S.E.M. for four to seven independent experiments. The statistical significance of the differences from the control was assessed by analysis of variance followed by Scheffé F test. *$P < 0.05$.

Addition to the medium	PI-3K activity (% of insulin-stimulated levels)
Control	32.6 ± 5.5
Vanadate	51.1 ± 9.7
Insulin + wortmannin	44.1 ± 6.7
Benzylamine + vanadate	81.6 ± 20.5*
Benzylamine + vanadate + wortmannin	48.6 ± 13.3
Tyramine + vanadate	67.9 ± 16.1*
Tyramine + vanadate + wortmannin	42.1 ± 10.8

Table 4 Effect of wortmannin on glucose transport stimulated by the combination of benzylamine and vanadate in isolated rat adipocytes

Rat adipocytes were incubated for 45 min in incubation medium without or with 100 nM insulin, a combination of 0.1 mM benzylamine and 0.1 mM vanadate or 1 mM vanadate alone. Incubations were performed in the absence (control) or presence of 1 μM wortmannin. Subsequently, uptake of 2-deoxyglucose was measured over a 10 min period. Results are means ± S.E.M. for six separate experiments. *Significant difference ($P < 0.05$) between control and wortmannin-treated groups (Student's t test).

	Uptake of 2-deoxyglucose (nmol/10 min per 100 mg of lipid)	
Addition to the medium	Control	Wortmannin
Basal	2.2 ± 0.1	1.2 ± 0.1
Insulin	12.1 ± 0.7	1.7 ± 0.1
Benzylamine + vanadate	6.6 ± 0.6	2.0 ± 0.1
Vanadate	8.8 ± 0.7	2.1 ± 0.1

for longer time (3.5 h) in 3T3-L1 adipocytes is shown in Table 2. Adipocytes responded to insulin with a large stimulation of glucose transport (14-fold stimulation). Vanadate (0.1 mM) or benzylamine (1 mM) alone moderately stimulated glucose transport (25% and 11% of the maximal stimulation caused by insulin) (Table 2); the combination of both benzylamine and vanadate caused a synergistic activation of glucose transport (73% of maximal insulin-stimulated glucose transport) (Table 2). The synergistic effect of benzylamine and vanadate in 3T3-L1 adipocytes was inhibited by the presence of 1 mM semicarbazide (results not shown). An analysis of GLUT4 abundance at the cell surface of 3T3-L1 adipocytes, as assessed by plasma membrane lawn assays, revealed that the combination of 1 mM benzylamine and 0.1 mM vanadate caused a marked enrichment in GLUT4 (Figure 2). In keeping with the glucose transport data, the recruitment of GLUT4 to the cell surface caused by benzylamine/vanadate was smaller than that triggered by insulin (Figure 2).

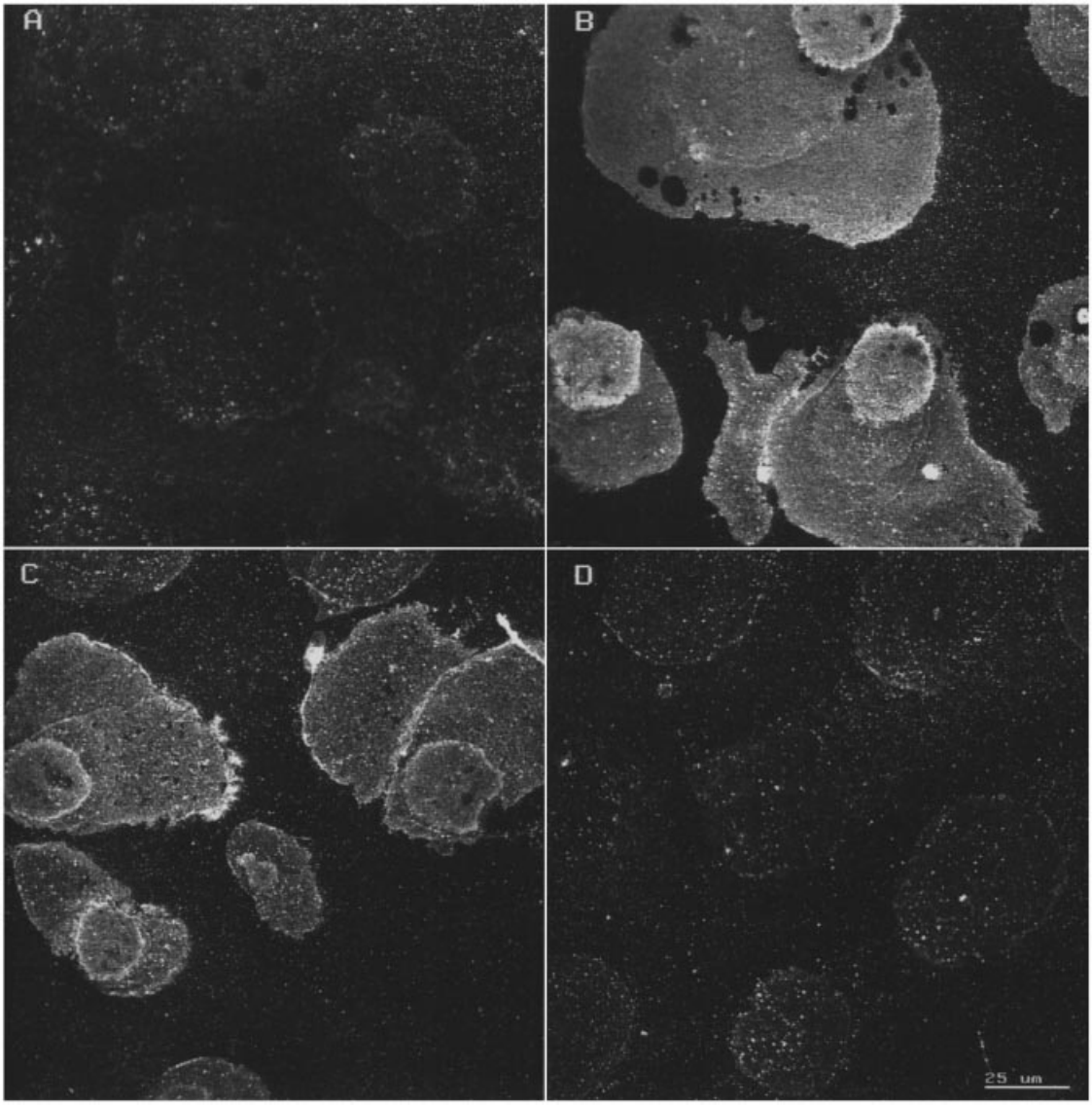

Figure 5 Wortmannin blocks the recruitment of GLUT4 to the cell surface in response to the combined addition of benzylamine and vanadate in isolated rat adipocytes

Adipocytes were incubated for 45 min in the absence (**A**) or in the presence (**B**) of 100 nM insulin, a combination of 0.1 mM benzylamine and 0.1 mM vanadate (**C**) or benzylamine and vanadate together with 1 μM wortmannin (**D**). Thereafter, cells were subjected to processing to obtain plasma membrane lawns. Immunofluorescence assays were performed on plasma membrane lawns by using specific anti-GLUT4 antibodies. Representative images are shown. Scale bar, 25 μm (for all panels).

Benzylamine and vanadate induce tyrosine phosphorylation of IRS proteins and activation of PI-3K in isolated rat adipocytes

To assess the nature of the mechanisms involved in the effects of SSAO substrates, we examined the effect of the combination of SSAO substrates and vanadate on insulin receptor, IRS proteins or PI-3K in isolated rat adipocytes. The effect of incubation for 30 min with insulin and the combination of 0.1 mM benzylamine and 0.1 mM vanadate on insulin receptor phosphorylation was determined by immunoprecipitation of cell lysates with anti-phosphotyrosine antibodies, followed by immunoblotting of insulin receptor β-subunit (Figure 3). Insulin caused a marked stimulation of tyrosine phosphorylation of insulin receptor β-subunit (Figure 3). Neither 0.1 mM benzylamine nor 0.1 mM vanadate alone enhanced the tyrosine phosphorylation of insulin receptor (Figure 3) and the combination of 0.1 mM benzylamine and 0.1 mM vanadate caused only a weak increase in the tyrosine phosphorylation of insulin receptor β-subunit in adipose cells (Figure 3); this amounted to 15% of the tyrosine phosphorylation detected after incubation with supramaximal insulin concentrations.

Next we studied whether the combination of SSAO substrates with vanadate stimulated the tyrosine phosphorylation of IRS proteins. First, IRS-1 was immunoprecipitated from whole-cell lysates with IRS-1 antibody; its phosphotyrosine content was analysed by Western blotting with PY20 antibody (Figure 4A). As expected, 1 nM insulin induced a marked tyrosine phosphorylation of IRS-1, whereas 0.1 mM vanadate or 1 mM tyramine did not (results not shown). However, the combination of 0.1 mM vanadate and 1 mM tyramine promoted a significant

increase in tyrosine phosphorylation of IRS-1 [means ± S.E.M. of tyrosine phosphorylation were 360 ± 69 ($n = 5$) and 1677 ± 511 ($n = 5$) arbitrary units in the basal and tyramine/vanadate groups respectively; $P < 0.05$) (Figure 4A). Moreover, the IRS-1 phosphorylation induced by the combination of vanadate and tyramine was blocked by 1 mM semicarbazide (Figure 4A).

Under stimulation with insulin, the regulatory subunit (p85) of PI-3K rapidly associates with IRS-1 as well as with IRS-2 and IRS-3 [33,34]. To determine whether p85 co-immunoprecipitates with IRS-1 and other IRS proteins in response to the combination of SSAO substrates together with vanadate, p85 was immunoprecipitated with an anti-p85 antibody and subjected to Western blotting with PY20 antibody. In keeping with previous observations [35,36], after stimulation with insulin, phosphorylated IRS-1 and IRS-3 co-immunoprecipitated with p85 (Figure 4B). The combination of vanadate and tyramine and also the combination of vanadate and benzylamine caused a greater tyrosine phosphorylation of IRS-3 than IRS-1 (Figure 4B). Moreover, SSAO substrates in combination with vanadate induced a stronger tyrosine phosphorylation of IRS-3 than did insulin (1 and 100 nM) (Figure 4B). Vanadate alone was able to induce the tyrosine phosphorylation of IRS-3 but only at high concentration (5 mM); at low concentrations (0.1 mM) the effects were not significantly different from basal levels (210 ± 65 and 100 ± 19 arbitrary units for vanadate and control groups respectively). The synergistic effect of SSAO substrates and vanadate on IRS proteins was blocked by 1 mM semicarbazide. Under these conditions, IRS-2 tyrosine phosphorylation was not detectable (results not shown).

On the basis of the fact that the association of PI-3K with IRS proteins has been shown to activate PI-3K in response to insulin [35] and because our results indicated that IRS-1 and IRS-3 co-immunoprecipitated with p85 in response to the combination of SSAO substrates with vanadate, we measured PI-3K activity in PY20 immunoprecipitates from adipocytes stimulated by insulin, vanadate or a combination of vanadate and benzylamine or tyramine (Table 3). Benzylamine plus vanadate as well as tyramine plus vanadate significantly increased PI-3K activity, whereas 0.1 mM vanadate did not. The activation of PI-3K induced by these different treatments was inhibited by 100 nM wortmannin, a potent inhibitor of PI-3K (Table 3).

In keeping with these observations, wortmannin caused a complete inhibition of insulin-stimulated glucose transport and also glucose transport induced by the combination of benzylamine and vanadate (Table 4). Under these conditions, wortmannin also inhibited the stimulation of glucose transport promoted by insulin or by 1 mM vanadate (Table 4). Furthermore, wortmannin fully prevented the recruitment of GLUT4 to the cell surface stimulated by 0.1 mM benzylamine plus 0.1 mM vanadate (Figure 5), suggesting that SSAO substrates activate glucose transport via a PI-3K-dependent pathway.

Effects of the combined administration *in vivo* of benzylamine and vanadate

Lastly we assessed whether the combination of benzylamine and vanadate could affect plasma glucose concentrations in normal rats. With this aim male Wistar rats were injected intravenously with different doses of benzylamine or vanadate; 30 min later, plasma glucose and insulin were analysed. The administration of 7 or 70 μmol/kg benzylamine alone or the intravenous injection of 10 or 20 μmol/kg vanadate alone [36] did not alter the plasma concentrations of glucose or insulin (Table 5). Again, the combination of 10 μmol/kg vanadate and 7 μmol/kg benzylamine did not alter glucose or insulin concentrations in plasma (Table 5). However, the combined intravenous administration of 7 μmol/kg benzylamine and 20 μmol/kg vanadate caused a significant decrease in plasma glucose levels (16% decrease), which occurred in the absence of alterations in circulating insulin (Table 5). The effects of the combined administration of 7 μmol/kg benzylamine and 20 μmol/kg vanadate on plasma glucose persisted for at least 75 min (results not shown).

Table 5 Effect of the administration of benzylamine and vanadate *in vivo* on plasma concentrations of glucose and insulin in rats

Postabsorptive male Wistar rats were administered intravenously with various doses of vanadate or benzylamine alone or in combination. At 30 min after the injection, blood samples were collected from the tail; plasma glucose and insulin concentrations were determined by standard methods. Results are means ± S.E.M. for four to nine independent observations. Abbreviations: n.d., not determined; b.w., body weight. *Statistically significant difference ($P < 0.05$) from the control group (Student's t test).

Injection	Glucose (mM)	Insulin (ng/ml)
None (control)	5.5 ± 0.2	0.7 ± 0.3
Vanadate (10 μmol/kg b.w.)	6.1 ± 0.4	0.9 ± 0.3
Vanadate (20 μmol/kg b.w.)	5.1 ± 0.4	0.8 ± 0.2
Benzylamine (7 μmol/kg b.w.)	5.4 ± 0.2	0.6 ± 0.1
Benzylamine (70 μmol/kg b.w.)	5.4 ± 0.4	0.9 ± 0.2
Benzylamine (7 μmol/kg b.w.) + Vanadate (10 μmol/kg b.w.)	5.4 ± 0.3	N. D.
Benzylamine (7 μmol/kg b.w.) + Vanadate (20 μmol/kg b.w.)	4.6 ± 0.3*	0.7 ± 0.2

DISCUSSION

The results of this study demonstrate that many different substrates of SSAO in combination with low vanadate concentrations stimulate glucose transport in rat and in 3T3-L1 adipose cells. This enhanced glucose transport is characterized by the recruitment of GLUT4 glucose transporters to the cell surface. As regards the nature of the mechanisms involved, we have demonstrated that SSAO substrates together with vanadate markedly stimulate the tyrosine phosphorylation of IRS-1 and IRS-3 and also PI-3K activity, and that this occurs in the presence of a modest insulin receptor phosphorylation. Taken together, our results suggest that SSAO substrates and vanadate synergistically stimulate one or several tyrosine protein kinases or inhibit protein tyrosine phosphatases, leading to the activation of an intracellular pathway similar to that triggered by insulin that leads to GLUT4 glucose-transporter translocation. Furthermore, we have found that the acute administration of benzylamine and vanadate *in vivo* lowers plasma glucose levels in the absence of alterations in plasma insulin, suggesting an effect on whole-body glucose disposal.

Interestingly, we have found that different SSAO substrates such as methylamine, benzylamine, n-decylamine, tyramine, β-phenylethylamine, histamine, *N*-acetylputrescine or tryptamine stimulate glucose transport in the rat adipose cell. This strengthens the view that SSAO substrates stimulate glucose transport as a consequence of H_2O_2 production rather than as a consequence of aldehyde production; this is in agreement with previous observations indicating that the effects of benzylamine or tyramine are blocked by the concomitant addition of catalase to the incubation medium [12,21]. Furthermore, and on the basis of the effect of the combined administration of benzylamine and vanadate decreasing plasma glucose levels in normal rats, we consider that the use of some SSAO substrates in synergism with

low doses of vanadate might be effective as a treatment in insulin-resistant states.

Here we have observed that the combination of SSAO substrates and vanadate stimulates the tyrosine phosphorylation of the insulin receptor and IRS proteins and also activates PI-3K, crucial components of the insulin signal transduction. Benzylamine and vanadate caused a weak stimulation of tyrosine phosphorylation of the insulin receptor, suggesting that the insulin receptor tyrosine kinase might not be the main tyrosine kinase involved in SSAO-dependent signalling. In addition, we have shown that the combination benzylamine/vanadate or tyramine/vanadate stimulates the tyrosine phosphorylation of IRS proteins. This is concluded from the following observations: (1) SSAO substrates and vanadate markedly stimulate the tyrosine phosphorylation of immunoprecipitated IRS-1, and (2) SSAO substrates and vanadate stimulate the tyrosine phosphorylation of IRS-1 and IRS-3 co-immunoprecipitated with the p85 subunit of PI-3K.

IRS-3, a 60 kDa phosphotyrosine protein, is a recently identified member of the IRS family [37,38] that interacts rapidly with the p85 subunit of PI-3K after stimulation by insulin in rat adipocytes [34]; it is predominantly involved in regulating PI-3K in the absence of IRS-1 [39]. In this regard, our results indicate that IRS-3 is the main IRS protein activated in response to SSAO substrates and vanadate, whereas IRS-1 is predominant after stimulation with insulin. To our knowledge this is the first report showing that insulin-like agents such as SSAO substrates in combination with vanadate potently induce the tyrosine phosphorylation of IRS-3. Our conditions did not allow us to detect the tyrosine phosphorylation of IRS-2 but this did not exclude the fact that IRS-2 could be involved in mediating transient signals, as reported previously [40].

The p85/p110 PI-3K activity is also stimulated by the combination of SSAO substrates and vanadate; this participates in the stimulation of GLUT4 recruitment to the cell surface. This conclusion is based on the following observations: (1) the p85 subunit of PI-3K associates with activated IRS-1 and IRS-3 after incubation with vanadate and benzylamine or tyramine, (2) PI-3K activity is enhanced by benzylamine/vanadate or tyramine/vanadate in p85 immunoprecipitates, and (3) wortmannin completely abolishes the stimulation triggered by benzylamine/vanadate on glucose transport or GLUT4 recruitment in adipose cells. Because p85 co-immunoprecipitates with IRS-1 and IRS-3, we also conclude that the PI-3K activation detected in our study must be due both to the association between p85 and IRS-3 and between p85 and IRS-1. The association between p85 and IRS-3 might be predominant in PI-3K activation in rat adipocytes treated with the combination of benzylamine and vanadate.

A critical question regarding the stimulation of GLUT4 transporter translocation to the cell surface in rat adipose cells or in 3T3-L1 adipocytes is the nature of the active molecules that are generated by the catalysis of SSAO in the presence of vanadate. On the basis of the fact that H_2O_2 production is crucial for the triggering of these effects, because catalase blocks the effects [12,21] and provided that peroxovanadate is a very potent insulin-like agent [41–43], it seems feasible to propose that peroxovanadate is formed either extracellularly or in the intracellular milieu and gives rise to the effects caused by the combination of vanadate and SSAO substrates. In this regard, a formation of peroxovanadate *in situ* offered by the combination of SSAO substrates and vanadate would be advantageous over the administration of exogenous peroxovanadate. Nevertheless, there are some difficulties in attributing the effects of SSAO substrates to merely the formation of peroxovanadate compounds because of the following observations: (1) insulin and peroxovanadate have been reported to cause additive effects on glucose transport in isolated rat adipocytes [44] and we found no additive effects on glucose transport in the presence of insulin and benzylamine/vanadate or tyramine/vanadate; and (2) peroxovanadate has been reported to activate insulin receptor kinase activity markedly in rat adipocytes, in contrast with vanadate, which does not [41,45–48], and the combination of benzylamine and vanadate causes a modest stimulation of insulin receptor kinase as assessed by tyrosine phosphorylation. In all, it might be that an active compound different from peroxovanadate is formed in the presence of SSAO substrates and vanadate, which leads to the activation of anabolic metabolic processes in adipose cells. In any case, we cannot rule out the possibility that SSAO activity stimulates the metabolic activity of adipose cells via vanadate-independent pathways.

In summary, our results are consistent with a model by which SSAO activity in the presence of vanadate generates unknown compounds that trigger the activation of insulin receptor kinase as well as the activation of other unknown protein tyrosine kinase activities or the inhibition of protein tyrosine phosphatases. This causes the activation of IRS-1 and IRS-3 and the concomitant activation of p85/p110 PI-3K. We propose that the activation of these signalling molecules by the combination of SSAO substrates and vanadate causes GLUT4 recruitment to the cell surface and the stimulation of glucose transport in adipose cells. We also indicate that substrates of SSAO might regulate whole-body glucose disposal and, in this connection, our results support the feasibility of the future design of SSAO-based therapy in insulin-resistant conditions.

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Combined Treatment With Benzylamine and Low Dosages of Vanadate Enhances Glucose Tolerance and Reduces Hyperglycemia in Streptozotocin-Induced Diabetic Rats

Luc Marti,[1] Anna Abella,[1] Christian Carpéné,[2] Manuel Palacín,[1] Xavier Testar,[1] and Antonio Zorzano[1]

Semicarbazide-sensitive amine oxidase (SSAO) is highly expressed in adipose cells, and substrates of SSAO, such as benzylamine, in combination with low concentrations of vanadate strongly stimulate glucose transport and GLUT4 recruitment in 3T3-L1 and rat adipocytes. Here we examined whether acute and chronic administration of benzylamine and vanadate in vivo enhances glucose tolerance and reduces hyperglycemia in diabetic rats. Acute intravenous administration of these drugs enhanced glucose tolerance in nondiabetic rats and in streptozotocin (STZ)-induced diabetic rats. This occurred in the absence of changes in plasma insulin concentrations. However, the administration of benzylamine or vanadate alone did not improve glucose tolerance. The improvement caused by benzylamine plus vanadate was abolished when rats were pretreated with the SSAO-inhibitor semicarbazide. Chronic administration of benzylamine and vanadate exerted potent antidiabetic effects in STZ-induced diabetic rats. Although daily administration of vanadate alone (50 and 25 μmol · kg^{-1} · day^{-1} i.p.) for 2 weeks had little or no effect on glycemia, vanadate plus benzylamine reduced hyperglycemia in diabetic rats, enhanced basal and insulin-stimulated glucose transport, and upregulated GLUT4 expression in isolated adipocytes. In all, our results substantiated that acute and chronic administration of benzylamine with low dosages of vanadate have potent antidiabetic effects in rats. *Diabetes* 50:2061–2068, 2001

The semicarbazide-sensitive amine oxidase (SSAO) belongs to the large family of copper-containing amine oxidases (EC 1.4.3.6) that convert primary amines to aldehydes, with the concomitant production of hydrogen peroxide and ammonia. These proteins are characterized by broad substrate selectivity among species, which makes it difficult to ascertain their biological function. The enzyme readily oxidizes exogenous (e.g., benzylamine, tyramine) or endogenous (e.g., phenylethylamine, histamine) aromatic primary amines, but also endogenous (e.g., methylamine, aminoacetone) aliphatic primary amines (1). More recently, a new function has been assigned to SSAO: the vascular adhesion protein-1 (VAP-1), found to be identical to SSAO, belongs to the family of adhesive proteins implicated in processes like inflammation or cell-to-cell interaction (2–4). The relation between the enzymatic and adhesive functions of SSAO/VAP-1 remains to be determined.

Adipose tissue contains high levels of SSAO (5–9), and an increase in the expression of the membrane-bound SSAO has been reported in adipocyte differentiation (8,10). As to the function of adipocyte SSAO, hydrogen peroxide, one of the reaction products, has insulinomimetic properties (11). Moreover, membrane fractionation and vesicle immunoisolation analysis have shown that a portion of the SSAO protein or enzymatic activity colocalizes with intracellular GLUT4-containing vesicles (5,12). We recently reported that substrates of SSAO can stimulate glucose transport in rat adipocytes (12–14). The amine-induced stimulation of glucose transport was observed in the presence of ineffective concentrations of vanadate and was abolished by semicarbazide and catalase (12,13). These observations suggested that the SSAO-dependent generation of hydrogen peroxide is responsible for the increased stimulation of glucose transport via a chemical interaction. The combination of vanadate and hydrogen peroxide can form peroxovanadate, a powerful insulinomimetic agent that may be partly responsible for this effect (13,15). In addition, the combination of SSAO substrates with vanadate stimulates glucose transport through translocation of GLUT4 to the adipocyte cell surface (12,13). Furthermore, the combination of SSAO substrates and vanadate stimulates phosphatidylinositol (PI) 3-kinase activity and tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and -3 in conditions in which the insulin receptors themselves are only slightly activated (14). All these data support the view that the combination of SSAO substrates and vanadate stimulates glucose transport via a pathway that does not involve the insulin receptor.

From the [1]Departament de Bioquìmica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain; and [2]Institut National de la Santé et de la Recherche Médicale (INSERM U 317), Toulouse, France.

Address correspondence and reprint requests to Antonio Zorzano, Ph.D., Departament de Bioquìmica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, E-08028 Barcelona, Spain. E-mail: azorzano@porthos.bio.ub.es.

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L.M. and A.A. contributed equally to the study.

2-DG, 2-deoxy-D-[1,2-^{3}H]glucose; IRS, insulin receptor substrate; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PTP, protein tyrosine phosphatase; SSAO, semicarbazide-sensitive amine oxidase; STZ, streptozotocin; VAP-1, vascular adhesion protein-1.

In this study, we examined the acute and chronic effects of benzylamine and vanadate on control and diabetic rats. We demonstrated potent antidiabetic properties of benzylamine and vanadate in streptozotocin (STZ)-induced diabetic rats.

RESEARCH DESIGN AND METHODS

Materials. We obtained 2-deoxy-D-[1,2-^{3}H]glucose (2-DG; 26 Ci/mmol) from NEN Life Science Products and [^{14}C]benzylamine (59 Ci/mmol) from Amersham Pharmacia Biotech (Arlington Heights, IL). Purified porcine insulin was a kind gift from Eli Lilly (Indianapolis, IN). STZ, 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) and collagenase type I from Worthington. The osmotic minipumps used in the chronic studies were from Alza. All electrophoresis reagents and molecular weight markers were obtained from Bio-Rad. Enhanced chemiluminescence reagents (super signal substrate) were obtained from Amersham. Anti-GLUT4 antibody (OSCRX) was produced from rabbit, as previously reported (15). Rabbit polyclonal antibodies against rat $\hat{a}_1$-integrin were kindly given by Dr. C. Enrich (Universitat de Barcelona).

Animals. Male Wistar rats weighing 180–220 g were purchased from Harlan (Interfauna Ibèrica S.A., Spain). The animals were housed in animal quarters at 22°C with a 12-h light/dark cycle and were fed ad libitum, unless otherwise stated. Type 1 diabetes was induced by a single intraperitoneal injection of a freshly prepared solution of STZ (45–70 mg/kg body wt dissolved in 50 mmol/l citrate buffer [pH 4.5]). Only diabetic animals with glycemia >300 mg/dl were used. Inhibition of SSAO activity was obtained in vivo by administration of semicarbazide (5 mg · kg^{-1} · day^{-1} i.p.) for 3 days. All procedures used were approved by the Ethics Committee of the University of Barcelona.

Glucose tolerance tests. Anesthetized (pentobarbital 80 mg/kg body wt) control rats or nonanesthetized diabetic rats were injected intravenously via the tail vein with vehicle (phosphate-buffered saline [PBS], pH 7.4) or with 7 μmol/kg body wt benzylamine and/or 20 μmol/kg sodium orthovanadate. In anesthetized control rats, an intravenous (saphenous vein) glucose tolerance test (0.8 g/kg body wt) (16) was carried out 30 min after administration of benzylamine, vanadate, or both. Blood samples were collected before (0 min) and 5, 10, 15, 20, 30, and 45 min after glucose administration. In other studies of diabetic rats, an oral glucose tolerance test (2 g/kg body wt) (17) was carried out 15 min after injection of benzylamine, vanadate, or both. Blood samples were collected before and 15, 30, 45, 60, 90, and 150 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 treatment of diabetic animals. Osmotic minipumps containing benzylamine (84 μmol · kg^{-1} body wt · day^{-1}) were implanted subcutaneously in STZ-induced 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 or 50 μmol/kg body wt) or the vehicle (PBS) was administered daily at 9:00 A.M. Food was removed after the administration of vanadate and replaced at 2:00 P.M. Glycemia was measured in arteriovenous blood collected from the tail vessels at 9:00 A.M., before the administration of vanadate, for 14 days. Insulin concentrations were determined before and after treatment.

Glucose transport measurements in isolated rat adipocytes and preparation of membrane extracts. Adipocytes were isolated from epididymal fat pads in nondiabetic and diabetic male Wistar rats (180–220 g), as previously reported (12,13). After a preincubation period of 45 min at 37°C, each vial, containing 400 μl of cell suspension in Krebs-Ringer bicarbonate buffer and the drugs being tested, received an isotopic dilution of 2-DG in a final concentration of 0.1 mmol/l, equivalent to ~1,300,000 dpm/vial. 2-DG transport assays were performed as previously reported (12,13). 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 previously reported (12). Protein concentrations were determined by the Bradford method (18), with γ-globulin as a standard.

Amine oxidase activity assays. The radiochemical determination of amine oxidase activity was performed basically as described by Fowler and Tipton (19), with slight modifications (13).

Electrophoresis and immunoblot analysis. SDS-PAGE was performed on membrane proteins following the method of Laemmli (20). Proteins were transferred to etorphine hydrochloride (Immobilon) in buffer consisting of 20% methanol, 200 mmol/l glycine, and 25 mmol/l Tris (pH 8.3). After transfer, the filters were blocked with 5% nonfat milk in PBS for 1 h at 37°C and then incubated with polyclonal antibodies raised against GLUT-4 or β_1-integrin. The immune complex was detected by enhanced chemiluminescence Western blot, in the linear response range.

Analytical methods. The glucose concentration in plasma or urine was determined by the glucose oxidase method (MPR-3 glucose/GOD-PAP Method; Boehringer) in glucose-tolerance experiments and with a rapid glucose analyzer (Accutrend Sensor Comfort; Roche) in chronic treatments. The plasma immunoreactive insulin concentration was determined with a sensitive rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO).

Calculations and statistical analysis. Insulin and glucose responses during the glucose tolerance tests were calculated as the incremental plasma values integrated over a 45-min period in control animals and 150-min period in diabetic animals after the injection of glucose. Areas under the curve for insulin and glucose responses were calculated using the Graph Prism program (GraphPad Software). Data are presented as means ± SE; an 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 analysis of variance and further post hoc Dunnett's or Tukey's *t* tests.

RESULTS

Acute administration of benzylamine and vanadate enhanced glucose tolerance in nondiabetic rats. We have previously reported that in vitro substrates of SSAO, such as benzylamine or tyramine, in combination with low concentrations of vanadate strongly stimulate glucose transport and GLUT4 recruitment in 3T3-L1 and rat adipocytes (12,13). In this study, we examined whether the combination of SSAO substrates and vanadate exhibit insulin-like effects in vivo.

Control anesthetized nondiabetic rats were injected via the tail vein with vehicle (PBS) or 7 μmol/kg benzylamine and/or 20 μmol/kg sodium orthovanadate. Glucose load (0.8 g/kg body wt by saphenous vein) was carried out 30 min after administration of benzylamine, vanadate, or both. Acute intravenous administration of vanadate did not alter glucose tolerance (Fig. 1*A*), and acute administration of benzylamine only caused reduced glycemia values 5 min after glucose injection, but the integrated glucose area under the curve remained unaltered (Fig. 1*B* and *D*). In the same conditions, administration of benzylamine plus vanadate reduced glycemia at different times after glucose injection (Fig. 1*C*) and increased glucose tolerance by 35% (Fig. 1*D*). No changes in glucose concentrations in urine were detected between the control and benzylamine + vanadate groups (0.38 ± 0.07 vs. 0.42 ± 0.09 mmol/l, respectively) during the glucose tolerance test (data not shown).

As expected, plasma insulin was maximal 5 min after glucose injection (Table 1), and returned to basal levels 30 min later (data not shown). The acute administration of benzylamine, vanadate, or both had no effect on plasma insulin levels (Table 1).

Semicarbazide treatment prevented the effects of benzylamine and vanadate on glucose tolerance in nondiabetic rats. To determine whether the effect of benzylamine plus vanadate on glucose tolerance was a consequence of the SSAO activity, rats previously treated for 3 days with the SSAO inhibitor semicarbazide (5 mg · kg^{-1} · day^{-1} i.p.) were subjected to a glucose tolerance test. Semicarbazide treatment caused >90% inhibition of SSAO activity in adipose tissue extracts (data not shown). In keeping with the inhibition of SSAO activity, isolated rat adipocytes from semicarbazide-treated rats did not respond to the combination of 0.1 mmol/l benzylamine plus 0.1 mmol/l vanadate by stimulating glucose transport (data not shown).

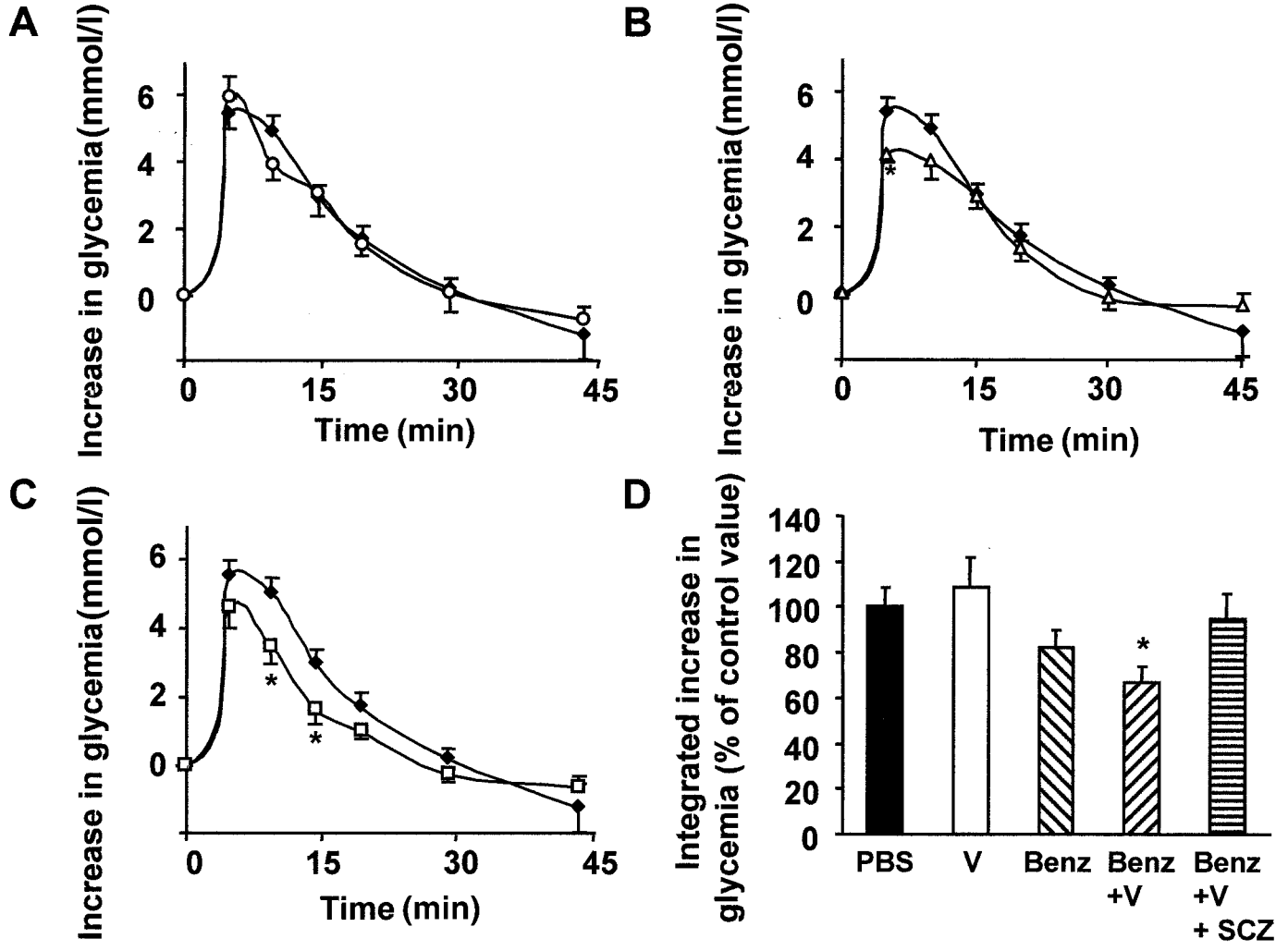

FIG. 1. Effect of acute administration of benzylamine and vanadate on glucose tolerance in control rats. Glucose was injected in the saphenous vein (0.8 g/kg body wt) 30 min after intravenous administration of vehicle (*A*, *B*, and *C*; ◆), 20 μmol/kg vanadate (*A*; ○), 7 μmol/kg benzylamine (*B*; △), or vanadate plus benzylamine (C; □). Results are expressed as increments in plasma glucose. Plasma glucose concentrations at time zero were 5.5 ± 0.2, 5.1 ± 0.4, 5.4 ± 0.2, and 4.6 ± 0.3 μmol/l in control, vanadate, benzylamine, and benzylamine plus vanadate groups, respectively. *D*: Integrated increase in glycemia. Data are means ± SE of six to nine observations presented in *A*, *B*, and *C* and are expressed as a percentage of the control (PBS) group. Last bar corresponds to the increase in glycemia from rats pretreated with semicarbazide (SCZ; 5 mg/kg) for 3 days and receiving benzylamine (Benz) plus vanadate (V) just before the glucose tolerance test. *Different from vehicle control at $P < 0.05$.

Acute administration of benzylamine (7 μmol/kg body wt) with vanadate (20 μmol/kg body wt) in semicarbazide-treated rats had no effect on glucose tolerance (Fig. 1*D*). In these conditions, plasma insulin levels were normal (data not shown).

Acute administration of benzylamine plus vanadate enhanced glucose tolerance in STZ-induced diabetic rats. Next, we tested the in vitro and in vivo effects of the combination of benzylamine and vanadate in STZ-induced diabetic rats. Adipose tissue from diabetic rats shows normal SSAO activity (21; G. Enrique-Tarancón, unpublished observations); however, we evaluated whether adipose cells from diabetic rats respond to SSAO substrates and vanadate by stimulating glucose transport.

As expected from previous reports (22,23), isolated adipocytes from diabetic rats showed a reduced maximal stimulation of glucose transport in response to insulin (Table 2). Interestingly, the combination of 0.1 mmol/l

TABLE 1
Effect of acute administration of benzylamine and vanadate on plasma insulin concentrations in control rats

	5 min	10 min	15 min	20 min	ΔI (ng · ml^{-1} · min^{-1})
PBS treated	5.4 ± 1	3.8 ± 0.8	2.6 ± 0.5	1.7 ± 0.3	78.9 ± 9.6
Benzylamine treated	4.2 ± 1.2	3.8 ± 1.3	2.2 ± 0.2	1.0 ± 0.4	63.3 ± 10.6
Vanadate treated	5.3 ± 2	2.6 ± 1	1.9 ± 1	1.2 ± 0.4	66.6 ± 19.1
Benzylamine + vanadate treated	5.9 ± 0.5	3.7 ± 0.9	2.6 ± 0.7	1.1 ± 0.3	65.8 ± 8.11

Data are means ± SE of four to eight rats per group and are given as nanogram of immunoreactive insulin per milliliter of plasma at different time periods or integrated increase in plasma insulin (ΔI) obtained during the glucose tolerance tests presented in Fig. 1 (see legend for experimental details). Plasma insulin concentrations at time zero were 0.8 ± 0.17, 0.52 ± 0.12, 1.1 ± 0.39, and 1.0 ± 0.1 mmol/l in the control (PBS-treated), vanadate, benzylamine, and benzylamine plus vanadate groups, respectively.

TABLE 2
Effect of benzylamine and vanadate on glucose transport in isolated adipocytes from control or STZ-induced diabetic rats, as measured by 2-DG uptake (nmol · 100 mg^{-1} lipid · 5 min^{-1})

	Nondiabetic rat	STZ-induced diabetic rat
Basal	0.48 ± 0.15	0.57 ± 0.08
Insulin	4.58 ± 0.88*	2.97 ± 0.33*
Vanadate	0.83 ± 0.31	0.80 ± 0.13
Benzylamine	0.58 ± 0.18	0.91 ± 0.14
Benzylamine + vanadate	3.25 ± 0.45*	2.57 ± 0.28*

Data are means ± SE of 2-DG transport. Isolated adipocytes from control rats (n = 5) or STZ-induced diabetic rats (n = 11) were incubated for 45 min in basal conditions or in the presence of 100 nmol/l insulin, 100 μmol/l vanadate, 100 μmol/l benzylamine, or benzylamine plus vanadate. Subsequently, 2-DG transport was measured over 5 min as described in RESEARCH DESIGN AND METHODS. *A significant stimulation of 2-DG uptake compared with basal transport value at $P < 0.01$.

benzylamine and 0.1 mmol/l vanadate stimulated glucose transport to a similar extent in adipocytes from diabetic and nondiabetic rats (Table 2). Moreover, in diabetic rats, this stimulation was comparable to that of insulin, although it reached only 70–80% of insulin's effect in control animals (Table 2).

Nonanesthetized diabetic rats were injected via the tail vein with vehicle or 7 μmol/kg body wt benzylamine and/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. 2). The acute intravenous administration of benzylamine plus vanadate reduced glycemia (Fig. 2*A*) and enhanced glucose tolerance in the STZ-induced diabetic rats (42% increase), but benzylamine or vanadate alone did not alter glucose tolerance (Fig. 2*B*). The effect of benzylamine plus vanadate on glucose tolerance in vivo was independent of changes in plasma insulin concentrations (Table 3). In addition, the improvement in

TABLE 3
Effect of the acute administration of benzylamine and vanadate on plasma insulin concentrations in diabetic rats

	Before glucose administration	At glucose peak
Nondiabetic rats—PBS	1.1 ± 0.3	6.2 ± 1.0*
Diabetic rats		
PBS	0.44 ± 0.26	0.28 ± 0.21
Benzylamine + vanadate	0.33 ± 0.05	0.56 ± 0.13

Data are means ± SE of three to six rats per group and are given as nanograms of insulin per milliliter of plasma. *A significant difference ($P < 0.05$) between insulin levels before glucose administration and at the peak of hyperglycemia (5 min in nondiabetic rats and 30 min in diabetic rats).

glucose tolerance induced by benzylamine plus vanadate was abolished in semicarbazide-treated diabetic rats, indicating that intact SSAO activity was required to improve glucose tolerance (Fig. 2*B*). No changes in glycosuria were detected in control or benzylamine + vanadate groups (515 ± 55 vs. 447 ± 43 mmol/l, respectively) during the tolerance test.

Chronic administration of benzylamine plus vanadate reduced hyperglycemia in diabetic rats. Based on the finding of potent insulin-like effects of acute administration of benzylamine plus vanadate, we tested chronic administration of these compounds in diabetic rats. To this end, STZ-induced diabetic rats were implanted subcutaneously with osmotic minipumps releasing benzylamine (84 μmol · kg^{-1} · day^{-1}) or were sham-operated. Preliminary studies have indicated that benzylamine is stable for 2 weeks in implanted osmotic minipumps, based on its capacity to further stimulate glucose transport in isolated adipocytes after this period (data not shown). Another group of diabetic animals was subjected to daily intraperitoneal injection of vanadate (50 μmol/kg body wt) for 2 weeks, as compared with a group of diabetic rate that

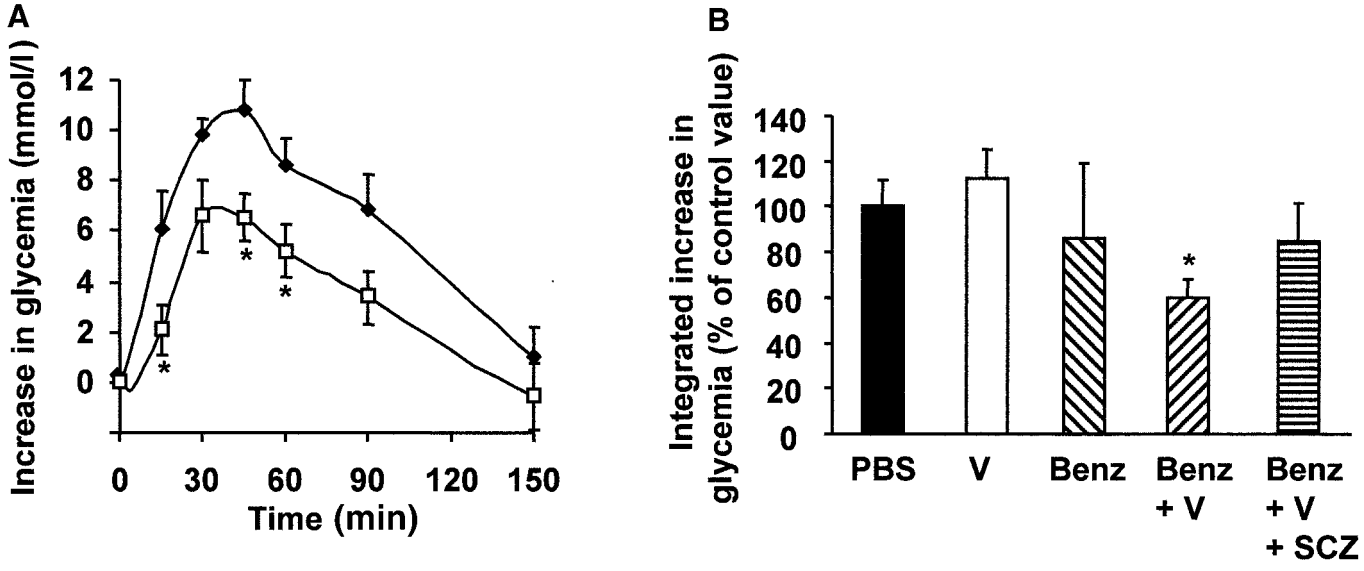

FIG. 2. Effect of the acute administration of benzylamine plus vanadate on oral glucose tolerance test in STZ-induced diabetic rats. *A*: Relative changes in glycemia with an oral glucose load (2 g/kg) starting 15 min after intravenous injection of vehicle (◆) or 20 μmol/kg vanadate + 7 μmol/kg benzylamine (□). Results are expressed as increments in plasma glucose. *B*: Integrated increase in glycemia. Concentrations of plasma glucose at time zero were 21.1 ± 1.2, 23.1 ± 2.3, 23.2 ± 3.9, 21.2 ± 1.3, and 26.2 ± 1.1 mmol/l in diabetic rats untreated (PBS) or treated with vanadate (V), benzylamine (Benz), Benz + V, and Benz + V + semicarbazide (SCZ), respectively. Data are means ± SE of 4–15 observations expressed as percentage, with 100% corresponding to the PBS group. *A significant difference versus PBS group at $P < 0.05$.

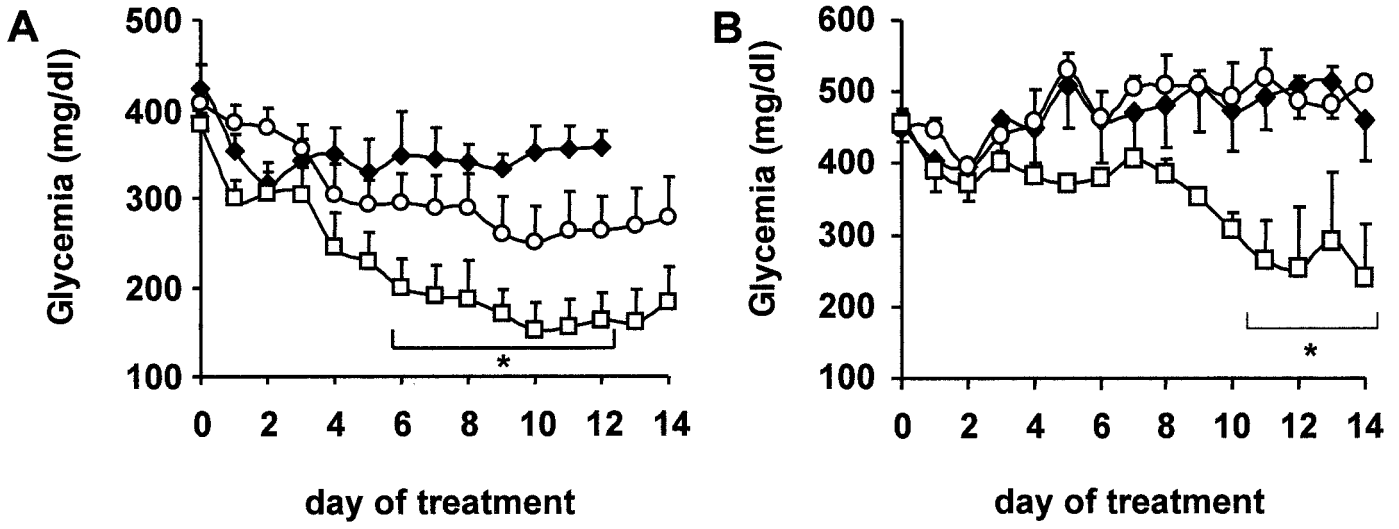

FIG. 3. Chronic treatment with benzylamine and vanadate ameliorates glycemia in STZ-induced diabetic rats. *A*: Diabetic rats received daily intraperitoneal injection of vanadate (50 μmol/kg) alone (○) or in combination with benzylamine (84 μmol · kg^{-1} · day^{-1}) (□) delivered by osmotic minipumps. Control animals (◆) were sham-operated diabetic rats that received a daily injection of the vehicle (PBS). Data are means ± SE of 4–12 observations. *Significantly different from sham-operated group from day 6 at $P < 0.05$. *B*: Rats received daily intraperitoneal injections of vanadate at 25 and 50 μmol/kg body wt for the 1st and 2nd weeks, respectively. Glycemia of diabetic rats untreated (PBS; ◆) or treated with vanadate (○) or benzylamine plus vanadate (□) is presented. Data are means ± SE of three to six observations per group. *Significantly different ($P < 0.05$) versus PBS group from day 11.

received both benzylamine and vanadate. Although treatment with vanadate caused a moderate reduction of glycemia, only the combination of benzylamine plus vanadate normalized glycemia after 1 week of treatment (Fig. 3*A*). Administration of benzylamine plus vanadate for 2 weeks did not alter SSAO activity in extracts from adipose cells (data not shown).

In other studies, diabetic rats were daily injected intraperitoneally with 25 μmol/kg vanadate for the 1st week and 50 μmol/kg vanadate for the 2nd week. These rats, as well as diabetic rats treated with 84 μmol/kg benzylamine alone, remained hyperglycemic (Fig. 3*B*; data not shown). However, the combination of benzylamine plus vanadate reduced hyperglycemia in diabetic rats from day 10 of treatment (Fig. 3*B*).

The chronic treatment with benzylamine plus vanadate caused a substantial decrease in food and water consumption to normal levels, and a 45% increase in the weight of epididymal adipose tissue (Table 4). All these variations occurred in the absence of changes in body weight. The effects on food and water intake were not detected when benzylamine or vanadate was administered alone (Table 4).

The normalization of glycemia caused by chronic treatment with both benzylamine and vanadate took place in the absence of changes in plasma insulin concentrations (Table 4).

Chronic administration of benzylamine plus vanadate stimulated glucose transport and GLUT4 expression in adipocytes from diabetic rats. To determine whether the antidiabetic effects caused by chronic treatment with benzylamine plus vanadate were a consequence of stimulation of peripheral glucose utilization, we analyzed glucose transport and glucose transporter expression in adipose cells obtained from rats treated for 2 weeks with different combinations of benzylamine and/or vanadate. Treatment for 2 weeks with benzylamine plus vanadate stimulated basal glucose transport in isolated adipocytes and normalized insulin-stimulated glucose transport (Fig. 4*A*). These effects were not detected in adipose cells obtained from rats treated with benzylamine or vanadate alone. Indeed, in diabetic rats there was a negative correlation between glycemia at the end of the experiment and basal glucose transport in adipose cells (Fig. 4*B*).

Treatment with benzylamine plus vanadate increased GLUT4 expression (by sevenfold) more than vanadate

TABLE 4
Effect of chronic treatment with benzylamine and vanadate on body weight, epididymal adipose tissue weight, food and water intake, and plasma insulin

	Body weight (g)	Adipose tissue weight (g)	Food intake (g)	Water intake (ml)	Insulin (ng/ml)
Diabetic rats					
PBS treated	235 ± 10	1.33 ± 0.31	40 ± 2	176 ± 29	0.45 ± 0.07
Vanadate treated	261 ± 11	1.42 ± 0.19	36 ± 7	158 ± 42	0.56 ± 0.08
Benzylamine treated	250 ± 10	1.41 ± 0.15	39 ± 3	154 ± 18	ND
Benzylamine + vanadate treated	259 ± 16	1.93 ± 0.13	22 ± 3†	50 ± 15†	0.59 ± 0.07
Nondiabetic rats	278 ± 2†	2.83 ± 0.21†	21 ± 1†	31 ± 2†	1.10 ± 0.30*

Data are means ± SE of three to seven rats per group. Diabetic rats correspond to groups presented in Fig. 3*B*. All groups were age-matched. *$P < 0.05$, †$P < 0.01$ vs. PBS group. ND, not determined.

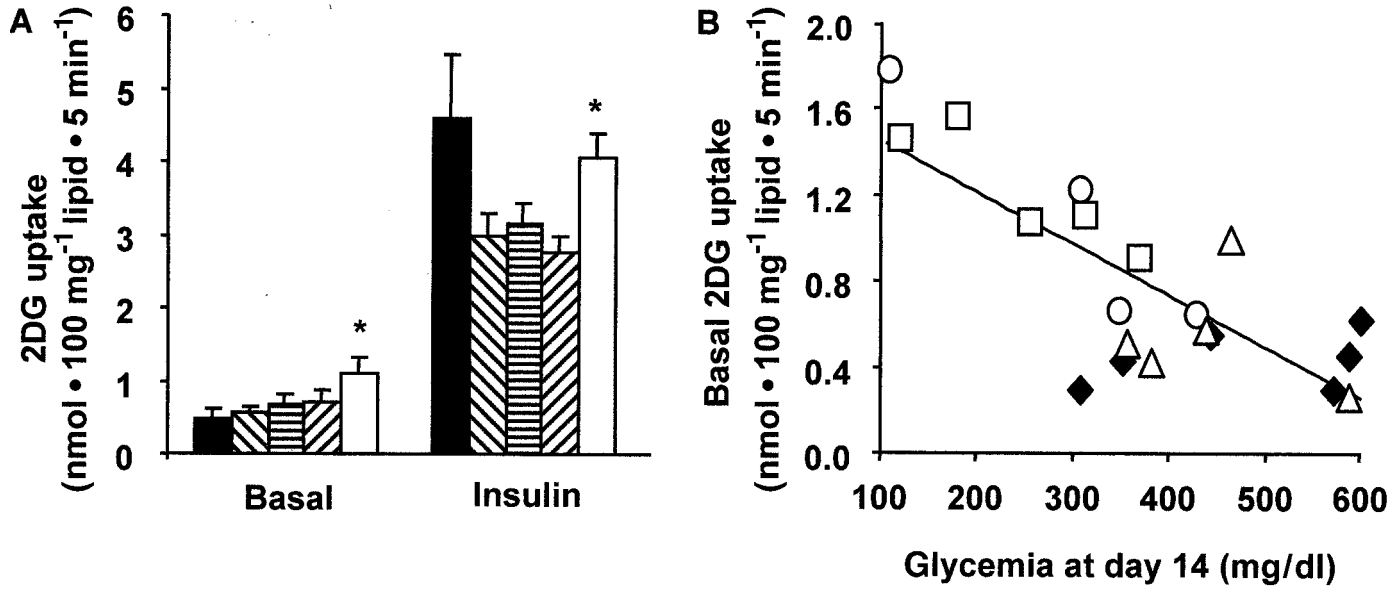

FIG. 4. Chronic treatment with benzylamine plus vanadate stimulates glucose transport in adipocytes from diabetic rats. *A*: STZ-induced diabetic rats were implanted subcutaneously with an osmotic pump containing benzylamine (84 μmol · kg^{-1} body wt · day^{-1}) or sham-operated. Rats received daily intraperitoneal injections of vanadate at 25 and 50 μmol/kg body wt for the 1st and 2nd weeks, respectively. After 2 weeks of treatment, isolated adipocytes from nondiabetic rats (■) and untreated (▧), vanadate-treated (▤), benzylamine-treated (▨), and benzylamine plus vanadate–treated diabetic rats (□) were obtained from epididymal adipose tissue. Adipose cells were incubated for 30 min in basal conditions or in the presence of 100 nmol/l insulin. Subsequently, 2-DG transport was measured for 5 min (expressed as nmol · 5 min^{-1} · 100 mg^{-1} lipid). Data are means ± SE of four to five observations per group. *$P < 0.05$. *B*: Correlation between basal glucose transport in isolated rat adipocytes and plasma glycemia of the rats ($r = 0.77$, $P < 0.01$). Data from rats treated with PBS (◆), vanadate (○), benzylamine (△), and benzylamine plus vanadate (□) are shown.

alone (Fig. 5). In addition, there was a correlation between the amount of GLUT4 and basal glucose transport in adipocytes (data not shown). No changes in GLUT4 expression were detected in soleus or extensor digitorum longus muscles in response to chronic treatment with benzylamine plus vanadate (data not shown).

DISCUSSION

Previous studies have demonstrated that many SSAO substrates, in combination with low vanadate concentrations, stimulate glucose transport in rat and 3T3-L1 adipose cells (14). The stimulation of glucose transport is dependent on SSAO activity and hydrogen peroxide production rather than on aldehyde production (13). The enhanced glucose transport induced by SSAO substrates plus vanadate is characterized by the recruitment of GLUT4 glucose transporters to the cell surface, as demonstrated by subcellular fractionation and plasma membrane lawn techniques (12,13). As to the nature of the mechanisms involved, SSAO substrates plus vanadate markedly stimulate tyrosine phosphorylation of IRS-1 and -3 as well as PI 3-kinase activity (14). On the basis of these data, we proposed that SSAO substrates and vanadate synergistically stimulate one or several tyrosine protein kinases or inhibit protein tyrosine phosphatases, leading to activation of an intracellular pathway similar to that triggered by insulin (14).

In keeping with these observations in vitro, we found that acute administration of an SSAO substrate, benzylamine, together with a low dosage of vanadate, enhances glucose tolerance in nondiabetic and diabetic rats. This effect did not alter the profile of plasma insulin concentrations after glucose challenge, was not associated with alterations in renal glucose reabsorption, and required SSAO activity. These data indicate that the combination of benzylamine and vanadate stimulates glucose disposal after a glucose challenge in vivo.

Because the benzylamine plus vanadate combinations stimulated glucose transport in isolated rat adipocytes from nondiabetic and diabetic rats, we suggest that adipose tissue participates in this response. In addition, given the low concentrations of insulin in diabetic rats, we favor the view that benzylamine and vanadate have insulin-like effects in adipose tissue.

Interestingly, the chronic administration of benzylamine plus vanadate reduced glycemia in STZ-induced diabetic rats, whereas in some experimental protocols, this combination normalized glycemia. As to the time-dependence of these effects, a moderate reduction of glycemia was already noted at day 5–6 after the onset of treatment, which is similar to the time-dependence shown by vanadate or peroxovanadate treatments (24,25). This antihyperglycemic effect of benzylamine plus vanadate was also accompanied by normalization in food and water intake and, as reported for vanadate alone (26), was not a consequence of any increase in plasma insulin.

The chronic administration of benzylamine plus vanadate stimulated glucose uptake in adipose cells. Thus, cells obtained after combined benzylamine and vanadate treatment showed enhanced rates of basal and insulin-stimulated glucose transport and GLUT4 expression. These effects were observed only when both compounds were given in combination but not when they were given separately. The enhanced basal glucose transport may have been a result of the acute effects of benzylamine and vanadate in promoting GLUT4 recruitment to the cell surface or the presence of a larger GLUT4 population. However, the enhanced glucose uptake by adipose cells

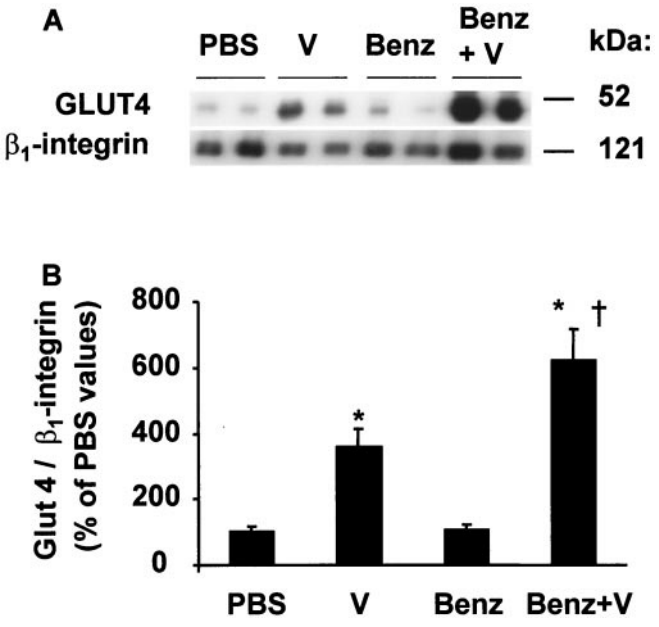

FIG. 5. Chronic treatment with benzylamine plus vanadate stimulates GLUT4 glucose transporter expression in adipocytes from diabetic rats. STZ-induced diabetic rats were implanted subcutaneously with an osmotic pump containing benzylamine (84 μmol · kg^{-1} body wt · day^{-1}) or were sham-operated (◆). Rats received daily intraperitoneal injections of vanadate at 25 and 50 μmol/kg body wt for the 1st and 2nd weeks, respectively. After 2 weeks of treatment, isolated adipocytes from untreated diabetic rats and diabetic rats treated with vanadate, benzylamine, and benzylamine plus vanadate were obtained from epididymal adipose tissue. Total adipocyte membranes were obtained and subjected to SDS-PAGE and further immunoblotting using specific antibodies directed against GLUT4 or β_1-integrin. *A*: Representative autoradiogram. *B*: GLUT4 abundance corrected by β_1-integrin. Data are means ± SE from six independent experiments. *Significant differences with the PBS group at $P < 0.05$; †significant difference with the vanadate-treated group at $P < 0.05$.

and the normalization of glycemia may also have been consequences of enhanced insulin sensitivity.

Our data indicate that the combination of benzylamine and vanadate was required for both acute and chronic effects; as previously observed in vitro (12,13), benzylamine or vanadate alone had little or no effect. Studies of adipose cells in vitro have suggested that hydrogen peroxide production is crucial for triggering the stimulation of glucose transport and GLUT4 recruitment to the cell surface, as catalase blocks the effects (12,13). Given that peroxovanadate is a potent insulin-like agent (27–31), it may be formed either inside or outside the cell and may be responsible for the effects of the combination of vanadate and SSAO substrates. Based on the fact that peroxovanadate inhibition of protein tyrosine phosphatases (PTPs) is irreversible, whereas the effect of vanadate is reversible (32), we have indirectly evaluated the generation of peroxovanadate by assessing PTP activity in the presence of 1 mmol/l EDTA and using extracts from adipose cells obtained from diabetic rats chronically treated with different compounds. Chronic vanadate treatment inhibited PTP activity, a result that is in keeping with prior observations (33); the administration of vanadate plus benzylamine also inhibited PTP activity, which was comparable to the effects of vanadate alone (data not shown). These data indicate that there is no correlation between total PTP activity and activation of basal glucose transport in adipose cells or reduction of hyperglycemia in diabetic rats treated with vanadate or benzylamine plus vanadate. Thus, although peroxovanadate may explain the effects of benzylamine plus vanadate, our data do not justify any conclusion on whether peroxovanadate is the only signal generated in adipose cells in response to benzylamine plus vanadate treatment.

Restoration of adipose glucose transport activity may be insufficient to normalize glucose levels in diabetic rats after benzylamine plus vanadate treatment. In adipose cells, benzylamine plus vanadate may generate molecules that have insulin-like effects in other insulin-sensitive tissues. In addition, amelioration of adipose tissue metabolism subsequent to benzylamine plus vanadate treatment, via molecules or hormones released from adipose cells, such as leptin, tumor necrosis factor-α, or resistin (34–36), may have a profound impact on muscle metabolism. It is important to determine whether skeletal muscle increases glucose disposal in response to benzylamine and vanadate.

ACKNOWLEDGMENTS

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

Anna Abella,[1] Luc Marti,[1] Marta Camps,[1] Marc Claret,[2] J. Fernández-Alvarez,[2] Ramon Gomis,[2] Anna Gumà,[1] Nathalie Viguerie,[3] Christian Carpéné,[3] Manuel Palacín,[1] Xavier Testar,[1] and Antonio Zorzano[1]

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-

From the [1]Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, and Parc Científic de Barcelona, Barcelona, Spain; the [2]Endocrinology and Diabetes Unit, IDIBAPS, Hospital Clinic de Barcelona, Barcelona, Spain; and the [3]Institut National de la Santé et de la Recherche Médicale (INSERM U 317), Toulouse, France.

Address correspondence and reprint requests to Antonio Zorzano, Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08028, Barcelona, Spain. E-mail: azorzano@porthos.bio.ub.es

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A.A. and L.M. equally contributed to the study.

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-^{3}H]-D-deoxyglucose (26 Ci/mmol) was from NEN Life Science Products (Boston, MA) and the [^{14}C]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 amylase 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 Ibèrica 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 μmol/kg body wt benzylamine and/or 20 μmol/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 μmol · kg body wt^{-1} · day^{-1}) were implanted subcutaneously in GK diabetic rats anesthetized by ketamine hydrochloride (95 mg/kg) and xylasine (10 mg/kg). Animals that did not receive benzylamine were sham operated. A single intraperitoneal injection of vanadate (25 μmol/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 coincubation 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/mmol/l), benzylamine hydrochloride (100 μ/mmol/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% O_2 5% CO_2. 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).

^{51}V nuclear magnetic resonance spectroscopy analysis. The ^{51}V 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 disolving samples with D_2O. 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 $VOCl_3$ (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 μmol/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 15,000*g* 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 μg 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 ± 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.

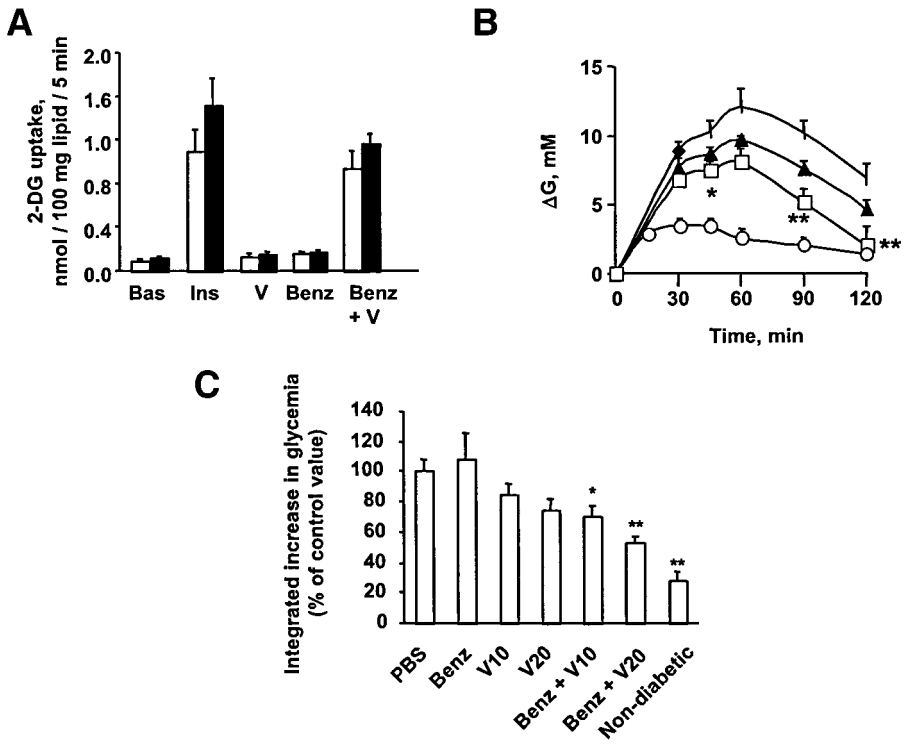

FIG. 1. Benzylamine and vanadate enhance glucose tolerance and stimulate glucose transport in adipose cells from GK diabetic rats. *A*: Adipose cells from GK diabetic rats (□) or nondiabetic Wistar rats (■) were incubated for 45 min in basal conditions (Bas) or in the presence of 100 nmol/l insulin (Ins), 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 tolerance 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 · min^{-1} · 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. 1*A*). In fact, benzylamine/vanadate-stimulated and maximally insulin-induced glucose transport was similar in control Wistar and in GK rats (Fig. 1*A*), 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. 1*B*). 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. 1*B* 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. 1*C*). In these conditions, administration of benzylamine or vanadate alone did not alter glucose tolerance (Fig. 1*B* 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 μmol · kg^{-1} · 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 unsignificant 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. 2*A*). Administration of benzylamine plus vanadate for 2 weeks did not alter SSAO/VAP-1 or GLUT4 protein expression in adipose cells (Fig. 2*B*). 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. 2*C*). 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. 2*C*). These effects on glucose transport were not detected in adipose cells obtained from rats treated with benzylamine or vanadate alone (data not shown).

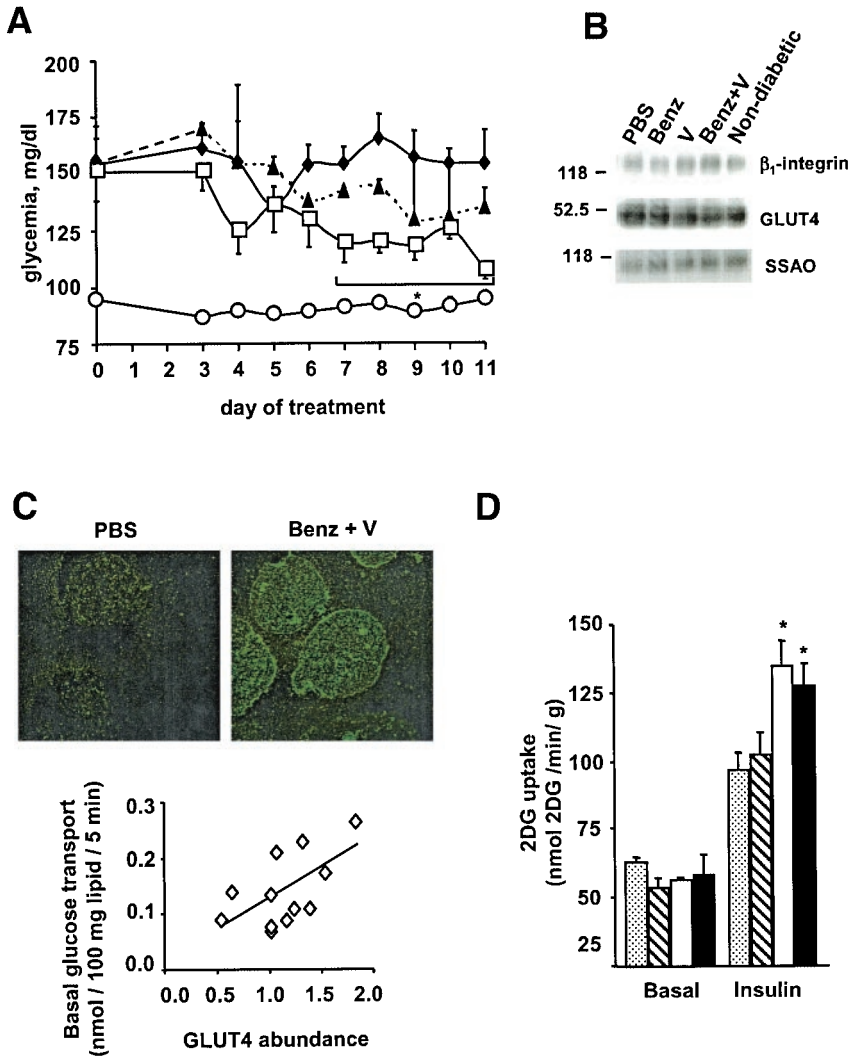

FIG. 2. Chronic treatment with benzylamine and vanadate ameliorates glycemia and enhance glucose transport in muscle and adipose cells. *A*: GK diabetic rats were treated by a daily intraperitoneal injection of vanadate (25 μmol/kg) alone (▲) or in combination with benzylamine delivered by osmotic minipumps (84 μmol · kg^{-1} · day^{-1}) (□) or received a daily injection of the vehicle (PBS) (♦). Nondiabetic rats treated with PBS were also studied (○). Values are mean ± 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 β1-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 (▨) vanadate-treated diabetic rats (▧), benzylamine- and vanadate-treated diabetic rats (□), and nondiabetic rats (■) were incubated in the absence (basal) or in the presence of 100 nmol/l insulin. Following 2-deoxyglucose uptake, muscles were digested and radiactivity 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. 2*D*), which is in keeping with previous observations (21,26). Chronic treatment with benzylamine/vanadate reversed the insulin response to normal levels (Fig. 2*D*). No effects were observed on muscle glucose transport after chronic administration with vanadate alone (Fig. 2*D*). 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 μmol/kg body wt) and/or vanadate (20 μmol/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. 3*A*). 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. 3*B*). 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. 3*B*), 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. 3*C*). 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-

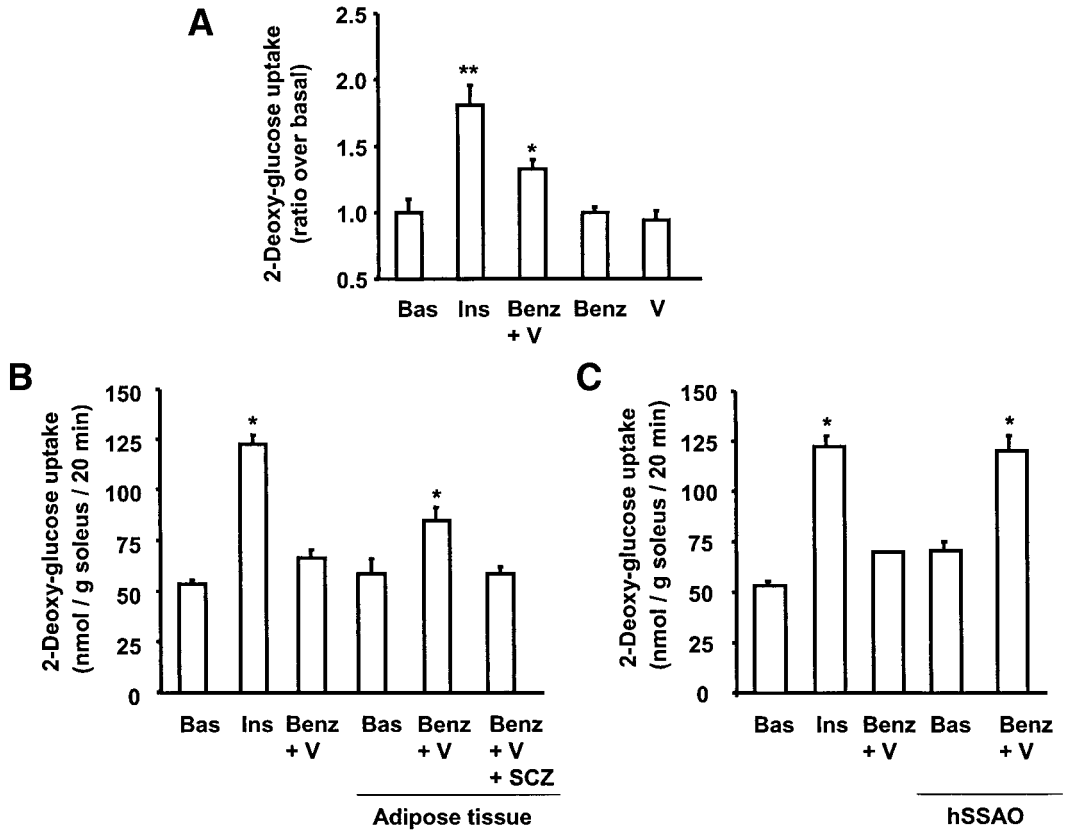

FIG. 3. Muscle glucose transport is stimulated by acute exposure to benzylamine, vanadate, and SSAO. *A*: Nondiabetic rats were intravenously injected with vehicle (Bas), 10 units/kg insulin (Ins), 7 μmol/kg benzylamine (Benz), 20 μmol/kg vanadate (V), or with benzylamine and vanadate (Benz + V); 30 min later, soleus muscles were incubated and glucose transport measured. Values are mean ± 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 radiactivity was measured. Values are mean ± 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 radiactivity was measured. Values are mean ± SE of seven observations per group. *$P < 0.05$ vs. the basal group.

centration of insulin and was also inhibited by semicarbazide (Fig. 3*C*). 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. 4*A*); in contrast, previous administration of benzylamine/vanadate showed a normalization of the profile of insulin concentrations after a glucose challenge (Fig. 4*A* and *B*). The acute treatment with vanadate alone showed no effect on plasma insulin concentrations (Fig. 4*B*). 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 incubated 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. 4*C* and *D*). In addition, the effect of benzylamine/vanadate on insulin secretion was blocked by semicarbazide (Fig. 4*D*). 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. 4*E*). The abundance of SSAO/VAP-1 protein was much higher in adipose tissue than in pancreatic islets (Fig. 4*E*). In addition, SSAO activity was nearly 300-fold greater in extracts from adipocytes than in pancreatic islets (data not shown). In all,

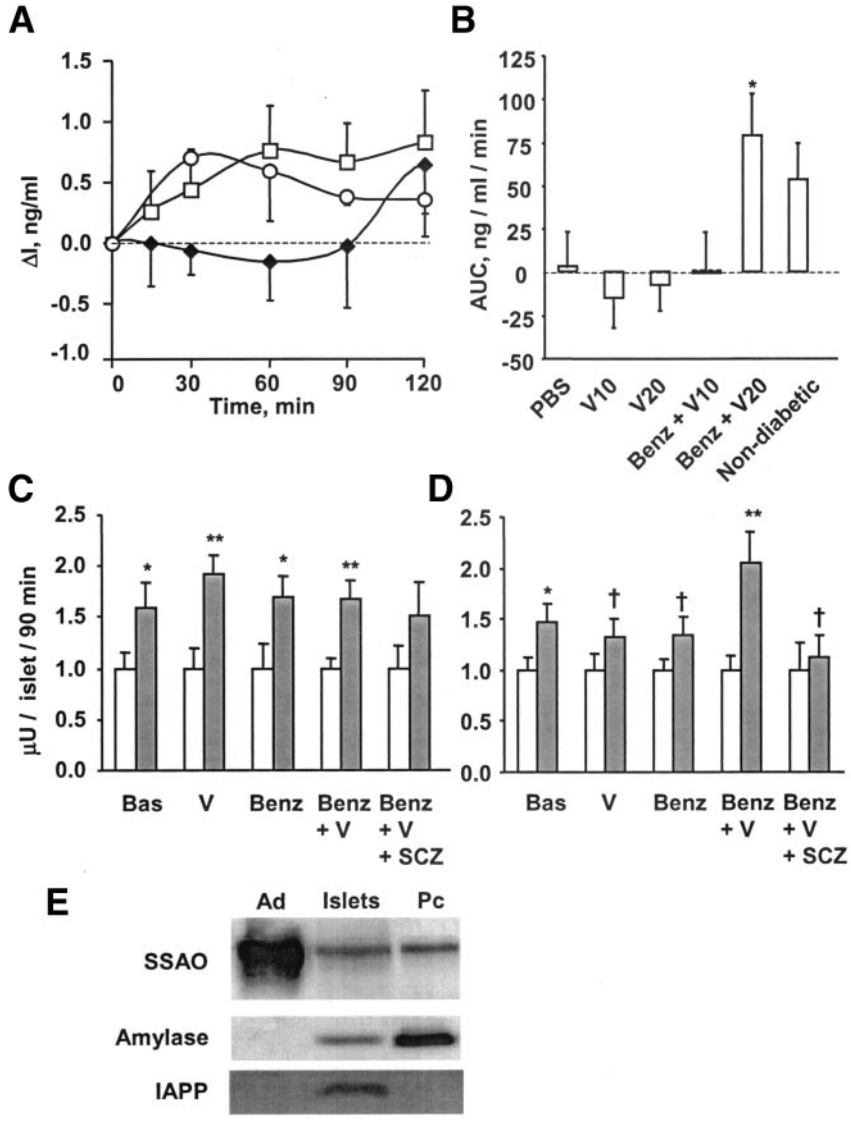

FIG. 4. Benzylamine and vanadate stimulate insulin secretion in GK diabetic rats. *A*: Plasma insulin concentrations after an oral glucose load (2 g/kg) starting 15 min after intravenous injection of vehicle (♦), 20 μmol/kg vanadate, and 7 μmol/kg benzylamine (□) in GK diabetic rats and vehicle in nondiabetic Wistar rats (○). Values are mean ± 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 μmol/kg vanadate (V10), 20 μmol/kg vanadate (V20), benzylamine/10 μmol/kg vanadate (Benz + V10), and benzylamine/20 μmol/kg vanadate (Benz + V20) in GK diabetic rats and in nondiabetic groups, respectively. Values are mean ± 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 ± 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. 5*A*), 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. 5*A*) (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. 5*A*). 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. 5*B*). The effect of benzylamine/vanadate was similar to that caused by peroxovanadate (Fig. 5*B*). 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. 5*C*). The effect of benzylamine/vanadate on protein tyrosine

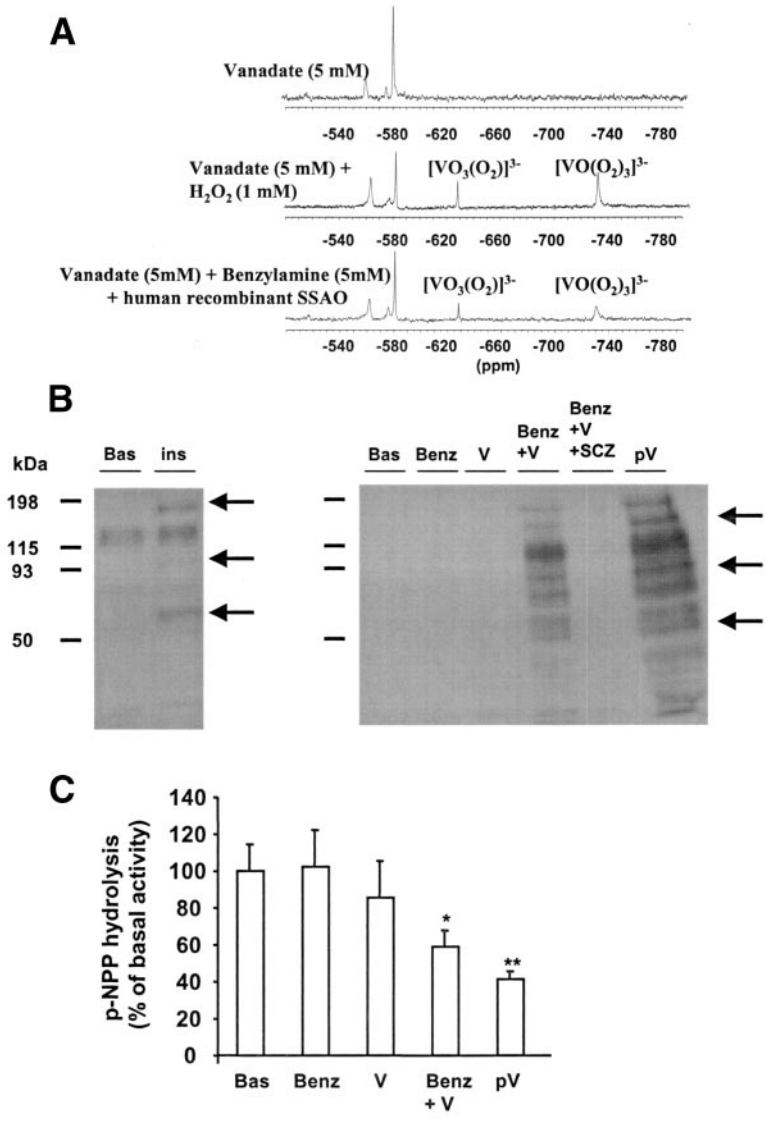

FIG. 5. Benzylamine, vanadate, and SSAO generate peroxovanadium and inhibit protein tyrosine phosphatase activity. *A*: Identification of vanadate and peroxovanadium compounds by resonance of V51 after incubation of 5 mmol/l sodium vanadate alone, 5 mmol/l sodium vanadate and 1 mmol/l hydrogen peroxide, 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 ± 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 μmol/l peroxovanadate (Fig. 5*C*).

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 monoamino 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 coincubation 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

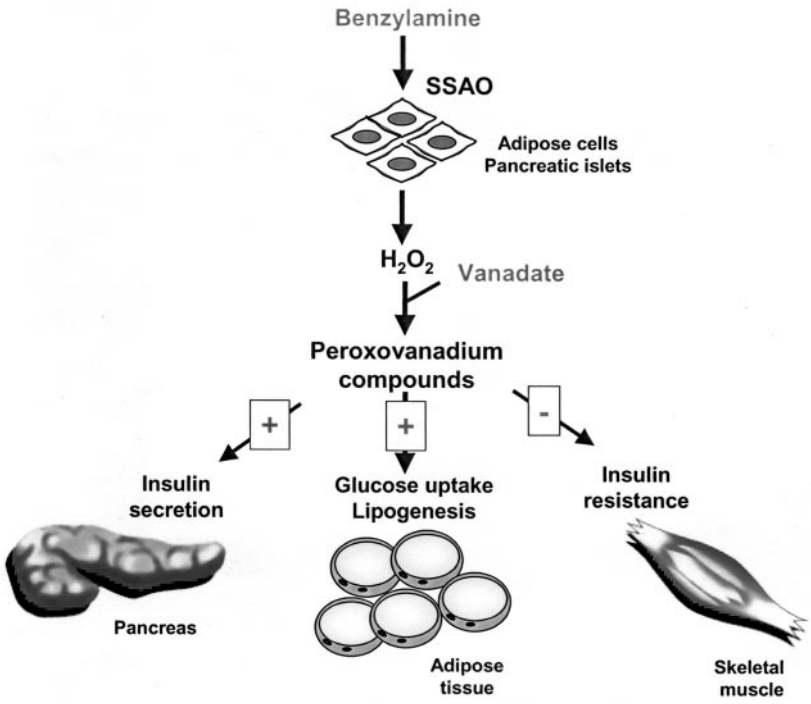

FIG. 6. Model on the cellular effects of SSAO/VAP-1 effects on glucose homeostasis.

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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Review

Semicarbazide-sensitive amine oxidase activity exerts insulin-like effects on glucose metabolism and insulin-signaling pathways in adipose cells

Antonio Zorzano[a,*], Anna Abella[a], Luc Marti[a], Christian Carpéné[b], Manuel Palacín[a], Xavier Testar[a]

[a] *Facultat de Biologia, Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona, Avda. Diagonal 645, 08028 Barcelona, Spain*
[b] *Institut National de la Santé et de la Recherche Médicale (INSERM U 317), IFR 31, C.H.U. Rangueil, 31403 Toulouse Cedex 4, France*

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Abstract

Semicarbazide-sensitive amine oxidase (SSAO) is very abundant at the plasma membrane in adipocytes. The combination of SSAO substrates and low concentrations of vanadate markedly stimulates glucose transport and GLUT4 glucose transporter recruitment to the cell surface in rat adipocytes by a mechanism that requires SSAO activity and hydrogen peroxide formation. Substrates of SSAO such as benzylamine or tyramine in combination with vanadate potently stimulate tyrosine phosphorylation of both insulin-receptor substrates 1 (IRS-1) and 3 (IRS-3) and phosphatidylinositol 3-kinase (PI 3-kinase) activity in adipose cells, which occurs in the presence of a weak stimulation of insulin-receptor kinase. Moreover, the acute administration of benzylamine and vanadate in vivo enhances glucose tolerance in non-diabetic and streptozotocin-induced diabetic rats and reduces hyperglycemia after chronic treatment in streptozotocin-diabetic rats.

Based on these observations, we propose that SSAO activity and vanadate potently mimic insulin effects in adipose cells and exert an anti-diabetic action in an animal model of type 1 diabetes mellitus.

Keywords: SSAO; VAP-1; Glucose transport; GLUT4; Phosphatidylinositol 3-kinase; Adipocyte

1. Introduction

Semicarbazide-sensitive amine oxidases (SSAOs) constitute a class of enzymes which catalyse the reaction by which a primary amine is converted into the corresponding aldehyde in the presence of oxygen with the concomitant production of hydrogen peroxide and ammonia. The activity is inhibited by semicarbazide due to the presence of the cofactor 2,3,5-trihydroxyphenylalanine quinone (TPQ) derived from a post-translationally modified tyrosine, and is resistant to the acetylenic compounds clorgyline, pargyline and selegiline, which are inhibitors of mitochondrial monoamine oxidases. SSAOs are found in a wide variety of tissues such as in the vasculature, both in smooth muscle cells and in endothelial cells [1–3], rat articular cartilage, bovine eye and pig dental pulp [4–9].

Several isoforms of SSAO have been cloned in human, rodent and bovine tissues. Thus, a human placenta SSAO (also termed AOC3, from amine oxidase, copper-dependent-3) and a human retina-specific amine oxidase (also termed AOC2) showing a 65% similarity at the protein level have been recently cloned and sequenced [10–12]. Human placental SSAO shows a significant, although lower, similarity (36%) to human kidney diamine oxidase (AOC1). Furthermore, two distinct bovine SSAO genes encoding a lung SSAO (blSSAO) and a serum SSAO (bsSSAO) have been identified [13,14]. The primary sequences of these two genes show a 91% similarity [14]. Human placental SSAO shows a high similarity with blSSAO and bsSSAO (81% and 79%, respectively).

Tissue forms of SSAO show a putative transmembrane domain at the N terminus, which is in agreement with its biochemical properties as a membrane protein [15,16]. SSAO represents an example of multifunctional membrane proteins that in addition to displaying amine oxidase activ-

Abbreviations: SSAO, semicarbazide-sensitive amine oxidase; VAP-1, vascular adhesion protein 1; IRS, insulin-receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase

* Corresponding author. Tel.: +34-93-4021519; fax: +34-93-4021559.
E-mail address: azorzano@porthos.bio.ub.es (A. Zorzano).

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ity, shows vascular adhesion properties [17,18]. The human vascular adhesive protein-1 (VAP-1) [19], which belongs to the large family of adhesive proteins implicated in fundamental processes, such as inflammation [20], is identical to human placental SSAO [17,18]. The interrelationship between both functions of SSAO/VAP-1, and the adhesive properties of SSAOs expressed in cell types distinct from endothelial cells, remains to be determined.

2. Adipose cells express high levels of SSAO

A high level of SSAO activity has been found in rat adipocytes [21]. Quantitative detection of SSAO in isolated rat adipocytes indicates that they contain 14×10^6 copies per cell [22]. A partial clone corresponding to SSAO expressed in rat adipose cells (vp97 clone) shows a 95% similarity to the mouse counterpart of human placenta SSAO [17,22,23]. In addition, mouse SSAO expressed in 3T3-L1 adipocytes is identical to mouse VAP-1 [23]. Based on these observations, it is concluded that adipocyte SSAO is the counterpart of human placental SSAO.

It has been demonstrated that SSAO is not expressed in 3T3-L1 fibroblasts and that SSAO gene expression is induced during adipogenesis [23]. 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 [24]. This suggests that SSAO is a member of the adipogenic gene program and, in addition, that SSAO may contribute to the acquisition of some final characteristics of fully differentiated adipose cells. The precise biological function of SSAO in adipose cells remains unknown. However, based on our studies and on recent data indicating that SSAO acts as an adhesion protein in endothelial cells [17,18], we propose that SSAO exerts a double biological function in adipose cells: on the one hand, it plays a regulatory role on anabolic metabolic processes and, on the other hand, it may control cell-to-cell interaction or cell–matrix interaction.

Most of the SSAO expressed in rat adipocytes is found in plasma membranes [22,25]. Thus, subcellular fractionation of membranes from 3T3-L1 adipocytes or isolated rat adipocytes has demonstrated that SSAO protein is far more abundant in the plasma membrane than in the light microsomes [22,25]. This suggests that adipose SSAO may metabolize extracellular amines. In addition, the distribution of SSAO 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 protein abundance or SSAO activity in intracellular membranes or in plasma membrane preparations [22,25].

SSAO co-localizes with GLUT4 in intracellular vesicles and this seems to be a rather general property of GLUT4-expressing tissues since this co-localization has been found in rat adipocytes and 3T3-L1 adipocytes [22,25]. We have found that the co-localization of GLUT4 and SSAO is partial; about 18–24% of total intracellular SSAO is present in the intracellular GLUT4 membrane population purified from isolated rat adipocytes or from 3T3-L1 adipocytes [25]. In addition, immunotitration experiments revealed that the co-localization of SSAO and GLUT4 was only detectable when maximal amounts of anti-GLUT4 antibody were used, suggesting that only a subset of GLUT4 vesicles, probably those in an endosomal compartment, contains SSAO [25]. Based on these results and on similar observations in 3T3-L1 adipocytes, skeletal muscle and cardiomyocytes supporting the view that GLUT4 is present in an endosomal compartment as well as in an exocytic compartment [26–28], we have proposed that SSAO co-localizes with intracellular GLUT4 in an endosomal population, rather than in the specific storage compartment. Co-localization of both proteins in an endosomal population would be followed by differential sorting to distinct membrane compartments.

3. SSAO substrates regulate glucose transport in adipose cells

Recently, we have demonstrated a stimulatory role of SSAO on glucose transport in isolated rat adipocytes [25,29]. Benzylamine, a synthetic substrate of SSAO and tyramine, an endogenous substrate of both SSAO and MAO, markedly stimulates glucose uptake. The effects of both substrates are only observed in the presence of low concentrations of vanadate, which is unable per se to modify basal glucose transport in adipose cells [25,29]. Because the response to amines plus vanadate is sensitive to semicarbazide and also to catalase, we have proposed that amines stimulate glucose uptake through a mechanism dependent on hydrogen peroxide production and in synergy with vanadate [25,29]. Stimulatory effects of SSAO substrates on glucose transport are also found in 3T3-L1 adipocytes. Thus, incubation of 3T3-L1 adipocytes for 3.5 h in the presence of 1 mM benzylamine and 0.1 mM vanadate causes a 10-fold stimulation of glucose transport [30].

We have also studied whether the effects promoted by benzylamine and tyramine are displayed by other SSAO substrates. To this end, we incubated isolated rat adipocytes in the presence of different concentrations (0.1 or 1 mM) of different SSAO substrates such as methylamine, *n*-decylamine, 2-phenylethylamine, histamine, *N*-acetyl putrescine or tryptamine, [5–9,21,31–33]. Glucose transport remained unaltered after incubation of adipose cells in the presence of 0.1 mM substrates (Fig. 1 and data not shown) and addition to the medium of 1 mM methylamine, *n*-decylamine or tryptamine caused a small increase in basal glucose transport (Fig. 1). The combination of SSAO substrates together with 0.1 mM vanadate led to a marked stimulation of glucose transport, which ranged from 25% to 88% of the maximal stimulation caused by insulin (Fig. 1). These data indicate that a variety of SSAO substrates markedly stim-

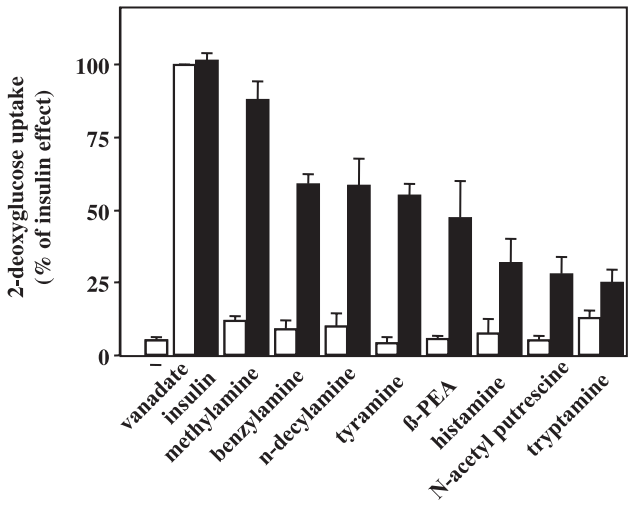

Fig. 1. Effects of SSAO substrates and vanadate on glucose transport by rat adipocytes. Rat adipocytes were incubated for 45 min in the absence or presence of 100 nM insulin or with the different SSAO substrates methylamine (1 mM), benzylamine (0.1 mM), *n*-decylamine (0.1 mM), tyramine (1 mM), 2-phenylethylamine (β-PEA) (1 mM), histamine (1 mM), *N*-acetyl putrescine (1 mM) and tryptamine (1 mM) without (open bars) or with 100 mM sodium orthovanadate (black bars). Data from cells incubated in the presence of 0.1 mM vanadate alone are also included (vanadate group). Thereafter, 2-deoxyglucose uptake was measured over a 10-min period. Basal and insulin-stimulated uptakes were equivalent to 1.19 ± 0.11 and 10.55 ± 0.67 nmol/100 mg lipid/10 min, respectively ($n = 53$). Results are mean values ± S.E.M. of 4–53 independent experiments and are expressed as a percentage of insulin-stimulated 2-deoxyglucose uptake. The effect of the combination of SSAO substrates and vanadate was significantly different from the vanadate group, at $P < 0.05$ (analysis of variance followed by Scheffé *F* test).

ulate glucose transport in isolated rat adipocytes when combined with low vanadate concentrations. In addition, these results strengthen the view that SSAO substrates stimulate glucose transport as a consequence of hydrogen peroxide production rather than as a consequence of aldehyde production, which is in agreement with prior observations indicating that the effects of benzylamine or tyramine are blocked by catalase [30].

As to the mechanisms involved, we have found that the combination of benzylamine–vanadate or tyramine–vanadate caused a marked enhancement in the GLUT4 present at the cell surface of rat adipocytes, as assessed by the plasma membrane lawn or by subcellular fractionation assays [25,29,30]. Analysis of the abundance of cell-surface GLUT4 in 3T3-L1 adipocytes, as assessed by plasma membrane lawn assays, has also revealed that the combination of benzylamine and vanadate recruits GLUT4 to the cell surface [30].

Interestingly, in some cell types such as in isolated human adipocytes or in mouse 3T3 F442A adipocytes, acute exposure to SSAO substrates causes activation of glucose transport in the absence of vanadate [34,35]. At present, the mechanisms by which some adipose cell types require both SSAO substrates and vanadate to acutely stimulate glucose transport whereas others respond to SSAO substrates independently of vanadate remains unknown.

4. SSAO substrates activate insulin-like signaling pathways in adipose cells

The nature of the mechanisms involved in the effects of SSAO substrates was assessed further by examination of the effect of the combination of SSAO substrates and vanadate on the insulin receptor, insulin-receptor substrate (IRS) proteins and phosphatidylinositol 3-kinase (PI 3-kinase) in isolated rat adipocytes. We observed that the combination of SSAO substrates and vanadate stimulates tyrosine phosphorylation of IRS proteins and activates PI 3-kinase [30], which are crucial components in insulin-signal transduction. In contrast, benzylamine and vanadate caused only a weak stimulation of tyrosine phosphorylation of the insulin receptor, suggesting that the insulin-receptor tyrosine kinase is not the main tyrosine kinase involved in SSAO-dependent signaling [30] (Fig. 2).

The stimulation of tyrosine phosphorylation of IRS-1 and IRS-3 in response to the combination of benzylamine–vanadate or tyramine–vanadate (Fig. 2) is concluded from the following observations: (a) SSAO substrates and vanadate markedly stimulate tyrosine phosphorylation of immunoprecipitated IRS-1, (b) SSAO substrates and vanadate stimulate the tyrosine phosphorylation of IRS-1 and IRS-3 co-immunoprecipitated with the p85 subunit of PI 3-kinase [30].

IRS-3, a 60-kDa phosphotyrosine protein, is a recently identified member of the IRS family [36,37] that rapidly interacts with p85 subunit of PI 3-kinase after insulin stimulation in rat adipocytes [38] and it is predominantly involved in regulating PI 3-kinase in the absence of IRS-1 [39]. In this regard, our data indicate that IRS-3 seems to be the main IRS protein activated in response to SSAO substrates and vanadate, whereas IRS-1 is predominant after insulin stimulation. Under our experimental conditions, we did not detect tyrosine phosphorylation of IRS-2 but this does not exclude the fact that IRS-2 could be involved mediating transient signals, as already reported [40].

The p85–p110 PI 3-kinase activity is also stimulated by the combination of SSAO substrates and vanadate and this participates in the stimulation of GLUT4 recruitment to the cell surface (Fig. 2). This is based on the following data: (a) the p85 subunit of PI 3-kinase associates with activated IRS-1 and IRS-3 after incubation with vanadate and benzylamine or tyramine, (b) PI 3-kinase activity is enhanced by benzylamine–vanadate or tyramine–vanadate in p85 immunoprecipitates, and (c) wortmannin completely abolishes the stimulation triggered by benzylamine–vanadate on glucose transport or GLUT4 recruitment in adipose cells. Because p85 co-immunoprecipitates with IRS-1 and IRS-3, we also conclude that the PI 3-kinase activation detected in our study must result from both the association between p85 and IRS-3 and between p85 and IRS-1.

A critical question regarding the stimulation of GLUT4 transporter translocation to the cell surface in rat adipose cells or in 3T3-L1 adipocytes is the nature of the active

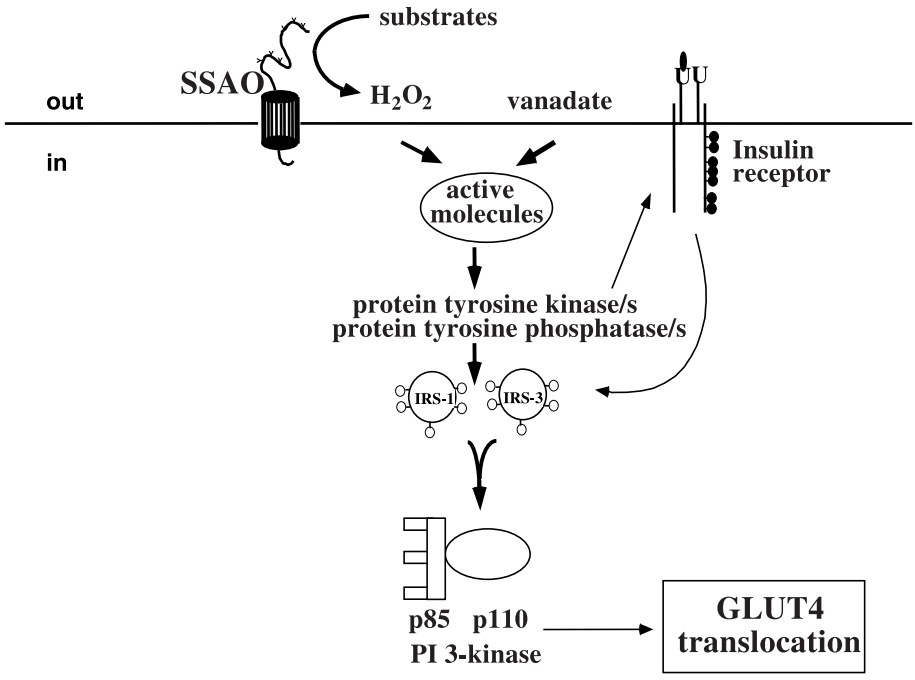

Fig. 2. Scheme of the putative mechanisms involved in the biological effects of SSAO activity in adipose cells. The model assumes the generation, either extracellularly or intracellularly, of unknown active compounds, derived from vanadate and the hydrogen peroxide produced via SSAO activity. Most likely, hydrogen peroxide may be produced extracellularly; however, we cannot rule out the possibility that intracellular SSAO also produces hydrogen peroxide. The active compounds may activate a protein-tyrosine kinase or inhibit a protein-tyrosine phosphatase, which in turn causes tyrosine phosphorylation of IRS-1 and IRS-3, stimulation of PI 3-kinase and GLUT4 recruitment to the cell surface. Activation of a protein-tyrosine kinase or inhibition of a protein-tyrosine phosphatase causes a weak activation of insulin-receptor kinase.

molecules that are generated by the catalysis of SSAO in the presence of vanadate. Based on the fact that hydrogen peroxide production is crucial for the triggering of these effects, since catalase blocks the effects [25,29] and, given that peroxovanadate is a very potent insulin-like agent [41–43], it seems feasible to propose that peroxovanadate is formed either extracellularly or in the intracellular milieu and that this gives rise to the effects observed with the combination of vanadate and SSAO substrates. In this regard, an in situ formation of peroxovanadate offered by the combination of SSAO substrates and vanadate would be advantageous over the administration of exogenous peroxovanadate. Nevertheless, there are some difficulties in attributing the effects of SSAO substrates just to the formation of peroxovanadate compounds, because: (a) insulin and peroxovanadate have been reported to cause additive effects on glucose transport in isolated rat adipocytes [44] and we found no additive effects on glucose transport in the presence of insulin and benzylamine–vanadate or tyramine–vanadate; (b) peroxovanadate has been reported to activate insulin-receptor kinase activity markedly in rat adipocytes, in contrast to vanadate which does not [44–48], and the combination of benzylamine and vanadate causes a modest stimulation of insulin-receptor kinase, as assessed by tyrosine phosphorylation. In any case, it is relevant to determine whether indeed peroxovanadate or other vanadate derivatives are generated in adipose cells in response to SSAO activity and in the presence of vanadate.

Taken together, our data suggest that SSAO substrates and vanadate generate unknown compounds that synergistically stimulate one or several protein tyrosine kinases or inhibit protein tyrosine phosphatases (Fig. 2). This causes the partial activation of insulin-receptor kinase, potent activation of IRS-1 and IRS-3 and activation of p85/p110 PI 3-kinase. We propose that the activation of these signaling molecules by the combination of SSAO substrates and vanadate causes GLUT4 recruitment to the cell surface and stimulation of glucose transport in adipose cells.

5. In vivo administration of benzylamine and vanadate enhances glucose tolerance and reduces hyperglycemia in streptozotocin-induced diabetic rats

The efficacy of the in vivo administration of benzylamine and vanadate has been assayed in non-diabetic and in streptozotocin-induced diabetic rats [49]. We found that acute administration of benzylamine together with a low dose of vanadate enhances glucose tolerance in non-diabetic and in streptozotocin-induced diabetic rats [49]. The effect of benzylamine–vanadate on glucose utilization after a glucose challenge was not obtained after the administration of benzylamine or vanadate alone. In addition, this effect required an intact SSAO activity and it was not associated with alterations in the profile of plasma insulin concentrations after a glucose challenge in non-diabetic or diabetic

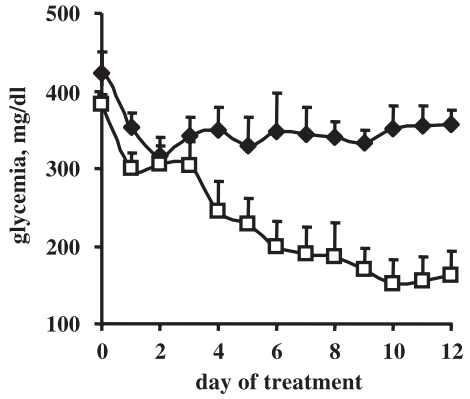

Fig. 3. Biological effects of chronic treatment with benzylamine and vanadate in streptozotocin-induced diabetic rats. Streptozotocin-induced diabetic rats were implanted subcutaneously with osmotic minipumps releasing benzylamine (84 μmol/kg/day) or were sham-operated. Rats receiving benzylamine were also subjected to a daily intraperitoneal injection of vanadate (50 μmol/kg body weight) for 2 weeks. The glycemia of diabetic rats either untreated (closed symbols) or treated with benzylamine–vanadate (open symbols) is shown. Data are mean ± S.E.M. of three to six observations per group and differences were statistically significant between control and benzylamine–vanadate groups from day 6. Data are redrawn from Ref. [49].

rats [49]. Because benzylamine–vanadate stimulates glucose transport in isolated rat adipocytes from non-diabetic rats, we suggest that adipose tissue participates in this response. Given the low concentrations of insulin present in streptozotocin-induced diabetic rats, we favour the view that benzylamine and vanadate have insulin-like effects in adipose tissue.

Interestingly, the chronic administration of benzylamine and vanadate normalized glycemia in streptozotocin-induced diabetic rats [49] (Fig. 3). As to the time dependence of these effects, a moderate reduction of glycemia was already noted at days 5 and 6 after the onset of treatment, which is similar to the time dependence shown by vanadate or peroxovanadate treatments [50,51]. We have also shown that the chronic administration of benzylamine and vanadate stimulates glucose uptake in adipose cells [49]. Thus, cells obtained after combined benzylamine and vanadate treatment showed enhanced rates of basal and insulin-stimulated glucose transport and GLUT4 expression [49]. The enhanced basal glucose transport may be a result of the acute effects of benzylamine and vanadate promoting GLUT4 recruitment to the cell surface or the presence of a larger GLUT4 population. However, the enhanced glucose uptake by adipose cells and normalization of glycemia may also be a consequence of enhanced insulin sensitivity.

Restoration of adipocyte glucose transport activity may be insufficient to normalize glucose levels in streptozotocin-induced diabetic rats following benzylamine–vanadate treatment. Based on the quantitative role of skeletal muscle on glucose homeostasis, it will be important to determine whether skeletal muscle increases glucose disposal in response to benzylamine and vanadate. In this connection, adipose cells may generate molecules with insulin-like effects in other insulin-sensitive tissues in response to benzylamine–vanadate treatment. Thus, we postulate that amelioration of adipose tissue metabolism due to benzylamine–vanadate treatment may have a profound impact on muscle metabolism via regulatory factors released from adipose cells such as leptin, TNFα, adiponectin or resistin [52–54].

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