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

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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.

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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.

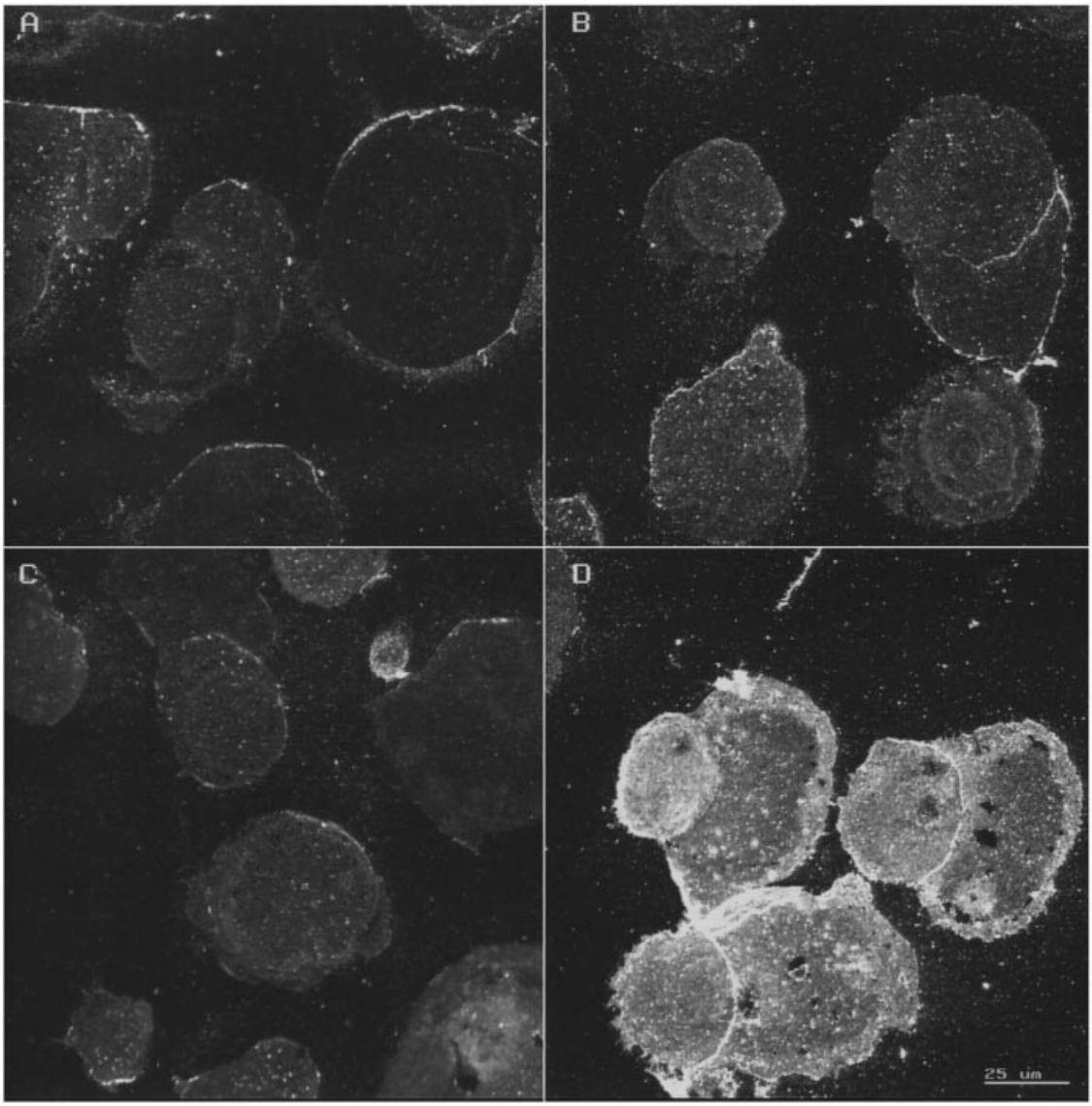

Figure 1 Benzylamine and vanadate recruit GLUT4 to the cell surface in isolated rat adipocytes

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*

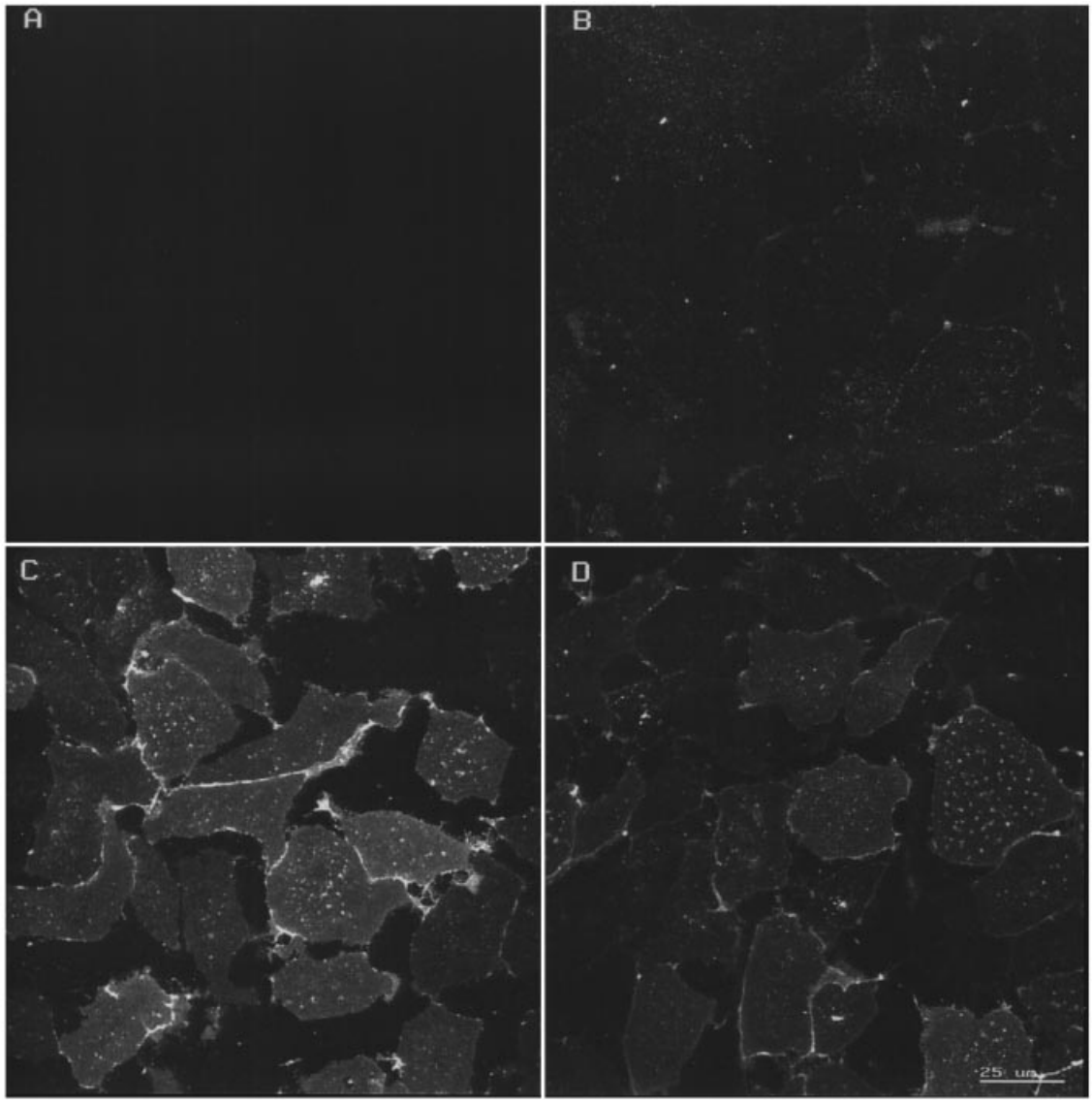

Figure 2 Benzylamine and vanadate recruit GLUT4 to the cell surface in 3T3-L1 adipocytes

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,

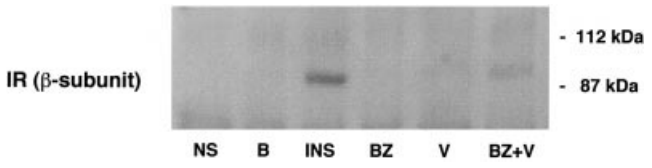

Figure 3 Combined addition of benzylamine and vanadate stimulates insulin receptor phosphorylation in isolated rat adipocytes

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.

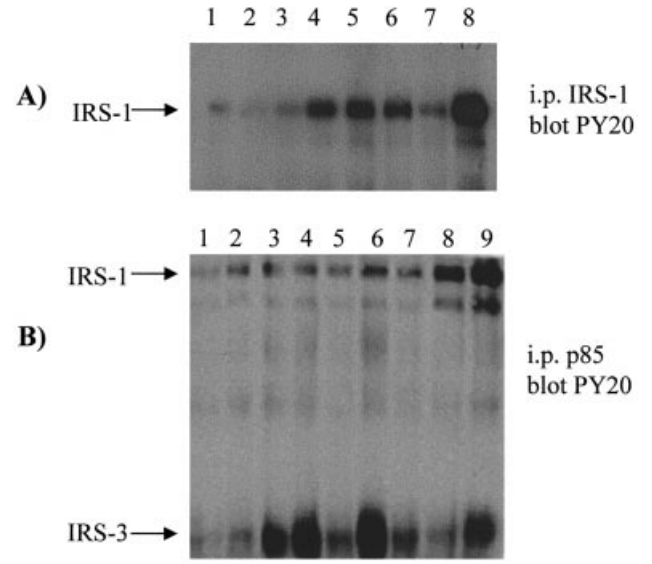

Figure 4 SSAO substrates and vanadate stimulate the tyrosine phosphorylation of IRS-1 and IRS-3

(**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).

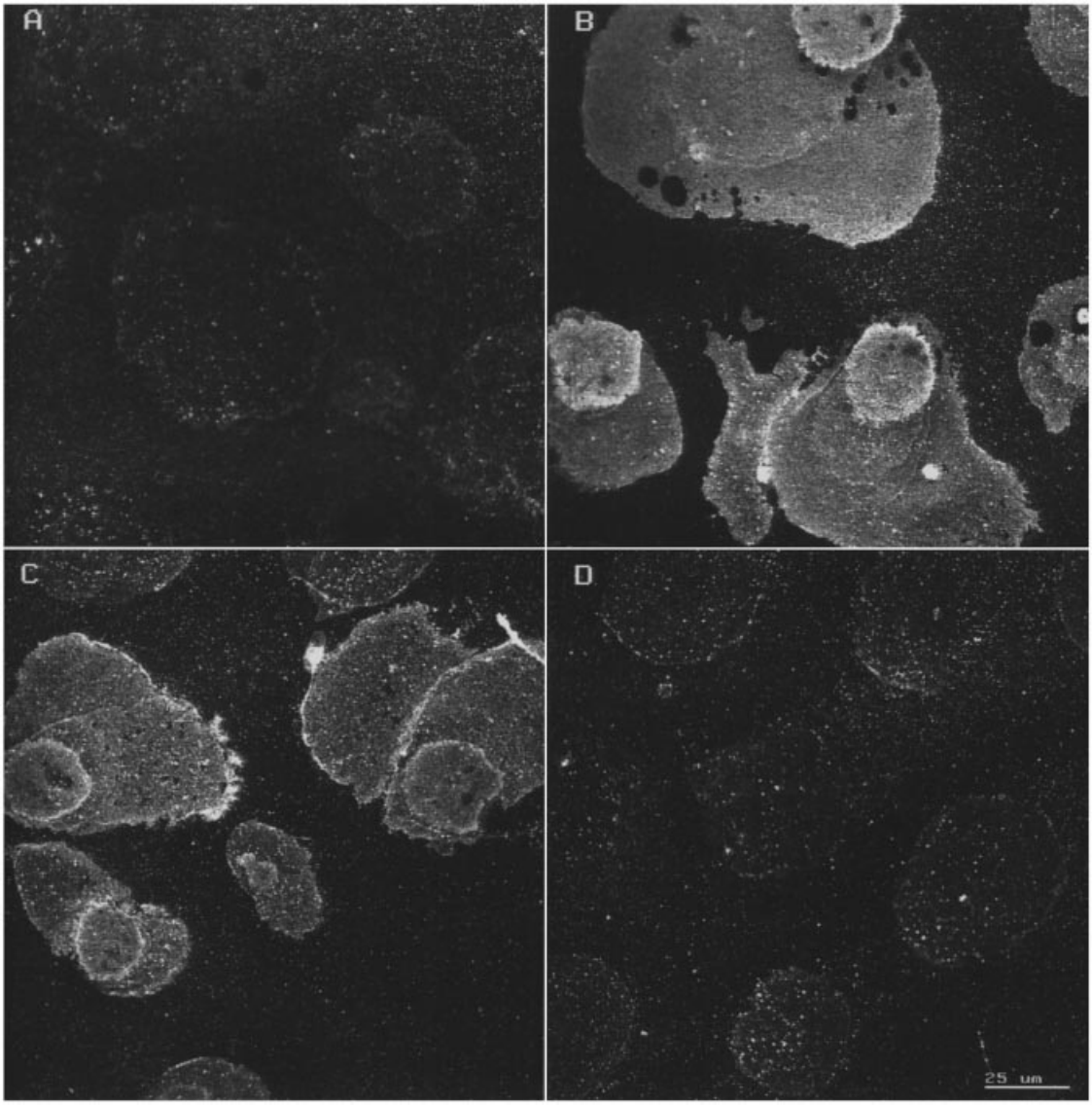

Figure 5 Wortmannin blocks the recruitment of GLUT4 to the cell surface in response to the combined addition of benzylamine and vanadate in isolated rat adipocytes

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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