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Tyramine and Vanadate Synergistically Stimulate Glucose Transport in Rat Adipocytes by Amine Oxidase-Dependent Generation of Hydrogen Peroxide[1]

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ABSTRACT

Nonadrenergic imidazoline I_2-binding sites colocalize with monoamine oxidase (MAO) in various tissues. As white adipocytes from various species have been reported to be very rich in I_2-sites, the authors consider whether these cells show a substantial MAO activity and explore its functional role. Oxidation of [^{14}C]tyramine by rat adipocyte membranes was dependent on both MAO and semicarbazide-sensitive amine oxidase (SSAO). Tyramine oxidation was identical in membranes and in intact adipocytes (V_{max}: 11-12 nmol/min/mg protein). A similar effect of MAO and SSAO inhibitors was obtained in both the intact cells and the membranes: half of the activity was sensitive to semicarbazide and the other half more easily inhibited by MAO-A than by MAO-B inhibitors. As the reaction catalyzed by amine oxidases generates H_2O_2, which mimicks certain insulin effects in adipocytes, we tested whether tyramine oxidation influences glucose transport in adipocytes. One mM tyramine weakly stimulated glucose transport. A clear potentiation of tyramine effect occurred in the presence of 0.1 mM vanadate, ineffective by itself, reaching half-maximal insulin stimulation. This stimulation was sensitive to MAO and SSAO inhibitors and to catalase. The 5-fold activation of glucose transport was accompanied by translocation of GLUT4 transporters to the plasma membrane. This shows that tyramine is readily oxidized by adipocytes and potentiates the effects of vanadium on glucose transport through release of hydrogen peroxide. The role of the amine oxidases, which are highly expressed in adipocytes, allows them to be considered as more than mere scavengers of circulating amines.

Among the various peripheral tissues, adipose tissue has been shown to be one of the richest in imidazoline binding sites (for review see Parini *et al.*, 1996). These binding sites are now pharmacologically classified as I_1- and I_2-sites (Ernsberger, 1992). They are characterized by their high affinity for imidazolinic ligands (clonidine for I_1 and idazoxan for I_2) but their biological function remains to be defined. Imidazoline-I_2 binding sites, which are labeled by [^{3}H]idazoxan, are particularly abundant in white adipocytes, at least in rats (Carpéné *et al.*, 1990; 1995b), hamsters (McKinnon *et al.*, 1989; Carpéné *et al.*, 1995b) and humans (Langin *et al.*, 1990). The imidazoline-I_2 sites have recently been localized on MAO (EC 1.4.3.4) by several observations (reviewed in Parini *et al.*, 1996). This I_2-site/MAO association, together with the abundance of I_2-sites in the fat cell, prompted us to reassess the monoamine oxidizing capacity of white adipocytes in which the presence and role of MAO is poorly documented. In fact, oxidation of monoamines or neurotransmitters has been more widely described in brown than in white adipocytes (Barrand and Callingham, 1982). Pioneering studies on the oxidation of tyramine and benzylamine by adipocyte preparations revealed the presence of another amine oxidase, resistant to blockade by the classical inhibitors of MAO-A and MAO-B, but inhibited by carbonyl reagents such as semicarbazide. It was thus called SSAO (EC 1.4.3.6) in both brown (Barrand and Callingham, 1982) and white adipocytes (Raimondi *et al.*, 1991, 1992). In our study, we describe the relative proportions of each of the adipose amine oxidases in tyramine oxidation. Their functional relevance was investigated by comparing their activities on crude membrane preparations (containing plasma, mitochondrial and vesicular membranes) and in intact fat cells. It is hypothesized that amine oxidases are not only scavengers for circulating endogenous and exogenous amines, but could also be involved in the regulation of glucose metabolism via the

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ABBREVIATIONS: MAO, monoamine oxidase; SSAO, semicarbazide-sensitive amine oxidase; 2-DG, 2-deoxyglucose; PM, plasma membranes; LDM, low density microsomes; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; KRBH, Krebs Ringer bicarbonate hepes.

hydrogen peroxide they all produce. In fact, hydrogen peroxide is considered as an insulinomimetic agent by several investigators (Heffetz *et al.*, 1992; Taylor and Halperin, 1979) but also as a second messenger in insulin signaling by others (May and De Ha 137 n, 1979; Mukherjee, 1980). Recently, we demonstrated in cardiomyocytes that serotonin-stimulated glucose uptake is mediated by the MAO-dependent degradation of the bioamine and the subsequent hydrogen peroxide formation (Fischer *et al.*, 1995). A similar approach has also shown that phenylephrine, a potent stimulator of cardiomyocyte glucose transport, exerts a biphasic effect: a fast receptor-mediated phase and a slow MAO-dependent phase (Fischer *et al.*, 1996). Moreover, hydrogen peroxide is also able to oxidize vanadyl into vanadate (Elberg *et al.*, 1994) and to transform vanadate into peroxovanadate, a most powerful insulin-mimicking agent (Shisheva and Shechter, 1993). In our study, the hydrogen peroxide generated by amine oxidases, was thus tested for its ability to activate glucose transport in white adipocytes, alone or in synergism with vanadate.

Even though the effects of MAO inhibitors have only been poorly studied in adipocytes, our results indicate that the oxidation of tyramine is far from negligible in these cells. The study also shows that tyramine and vanadate synergistically stimulate glucose transport via activation of SSAO and MAO-A; MAO-B being involved to a lesser extent. Thus, the highly active peroxovanadate can be generated not only by exogenous hydrogen peroxide, but also by the hydrogen peroxide endogenously produced by fat cells during amine oxidation.

Materials and Methods

Adipocyte isolation and crude membrane preparation. Male Wistar rats (200-260 g) were killed in a fed state, and the internal white adipose tissues (perirenal, epididymal and retroperitoneal fat depots) were dissected out and digested in Krebs-Ringer buffer containing albumin (3.5% w/v), 15 mM sodium bicarbonate, 10 mM Hepes, 2 mM sodium pyruvate and 1.5 mg/ml collagenase. After digestion for 35 to 45 min at 37°C under shaking, isolated fat cells were filtered and washed three times in the same buffer without collagenase (KRBH buffer). Freshly isolated adipocytes were adjusted to a suitable dilution (around 30 mg cell lipid/ml of KRBH) and dispensed in plastic vials (final volume 400 μl) for parallel determinations of amine oxidase and glucose transport activities. In other sets of experiments, isolated fat cells were disrupted for crude membrane preparation by hypo-osmotic lysis and centrifugation (40,000 $\times$ *g*, 20 min, 15-20°C) in a 2 mM Tris buffer containing 2.5 mM $MgCl_2$, 1 mM $KHCO_3$ and an antiprotease cocktail as previously described (Carpéné *et al.*, 1990). The pellets were immediately stored at -80°C until amine oxidase assay.

Determination of amine oxidase activity. Amine oxidase activity was measured according to the method of Yu (1986) using [^{14}C]tyramine as a substrate. For the measurement of tyramine oxidation by crude membranes, thawed pellets were washed in 200 mM phosphate buffer, pH 7.4 and repelleted by centrifugation (40,000 $\times$ *g*, 15 min, 4°C). Then, unless otherwise stated, approximately 50 μg of membrane proteins were incubated for 30 min at 37°C in 200 μl phosphate buffer containing 0.5 mM tyramine (approx. 120,000 d.p.m./vial). Tyramine oxidation by intact adipocytes was carried out in KRBH buffer with an incubation period of 45 min at 37°C in the presence of 1 mM tyramine (130,000 d.p.m./vial) except for the kinetic experiments. For the determinations of K_m and V_{max} values, membrane and adipocyte preparations were incubated with 44 $\pm$ 16 and 40 $\pm$ 3 μg membrane protein/vial for 15 and 30 min, respectively, with six different tyramine concentrations, ranging from 40 μM to 1 mM. Reactions were stopped with 50 μl of 4 M HCl. The reaction products were extracted by subsequent addition of 1 ml of solvent (toluene:ethyl acetate, v/v). An aliquot (0.7 ml) of the organic phase was transferred into scintillation vials and counted in a Packard 1900TR liquid scintillation analyzer (Rungis, France). In these conditions, product formation proceeded linearly and the initial rate was a linear function of enzyme concentration. Kinetic constants were estimated from double reciprocal plots. In both membrane and intact cell preparations, oxidation activities were expressed in nanomoles of tyramine oxidized per min and per mg of membrane proteins. For the whole cells, the protein content was determined on the pellet obtained after centrifugation (40,000 $\times$ *g*, 15 min) of 5 ml of the same cell suspension as that used with [^{14}C]tyramine in the conditions described above. The proportion of membrane proteins (in mg) per 100 mg of cellular lipid was around 0.24.

Hexose transport. After a preincubation period of 45 min at 37°C in vials containing 400 μl of cell suspension in KRBH with the tested drugs, [^{3}H]-2-deoxyglucose was added at a final concentration of 0.1 mM (approx. 1,300,000 d.p.m./vial). The assays were further incubated for 5 or 10 min and then stopped with 100 μl of 100 μM cytochalasin B. Aliquots (200 μl) of the cell suspension were centrifuged in microtubes as described by Olefsky (1978). After centrifugation, the fat cells (upper part of the tubes) were placed in scintillation vials and the incorporated radioactivity was counted. The extracellular 2-DG present in the cell fraction was determined using adipocytes whose transport activity had been previously blocked by cytochalasin B. It did not exceed 1% of the maximum 2-DG uptake in the presence of insulin.

Subcellular fractionation of adipocytes and immunoblot analysis. Fat cell suspensions obtained from a pool of nine rats were incubated for 45 min in plastic vials containing 20 ml of KRBH buffer and the tested compounds. The cells were then homogenized, and subcellular membrane fractions were prepared as previously described (Simpson *et al.*, 1983). PM and LDM were assayed for protein content and aliquots were subjected to SDS-PAGE and transferred to Immobilon-P membranes as already reported (Camps *et al.*, 1992). Briefly, membrane proteins were transferred to Immobilon in buffer consisting of 20% methanol, 200 mM glycine, 25 mM Tris, pH 8.3. After transfer, the filters were blocked for 1 hr at 37°C with 5% nonfat dry milk, 0.02% sodium azide in 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.7 mM KH_2PO_4 and were incubated with antibodies. The immune complex with the rabbit polyclonal antibodies was detected using [^{125}I]-protein A for 4 hr at room temperature and autoradiography. The areas at 45 kDa and 110 kDa corresponding to the GLUT4 and β1-integrin bands, respectively, were quantified using scanning densitometry (Ultrascan enhancer laser densitometer, LKB, Les Ulis, France).

Stastical analysis. The number of independent experiments is given by *n*. Unless otherwise stated, Student's *t* test for unpaired samples was used for detect the significant difference between the compared results, given as mean $\pm$ S.E.M. NS corresponds to nonsignificant difference ($P > .05$).

Chemicals. [^{14}C]tyramine (45 Ci/mmol) and 2-[1,2-^{3}H]-deoxyglucose (2-DG, 26 Ci/mmol) came from NEN (Boston, MA). [^{125}I]-Protein A was purchased from Amersham (Les Ulis, France). Selegiline was from RBI (Natick, MA). Sodium orthovanadate, pargyline, semicarbazide, clorgyline, phenelzine, collagenase, cytochalasin B, tyramine, γ-globulin, albumin (bovine serum albumin, fraction V) and other chemicals were purchased from Sigma Chemicals Co. (St. Quentin, France). Immobilon-P membranes were obtained from Millipore (Saint Quentin en Yvelines, France). All electrophoresis reagents and molecular weight markers were obtained from Bio-Rad (Ivry sur Seine, France). Anti-GLUT4 antibodies were produced from rabbit after immunization with a peptide corresponding to the final 15 amino acids of the carboxy terminus (Camps *et al.*, 1992). Rabbit

polyclonal antibodies against rat β_1-integrin were kindly given by Dr. C. Enrich (Barcelona University, Barcelona, Spain).

Results

Tyramine oxidation by crude membranes and by intact white adipocytes. In crude membranes from rat adipocytes, tyramine oxidation was characterized by a V_{max} of 11.9 ± 1.4 nmol tyramine oxidized/min/mg protein (n = 4), a value similar to that previously reported for liver membranes (Carpéné *et al.*, 1995a). The same V_{max} was reached with intact fat cells: 11.4 ± 1.5 nmol/min/mg membrane protein (n = 4, NS). The K_m values were 85 ± 18 and 138 ± 22 μM for crude membranes and isolated adipocytes, respectively (n = 4, *NS*). The K_m values for tyramine, a common substrate of MAO-A, MAO-B and SSAO, are in accordance with those found in the literature (Barrand and Callingham, 1982; Lyles, 1995). Thus, the Michaelis kinetic constants were similar in both disrupted and intact cells suggesting that tyramine internalization was not a limiting step for its oxidation. To further characterize the relative importance of the enzymes involved, experiments were carried out with increasing concentrations of amine oxidase inhibitors. The MAO-A inhibitor clorgyline was the most potent inhibitor in both membrane and intact cell preparations (fig. 1, A and B). In membranes, the MAO-B inhibitor selegiline shared the same low potency as pargyline, a less-selective MAO inhibitor (half-maximal inhibitions being obtained at around 20 μM). Selegiline was the weakest inhibitor when tested on intact cells. Semicarbazide, which selectively blocks SSAO but not MAO, was able to diminish tyramine oxidation in both preparations. However, complete inhibition of tyramine oxidation was obtained neither on membranes nor on fat cells, even with each inhibitor present at millimolar concentrations. The inhibitor-resistant oxidation (*ca.* 50% of total oxidation) was not due to nonenzymatic tyramine oxidation because blanks, obtained without any biological material or with denatured samples gave values of less than 5% of total oxidation (not shown). Figure 2 clearly shows that the inhibitions obtained with 1 mM pargyline and with 1 mM semicarbazide were additive. When both inhibitors were present in the incubation medium, they almost totally abolished tyramine oxidation. This was also observed with clorgyline plus selegiline when mixed with semicarbazide (not shown). SSAO and MAO equally participated in the tyramine oxidation by crude membranes or intact cells (fig. 2).

To further compare the amine oxidase activity between membranes and intact cells, various putative substrates were used as tyramine competitors. As expected, unlabeled tyramine equally inhibited oxidation in both preparations whereas serotonin and phenylephrine, considered as MAO-A substrates, were less active in intact cells than in membranes. Agmatine, the putative endogenous imidazoline I_2-site ligand (Li *et al.*, 1994) albeit possessing an amine function, did not seem to be oxidized at all in our model (table 1).

Effect of amine oxidase substrates on glucose transport in isolated fat cells. Because some amines behaved as substrates of amine oxidases in intact adipocytes and because it is known that amine oxidases produce hydrogen peroxide during the oxidative deamination reaction (Yu, 1986), we tested whether amine oxidation could mimick the action of insulin on glucose transport through the generation

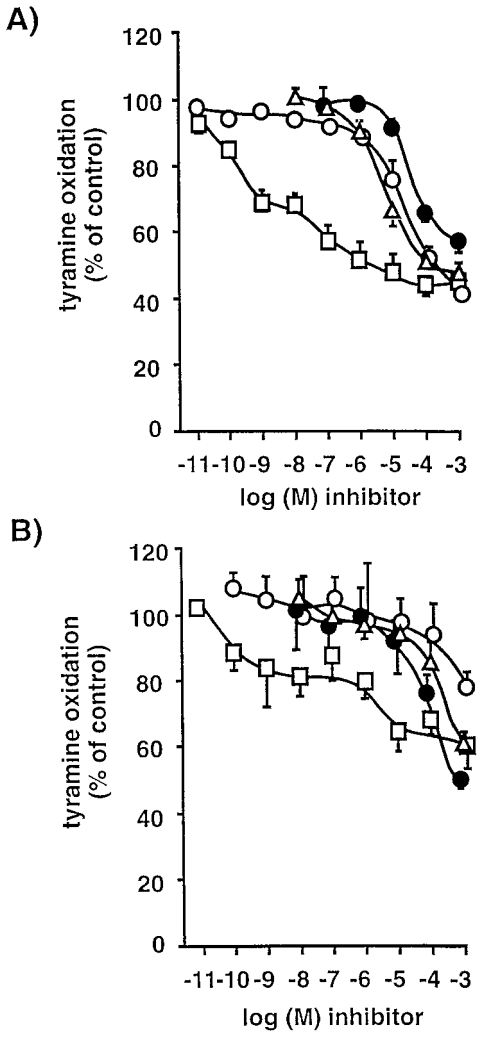

Fig. 1. Inhibition of tyramine oxidation in crude adipocyte membranes and in intact adipocytes. Crude membranes (A) or intact cells (B), containing 70 ± 9 and 30 ± 4 μg membrane proteins/vial, respectively, were incubated with a fixed tyramine concentration, 0.5 mM for membranes and 1 mM for intact cells, without inhibitor (100%) or with increasing concentrations of clorgyline (selective MAO-A inhibitor, squares), selegiline (selective MAO-B inhibitor, open circles), pargyline (mixed MAO inhibitor, triangles) and semicarbazide (SSAO inhibitor, closed circles). Total tyramine oxidation (100%), measured as described in "Material and Methods" after 30 and 45 min incubations, was equivalent to 8.6 ± .4 and 11.1 ± 1.3 nmol oxidized/min/mg protein, respectively, for membranes and intact adipocytes. Zero percent was assessed by blank assays with addition of HCl before addition of substrate. Means ± S.E.M. of three to seven different membrane preparations or three batches of intact cells.

of hydrogen peroxide. Indeed, it has been reported that exogenously added hydrogen peroxide stimulates protein phosphorylation and glucose transport in rat adipocytes (Heffetz *et al.*, 1992; Taylor and Halperin, 1979) but similar responses to endogenously produced H_2O_2 have never been reported. Glucose transport was not activated after 45 min preincubation of fat cells with increasing concentrations of tyramine, serotonin or phenylephrine whereas, in the same conditions (5-min 2-DG uptake), insulin increased basal uptake 12-fold (fig. 3). When the period of 2-DG transport was extended to 10 min, 1 mM tyramine slightly increased basal transport from 1.0 ± 0.2 to 1.7 ± 0.3 nmol 2-DG/100 mg lipid (n = 7, $P < .01$, paired t test) whilst 100 nM insulin stimulated up to 11.6 ± 1.6 nmol 2-DG/100 mg lipid (n = 7, $P < .001$). Increas-

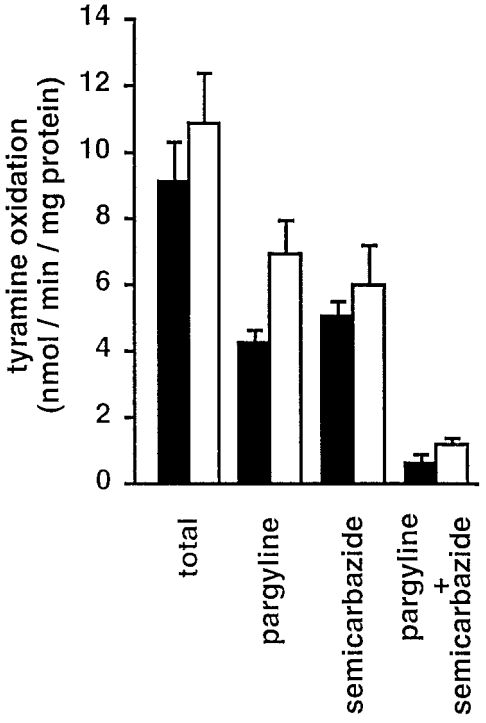

Fig. 2. Oxidation of tyramine by both MAO and SSAO in membranes and in intact fat cells. Crude membranes (dark columns) or intact adipocytes (white columns) were incubated in the conditions described in figure 1. Total tyramine oxidation was measured in the absence (total) or in the presence of 1 mM of the MAO inhibitor pargyline or the SSAO inhibitor semicarbazide alone or in combination. Mean ± S.E.M. of 11 different membrane or intact cell preparations.

TABLE 1
Competition of tyramine oxidation by amine substrates in membrane and fat cell preparations

	Membranes	Intact Fat Cells
Tyramine		
% of control oxidation	37 ± 1	38 ± 6
IC_{50}, μM	(625 ± 30)	(444 ± 124)
Serotonin		
% of control oxidation	18 ± 1	62 ± 4[a]
IC_{50}, μM	(392 ± 23)	(2990 ± 290)[a]
Phenylephrine		
% of control oxidation	41 ± 4	85 ± 5[a]
IC_{50}, μM	(7000 ± 156)	(und)
Agmatine		
% of control oxidation	102 ± 2	95 ± 5
IC_{50}	(und)	(und)

Amine oxidation was measured in crude membranes and isolated adipocytes incubated for 30 and 45 min with 0.5 and 1 mM [^{14}C]tyramine, respectively. Tyramine, serotonin (5-HT), phenylephrine and agmatine, were added from 10 μM to 10 mM. Shown is the residual tyramine oxidation found in the presence of 1 mM cold amines, expressed as a percentage of control activity. IC_{50} values, corresponding to the concentration in μM of cold amine inhibiting 50% the control tyramine oxidation are in parentheses (und: undeterminable). Means ± S.E.M. of four experiments.
[a] Different from corresponding percentage in membranes at $P < .001$.

ing the preincubation period from 45 to 120 min did not cause the amines to further stimulate glucose transport except for 0.1 mM tyramine which just increased transport up to 13% of the maximal insulin value (fig. 3). However, exogenously added H_2O_2 dose-dependently stimulated hexose transport. When preincubation lasted 45 min, 1 mM H_2O_2 multiplied the basal transport by a 5.0 ± 0.8 fold factor, an effect equivalent to 46% that of the maximal insulin effect ($n = 23$). The response then declined at concentrations higher than 1 mM (not shown). Thus, in the presence of tyramine, the H_2O_2 produced by adipocyte amine oxidases was insufficient or was immediately counteracted by endogenous scavenger systems and did not reproduce the effects of high doses of exogenous H_2O_2.

Nevertheless, hydrogen peroxide, when associated with vanadate, is well known to generate peroxovanadate, one of the most potent insulinomimetic compounds (Shisheva and Shechter, 1993). We tested whether the amine oxidation by intact fat cells generated sufficient H_2O_2 to promote the formation of peroxovanadate and subsequently stimulate glucose transport.

Potentiation by vanadate of tyramine effect on 2-deoxyglucose uptake. Figure 4 shows that vanadate potentiates the effect of tyramine as well as that of hydrogen peroxide. Vanadate alone (0.1 mM) did not affect basal or insulin-stimulated glucose transport, whereas, when combined with 1 mM hydrogen peroxide it provoked a substantial activation equivalent to three quarters of the maximal effect of insulin. Combination of vanadate with 1 mM serotonin or phenylephrine resulted in only weak activation of glucose transport that hardly doubled basal activity and represented 8 ± 2 and 12 ± 2% of maximal insulin effect ($n = 5$) whereas agmatine was inefficient (not shown). On a larger number of observations ($n = 40$), the stimulation of glucose transport induced by 1 mM tyramine plus 0.1 mM vanadate reached 54 ± 5% that of 100 nM insulin whilst vanadate alone reached only 5 ± 1% of the maximal insulin effect (vanadate plus insulin giving the same response as insulin alone: 102 ± 3%, $n = 40$). Moreover, there was no synergism between tyramine and low, ineffective, doses of insulin (10 pM) or hydrogen peroxide (0.01 mM) (not shown).

It was thus hypothesized that the vanadate/tyramine synergism works in a similar manner to that of vanadate/hydrogen peroxide via the formation of peroxovanate. Next, the effects of amine oxidase inhibitors and hydrogen peroxide scavengers were tested.

Prevention of vanadate/tyramine synergism by blockade of amine oxidase activity and by hydrogen peroxide degradation. The dose-dependent stimulation of glucose transport by tyramine plus vanadate was inhibited by most of the amine oxidase inhibitors tested, whereas none of them altered basal transport (fig. 5). Selegiline, which selectively inhibits MAO-B (seen to be a minor component of tyramine oxidation in figs. 1 and 2), was the only inhibitor unable to block the tyramine-dependent transport. When present at 0.1 mM, semicarbazide or clorgyline inhibited 59 ± 7 and 42 ± 3% of the transport promoted by 1 mM tyramine plus vanadate, but they inhibited less than 30% when tested at 10 μM ($n = 4$, not shown). Glucose transport stimulated by 1 mM hydrogen peroxide plus 0.1 mM vanadate was unaffected by 1 mM of the amine oxidase inhibitors tested alone (fig. 5) or in combination (not shown).

Addition of increasing amounts of catalase to the incubation medium did not modify basal or insulin-stimulated glucose transport in the absence (fig. 6) or presence of vanadate (not shown). When hydrogen peroxide and vanadate were mixed in the incubation before the addition of cell suspension and catalase, the peroxovanadate generated remained an efficient stimulator of glucose transport (fig. 6). However, when catalase was mixed with H_2O_2 before the addition of cell suspension and vanadate, the enzyme hydrolyzed its substrate, and thus prevented the formation of peroxovana-

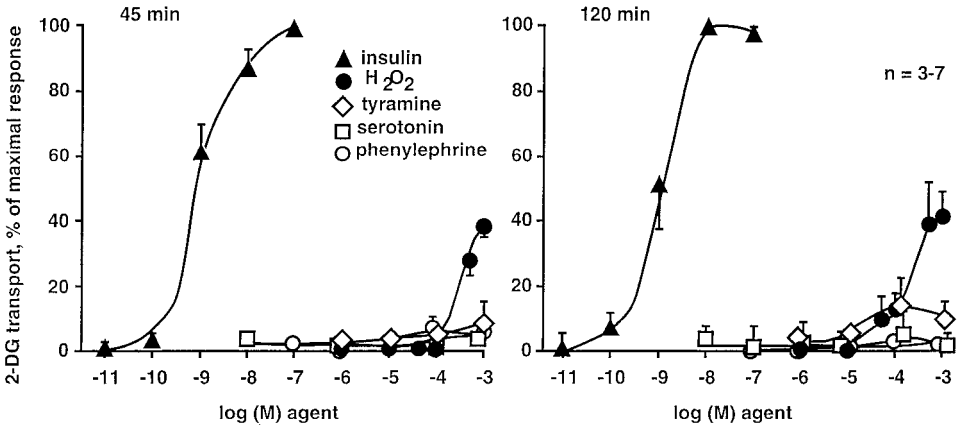

Fig. 3. Effect of amine oxidase substrates on glucose transport in rat adipocytes: influence of incubation time. Rat adipocytes were incubated for 45 (left panel) or 120 min (right panel) with the indicated concentrations of insulin (triangles), tyramine (diamonds), serotonin (squares), phenylephrine (open circles) or H_2O_2 (closed circles) and 2-DG transport was determined over an additional 5-min period. Results are expressed as percentages of maximal 2-DG transport that reached 6.72 ± 0.17 and 5.14 ± 1.30 nmol 2-DG/100 mg lipid/5 min for 45 and 120 min incubation, with insulin at 100 and 10 nM, respectively. Basal transport, taken as 0%, averaged 0.45 ± 0.06 and 1.06 ± 0.45 nmol 2-DG/100 mg lipid/5 min after 45 and 120 min preincubation. Mean ± S.E.M. of three to seven observations.

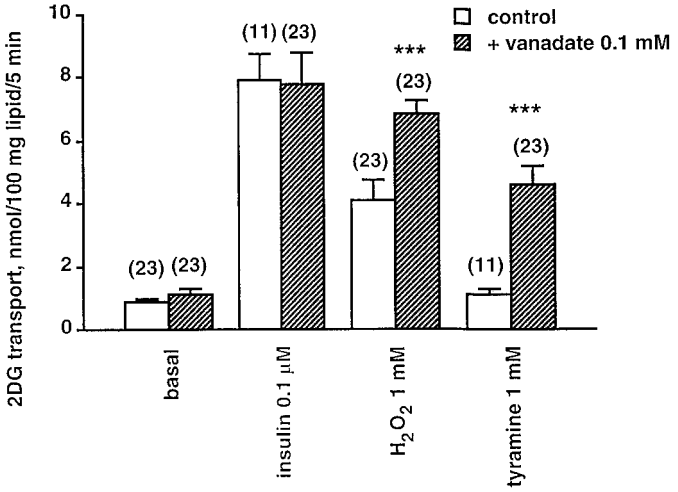

Fig. 4. Potentiation by vanadate of the effects of hydrogen peroxide or tyramine on glucose transport in rat adipocytes. Rat adipocytes were incubated for 45 min in KRBHA buffer without (control, white columns) or with vanadate 0.1 mM (hatched columns) alone (basal) and in the presence of the indicated concentrations of insulin, hydrogen peroxide or tyramine. Subsequently, 2-deoxyglucose transport was measured over a 5-min period. Mean ± S.E.M. of the number of experiments indicated in parentheses. Different from incubation without vanadate at ***P < .001.

date and the subsequent activation of glucose transport. When catalase was added simultaneously with tyramine plus vanadate, it inhibited the synergistic effect of these agents on glucose transport in a dose-dependent manner. When tyramine plus vanadate were mixed with fat cells 30 min before the addition of catalase, there was no more inhibition of glucose transport (not shown). Thus, the catalase-dependent degradation of hydrogen peroxide (exogenously added or endogenously generated) impeded the formation of peroxovanadate and was very likely the cause of the observed inhibitions.

GLUT4 translocation induced by tyramine plus vanadate. To verify whether the stimulation of glucose transport in rat adipocytes may be explained by a translocation of the insulin-sensitive glucose transporters from an internal site to the plasma membrane, as already demonstrated for insulin (Simpson *et al.*, 1983), the amount of GLUT4 protein was determined in PM and LDM fractions. Western blot analysis showed that, in conditions where insulin increased the amount of GLUT4 protein in the PM by 252 ± 43% (n = 3), tyramine plus vanadate increased it by 163 ± 9% (around half the insulin effect) whereas vanadate at 0.1 mM alone was inefficient (103 ± 8%). These increases in GLUT4 content occurred without any significant change in the content of β1-integrin, a constitutive PM protein (fig. 7). Changes in the GLUT4 content of LDM mirrored, to a lesser extent, those reported in the PM (basal LDM content was decreased by 29 and 12% with insulin and tyramine plus vanadate respectively, not shown).

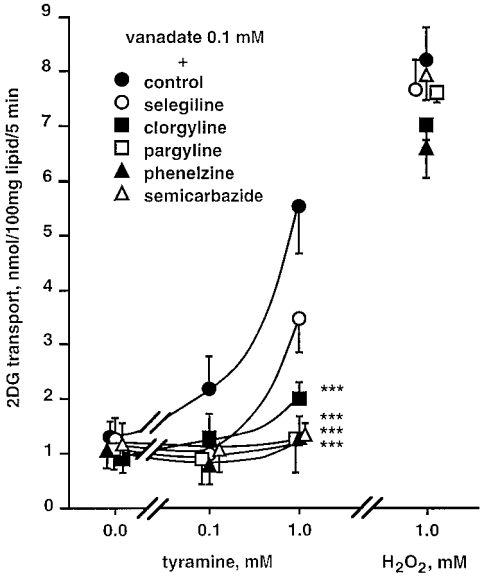

Fig. 5. Effect of inhibitors of amine oxidases on the glucose transport stimulated by tyramine plus vanadate. Rat adipocytes were incubated for 45 min with 0.1 mM vanadate and the indicated concentrations of tyramine, or hydrogen peroxide, and in the absence (control) or presence of 1 mM of the following amine oxidase inhibitors: semicarbazide (SSAO selective), selegiline (MAO-B selective), clorgyline (MAO-A selective), pargyline and phenelzine (mixed MAO). 2-DG transport was equivalent to 1.0 ± 0.4 nmol 2-DG/100 mg lipid/5 min in basal conditions (no addition). Mean ± S.E.M. of 6 to 10 observations. Different from vanadate plus tyramine at ***P < .001.

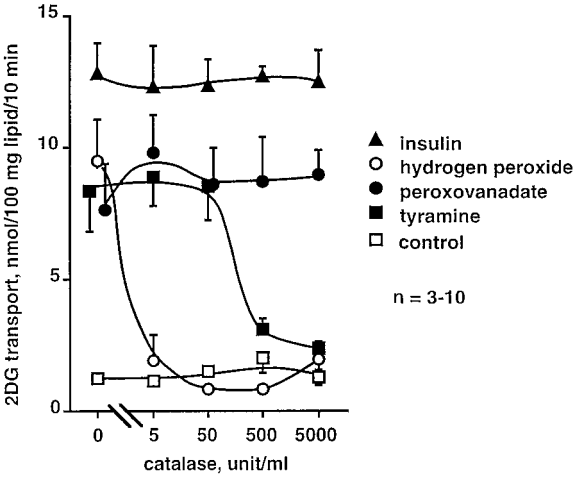

Fig. 6. Effect of catalase on basal or stimulated glucose transport in rat adipocytes. Glucose transport was measured over a 10-min period after a 45-min incubation of the fat cells in the presence of increasing doses of catalase without (control, open squares) or with 100 nM insulin (triangles) or 1 mM tyramine plus vanadate (closed squares). Aliquots of hydrogen peroxide and vanadate were mixed and allowed to generate peroxovanadate (closed circles) for 30 min before the addition of fat cells and catalase for a 45-min incubation. Hydrogen peroxide (open circles) was mixed with the indicated doses of catalase before the 45-min incubation with cell suspension and vanadate. When present, the final concentration of vanadate was 0.1 mM and that of hydrogen peroxide was 1 mM, except when degraded by catalase. Results are expressed as nmol of 2-DG transported into 100 mg of cellular lipids during 10 min.

Discussion

The relatively high level of amine oxidase activity reported here for white adipocytes is rather surprising because adipose tissue does not belong to the classical group of tissues known to participate in the clearance of endogenous or exogenous amines, essentially constituted by the brain and peripheral organs such as liver, kidney, lung and gut. Actually, the V_{max} values found for tyramine oxidation in rat adipose tissue (around 12 nmol tyramine oxidized/min/mg protein) are close to those previously reported for rat liver membranes (Carpéné *et al.*, 1995a). In fact, a substantial capacity to oxidize bioamines was already documented a decade ago in brown and white adipocytes in which the presence of SSAO, an oxidase mainly characterized by its resistance to classical MAO inhibitors and its complete inhibition by semicarbazide, was described by both the groups of Callingham and Buffoni (Barrand and Callingham, 1982, 1984; Raimondi *et al.*, 1991, 1992). However, both the high level of oxidative deamination and the diversity of the enzymes involved in this activity brought us to question the functional relevance of amine oxidases, especially in the white adipose tissue which has never been considered in studies of the peripheral effects of MAO inhibitors.

First, the high level of MAO activity observed in rat white adipocytes could be suspected from the high density of I_2-sites found in the adipose tissues of various animal species, including man (Langin, 1990) since these sites are now believed to be located on the MAOs on the basis of the following observations (reviewed in Parini *et al.*, 1996).

Second, maximal tyramine oxidation in adipocyte crude membranes was comparable to that observed in intact cells when calculated on a per milligram membrane protein basis. Moreover, K_m values were close in membranes and in intact

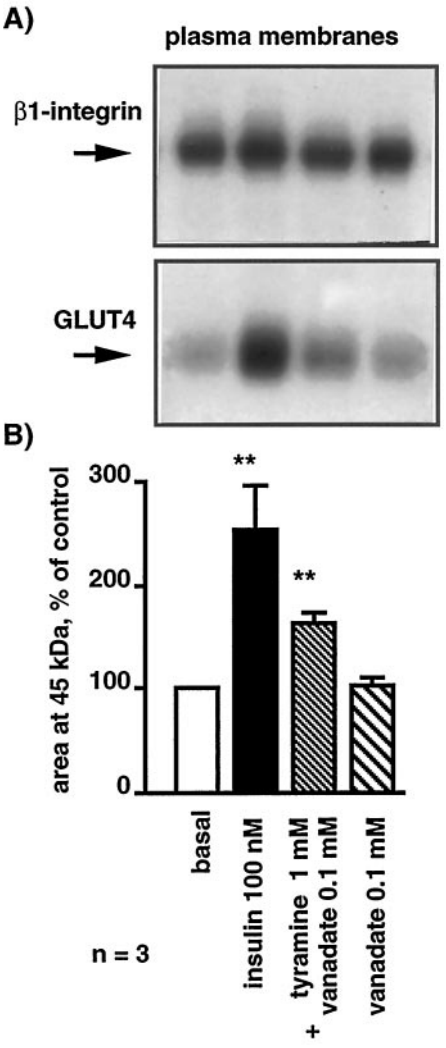

Fig. 7. Influence of tyramine, vanadate and insulin on the amount of glucose transporter GLUT4 in the plasma membrane of adipocytes. Adipocytes were incubated for 45 min in the absence (basal) or presence of insulin (100 nM) vanadate (0.1 mM) alone or in combination with tyramine (1 mM) before they were subjected to subcellular fractionation. Western blot analyses of GLUT4 transporter and of β1-integrin were carried out as reported in "Materials and Methods." Upper panel, Immunoblot of a representative experiment. Lower panel, Densitometric analyses of the bands at 45 kDa. Data, expressed in relative units, are means ± S.E.M. of three independent experiments. Different from vanadate alone at $^{**}P < .02$.

cells. These findings suggest that internalization of tyramine in adipocytes is not a limiting step for its degradation by the mitochondrial MAO. Nevertheless, this was not the case for all the amines because serotonin, a good MAO-A substrate, was apparently less oxidized in intact fat cells than in membranes. Tyramine is known to be catabolized by both MAOs and SSAO, but this is not the case for serotonin. In our model, the relative proportions of the enzymes involved in the tyramine oxidation look similar in membranes and in intact cells. It can be deduced from the experiments with inhibitors that around half of the oxidation is catalyzed by SSAO, mainly located in the plasma membrane (Barrand and Callingham, 1984; Morris *et al.*, 1997) whereas MAO-A predominates over MAO-B for the other half of oxidation. Evidence that tyramine can readily be oxidized by crude fat cell membranes but also by intact fat cells and that both oxidations can be inhib-

ited by MAO inhibitors or semicarbazide raises the question of the role of amine oxidation in adipose tissue biology.

In addition to their scavenging action on circulating amines, the amine oxidases are involved in oxidative stress and apoptosis in various cell types via their oxidation products that are aldehydes, acids, ammonium and hydrogen peroxide. However, concerning adipocytes, only one group has mentioned apoptosis (Prins and O'Rahilly, 1997) whereas the insulin mimicking effect of hydrogen peroxide has previously been frequently described (Heffetz *et al.*, 1992; May and De Haën, 1979; Mukherjee, 1980; Taylor and Halperin, 1979). Hydrogen peroxide can act directly or via the catalysis of vanadate transformation into peroxovanadate (Shisheva and Shechter, 1993). We thus tested in our work, whether the hydrogen peroxide generated by the amine oxidase activity could modulate a biological response highly sensitive to insulin: glucose uptake. When tested alone, tyramine barely stimulated glucose transport. The neurotransmitter serotonin and the adrenergic agonist phenylephrine, which behave as stronger competitors of tyramine oxidation in membranes than in intact fat cells, were totally inefficient. We estimated, from the apparent kinetic constants of oxidation, that 1 mM tyramine can generate up to approximately 40 μM H_2O_2 in the incubation medium, at least in our conditions (45-min oxidation with cells equivalent to 30-40 μg protein) and assuming that the reaction stoichiometry is one mole of hydrogen peroxide formed per mole of tyramine oxidized (Yu, 1986), and that no H_2O_2 degradation occurred. In view of this overestimate, it is likely that tyramine oxidation did not produce sufficient hydrogen peroxide to activate glucose transport since the addition of exogenous H_2O_2 stimulates glucose transport only at doses higher than 100 μM. An attempt to increase H_2O_2 accumulation by increasing the duration of tyramine oxidation was unsuccessful. However, incubation of tyramine with vanadate at doses inefficient by themselves, revealed a powerful synergism on glucose transport that appeared to be dependent on hydrogen peroxide formation. Serotonin, phenylephrine and agmatine did not markedly stimulate glucose transport in the presence of vanadate. Thus, it appears that the capacity of a given monoamine to stimulate glucose transport depends on its oxidation by intact fat cells. Whether the lack of effect of serotonin or phenylephrine on glucose transport was due to low internalization into the cell, to limited selective oxidation by MAOs, to a lack of oxidation by SSAO, or to a counterregulatory mechanism mediated by the stimulation of their respective receptors, remains to be established. The lack of effect of agmatine fits with the findings of Weitzel *et al*. (1980) who reported that this substance was able to mimick the antilipolytic effect of insulin but was devoid of any action on glucose metabolism and of hypoglycemic effects.

Evidence for the involvement of H_2O_2 in the tyramine-vanadate synergism was supplied by the inhibitory action of catalase. Catalase treatment did not affect the basal or insulin-stimulated glucose transport regardless of the presence of vanadate, but totally abolished the action of H_2O_2 only when added before vanadate. When H_2O_2 and vanadate were mixed to form peroxovanadate before the addition of catalase, the action of the powerful insulinomimetic compound was unaffected. The removal by catalase of the remaining H_2O_2 from the H_2O_2-vanadate mixture, which is generally included in the protocol of peroxovanadate preparation by various investigators (Fantus *et al.*, 1989; Shisheva and Shechter, 1993), was therefore without any effect on peroxovanadate-dependent transport. However, when tyramine, vanadate and catalase were added together with the fat cells for 45 min, there was a complete inhibition of glucose transport with high doses of the enzyme. It could be suspected that, in these conditions, hydrogen peroxide was divided between its interaction with vanadate and its degradation by catalase. Finally, it can be argued that hydrogen peroxide formation was exclusively due to the fat cell oxidases because no tyramine oxidation occurred in the incubation medium alone (see "Materials and Methods"). This excludes a putative stimulation of glucose transport by hydrogen peroxide generated from contaminants of albumin preparations such as spermine oxidase as previously described (Livingston *et al.*, 1977). Thus, the large stimulation of glucose transport obtained with the mixture of vanadate and tyramine seemed to be due to peroxovanadate. It is noteworthy that tyramine mixed with an inefficient dose of vanadate induced half of the GLUT4 translocation maximally obtained with insulin. Alternatively, the reduction of vanadate into vanadyl by fat cells, which has been shown to be prevented by H_2O_2, could also be a mechanism by which tyramine potentiates the effects of vanadate, since vanadyl, but not vanadate, is able to inhibit several kinases and even to inhibit the lipogenic effect of insulin (Elberg *et al.*, 1994). Whatever the chemical form of vanadium mediating the synergism observed, the H_2O_2 generation induced by adipocyte amine oxidases appears to be involved in the tyramine-dependent activation of glucose transport.

To further assess whether the oxidases involved in tyramine oxidation were also involved in tyramine-induced glucose transport, we compared inhibitions of the two phenomena. Concerning tyramine oxidation by intact fat cells, the inhibition curves of the MAO inhibitors were clearly shifted to the right when compared to the curves obtained with the membrane preparations. However, semicarbazide equally inhibited oxidation in disrupted or intact cells, with half-maximal inhibitions occurring in the 75 to 125 μM range. The potency of semicarbazide in inhibiting the tyramine-dependent glucose transport was also of the same order of magnitude: half-maximal inhibition was attained with 100 μM of the carbonyl reagent. These observations indicate that SSAO was readily inhibited by semicarbazide in intact cells, an observation in agreement with the studies of Barrand (Barrand and Callingham, 1984) and the recent localization of SSAO mainly in plasma membranes but also in microsomal membranes of rat adipocytes (Morris *et al.*, 1997; Enrique Tarancon *et al.*, 1998). However, it is important to note that, although it inhibits only half the tyramine oxidation by fat cells, semicarbazide totally blocked tyramine-dependent glucose transport (see figs. 1 and 5). This means either that 1) only the H_2O_2 generated by SSAO was involved in the recruitment of glucose transporters or 2) lowering endogenous H_2O_2 production under a given threshold was sufficient to avoid subsequent vanadate oxidation and activation of glucose transport. Detracting from the first hypothesis is the fact that tyramine-dependent glucose transport was also inhibited by the MAO inhibitors clorgyline, phenelzine and pargyline, thus also implicating the involvement of MAO in the observed transport activity. In addition, the relative blocking properties of clorgyline (MAO-A inhibitor) and sele-

giline (MAO-B inhibitor) were the same for tyramine oxidation and tyramine-dependent glucose transport. These results suggest that MAO-A was more involved than MAO-B in both phenomena. In support of the second "threshold" hypothesis, it should be mentioned that, in the presence of 0.1 mM vanadate, exogenous hydrogen peroxide did not stimulate glucose uptake when added at 10 μM but activated it by up to 62 ± 16% of the maximal insulin effect when tested at 50 μM (n = 3). Moreover, the difference in the maximal inhibitions induced by clorgyline between oxidation (partial inhibition) and transport (complete inhibition) suggests, as was the case with semicarbazide, the existence of a threshold hydrogen peroxide level necessary to oxidize vanadium and activate glucose uptake. Otherwise, a loss of selectivity of the inhibitors tested at 1 mM could be evoked to eliminate both hypotheses, but this would be contradictory with: 1) the additivity of pargyline and semicarbazide for the inhibition of tyramine oxidation in disrupted and intact cells and 2) the low efficiency of selegiline which was the weaker inhibitor for both oxidation and hexose transport. Although it is difficult to determine on the relative importance of SSAO and MAO in the tyramine-induced glucose transport from our results, we have already mentioned that, in rat cardiomyocytes, the serotonin stimulation of glucose transport was reduced by the MAO inhibitors clorgyline and tranylcypromine but unaffected by semicarbazide (Fischer *et al.*, 1995). To summarize, some amines can stimulate glucose uptake via both MAO-A and SSAO in adipocytes and via essentially MAO-A in cardiomyocytes.

Whether the relative importance of MAO-A, MAO-B and SSAO in amine-dependent glucose transport depends on their relative expression in a given cell type remains to be elucidated. Another complexity in the investigation of the respective roles of the different amine oxidases is that most of the substrates are poorly selective and only few amines are exclusively degraded by one type of oxidase. Moreover, this substrate selectivity is species-dependent (for review, see Lyles, 1995) and gives more complexity to the definition of the relative involvement of each amine oxidase in cellular H_2O_2 production.

Our results show that tyramine is readily oxidized by adipocytes and strongly potentiates the effects of vanadium on glucose transport through the generation of hydrogen peroxide. Both vanadium and hydrogen peroxide are well known for their insulin-mimicking properties but also for their toxic side-effects. Simultaneously administering amines and vanadate to the adipocytes induces a large insulin-like effect via the amine oxidase-generated H_2O_2. So, MAO and SSAO could then act to increase the therapeutic benefits of vanadate rather than being mere scavengers of circulating amines. Indeed, an individual being treated with vanadate as an insulin substitute could be given tyramine to generate peroxovanadate in the target cells thus enhancing the antidiabetic effects.

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