Amine oxidase substrates mimic several of the insulin effects on adipocyte differentiation in 3T3 F442A cells

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We have previously reported that substrates of monoamine oxidase (MAO) and semicarbazide-sensitive amine oxidase (SSAO) exert short-term insulin-like effects in rat adipocytes, such as stimulation of glucose transport. In the present work, we studied whether these substrates could also mimic long-term actions of insulin. Adipose differentiation of 3T3 F442A cells, which is highly insulin-dependent, served as a model to test the effects of sustained administration of amine oxidase substrates. Daily treatment of confluent cells with 0.75 mM tyramine (a substrate of MAO and SSAO) or benzylamine (a substrate of SSAO) over 1 week caused the acquisition of typical adipocyte morphology. The stimulation of protein synthesis and triacylglycerol accumulation caused by tyramine or benzylamine reached one half of that promoted by insulin. This effect was insensitive to pargyline (an MAO inhibitor), but was inhibited by semicarbazide (an SSAO inhibitor) and by *N*-acetylcysteine (an antioxidant agent), suggesting the involvement of the H₂O₂

generated during SSAO-dependent amine oxidation. Chronic administration of amine oxidase substrates also induced the emergence of adipose conversion markers, such as aP2, glycerol-3-phosphate dehydrogenase, the glucose transporter GLUT4, and SSAO itself. Moreover, cells treated with amines acquired the same insulin sensitivity regarding glucose transport as adipocytes classically differentiated with insulin. In all, most of the adipogenic effects of amines were additive to insulin. Our data reveal that amine oxidase substrates partially mimic the adipogenic effect of insulin in cultured preadipocytes. Furthermore, they suggest that SSAO not only represents a novel late marker of adipogenesis, but could also be directly involved in the triggering of terminal adipocyte differentiation.

Key words: benzylamine, glucose transport, monoamine oxidase, semicarbazide-sensitive amine oxidase, tyramine.

INTRODUCTION

The role of white adipose tissue as the major site of energy storage in the organism is achieved by the recruitment and differentiation of precursor cells into fat cells. The hormonal regulation of energy storage in adipocytes is a complex process in which insulin plays an important role. In mature adipocytes, insulin exerts a combination of rapid effects, such as stimulating glucose transport, lipogenesis and anti-lipolysis, as well as long-term actions co-ordinating the expression of multiple genes involved in glucose utilization, and lipid synthesis and storage. Insulin also enhances preadipocyte differentiation. We recently reported in rat adipocytes that the acute effect of insulin on glucose transport can be mimicked by substrates of monoamine oxidase (MAO) or semicarbazide-sensitive amine oxidase (SSAO), such as tyramine [1] and benzylamine [2]. Tyramine and benzylamine also stimulate phosphorylation of insulin-receptor substrates, phosphoinositide 3-kinase activation and translocation of the glucose transporter GLUT4 [3]. These substrates are able to stimulate glucose transport in the presence of low concentrations of vanadate, via the H₂O₂ generated during deaminative oxidation. In order to test whether amine oxidase substrates could also reproduce long-term effects of insulin in adipose tissue, we investigated, in the present work, the influence of tyramine and benzylamine on adipocyte differentiation in murine 3T3 F442A preadipocytes.

The study of preadipocyte lineages, such as 3T3 F442A and 3T3 L1, which differentiate into adipocytes [4], has shown that adipocyte differentiation is regulated by many agents, such as insulin, glucocorticoids, prostaglandins and cytokines (for review see [5,6]). Adipose conversion involves early events, such as cytoskeletal remodelling and induction of several adipose-specific genes, followed by several rounds of mitoses corresponding to clonal expansion [5,7]. Terminal differentiation results in the appearance of the functional characteristics of mature adipocytes, such as increased glucose transport capacity through expression of the insulin-responsive glucose transporter GLUT4 [8,9], and progressive accumulation of lipid droplets in the cytoplasm. In 3T3 F442A preadipocytes, insulin elicits adipogenesis by acting at diverse steps: increasing the transactivating action of peroxisome-proliferator-activated receptor (PPAR) γ and PPAR α [10], enhancing the expression of CCAAT-enhancer-binding protein (C/EBP) β , C/EBP δ and adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element-binding protein 1 ('ADD1/SREBP1') [6], and increasing the amount of various proteins involved in lipid storage, such as glycerol-3-phosphate dehydrogenase (GPDH) [11] and adipocyte lipid-binding protein (ALBP), the product of the aP2 gene [12].

SSAO, an enzyme previously known to scavenge biogenic or exogenous amines [13,14], has been recently proposed as a novel late adipogenesis marker, since its mRNA and activity are

Abbreviations used: ALBP, adipocyte lipid-binding protein; C/EBP, CCAAT-enhancer-binding protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GPDH, glycerol-3-phosphate dehydrogenase; MAO, monoamine oxidase; PPAR, peroxisome-proliferator-activated receptor; SSAO, semicarbazide-sensitive amine oxidase.

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strongly induced during adipocyte differentiation in 3T3 F442A and 3T3 L1 cells [15]. Moreover, convergent findings indicate that SSAO is strikingly related to glucose transport in rat adipocytes. On one hand, SSAO is highly expressed at the plasma membrane but is also located in GLUT4-containing intracellular vesicles [2,16]. On the other hand SSAO oxidative activity generates H_2O_2 , a compound known for its action on tyrosine phosphorylation of intracellular proteins [17], and its stimulation of glucose transport [18]. More recently, we have observed that, in the presence of vanadate, benzylamine stimulates glucose uptake and recruitment of GLUT4 to the cell surface in both rat fat cells and 3T3 L1 adipocytes [3]. MAO, another member of the amine oxidase family, is also present in rodent adipocytes [19], and is highly expressed in human adipose tissue [20]. Although MAO is different from SSAO regarding its cofactor, intracellular location, and substrate and inhibitor specificities [21], it also generates H_2O_2 during oxidative deamination [22]. MAO is involved in the stimulation of glucose transport by tyramine and serotonin in adipocytes [1] and cardiomyocytes [23] respectively. This novel interplay between amine oxidase and glucose uptake activities led us to search for other putative insulin-like effects of SSAO and MAO substrates, such as regulation of adipocyte differentiation.

The present work characterizes MAO and SSAO activities in 3T3 F442A cells and shows that tyramine (a substrate of MAO and SSAO) and benzylamine (an SSAO substrate) exert both short-term and long-term insulin-like effects on glucose transport and adipogenesis. The results suggest that amine oxidation may play an important role in adipocyte biology.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM) and calf serum (FCS) were obtained from Life Technologies. [^{14}C]Benzylamine was purchased from Amersham, and 2-deoxy[3H]glucose and [^{14}C]tyramine were obtained from DuPont NEN Life Science Products. The polyclonal antibody OSCRX was raised against the C-terminus of GLUT4 [24], and the anti-GLUT1 polyclonal antibody was purchased from Diagnostic International (Karlsdorf, Germany). The anti-SSAO antibody was produced from rabbit after immunization with purified bovine lung SSAO [25], and cross-reacted with the human and murine enzymes. All other reagents or chemicals were obtained from Sigma-Aldrich unless otherwise stated. Cell culture plasticware was purchased from Falcon (Becton Dickinson Labware, NJ, U.S.A.).

Cell culture and chronic treatments

3T3 F442A cells [4] were seeded at a density of 4000 cells/cm² and grown at 37 °C in a 7% CO₂ atmosphere. Cells were kept in DMEM supplemented with 5% (v/v) donor calf serum until confluence. Confluent cultures were induced to differentiate in DMEM in the presence of 5% (v/v) fetal calf serum (FCS) and 50 nM insulin for 8 days. When differentiation was induced by insulin, or other chronic treatments, fresh medium containing the tested compounds was changed daily. Post-confluent cells kept for the same period in DMEM containing 5% FCS served as control cells. Cell number was determined using a cell counter (Coulter Electronics). Differentiation of adipose cells was assessed by evaluating both cell morphology, using phase contrast microscopy, and biochemical parameters, such as triacylglycerol and protein content, and GPDH activity. For these procedures, 3T3 F442A cells were first washed twice with PBS, then scraped, resuspended in 50 mM Tris/HCl (pH 7.5)/1 mM EDTA buffer

and homogenized. Triacylglycerol content was quantified by a colorimetric reaction using the Triglyceride Enzymatic Trinder kit (Biotrol Diagnostic, Chennevières, France). Protein content was determined using the Bio-Rad DC Protein Assay kit. GPDH activity was evaluated by spectrophotometric assays conducted as previously reported [11].

RNA extraction and Northern-blot analysis

Total RNA was extracted using the Qiagen RNeasy kit. RNA (25 µg/lane) was electrophoresed in a 1% (w/v) agarose/2.2 M formaldehyde gel, then transferred on to a nylon membrane (Schleicher and Schuell, Dassel, Germany) and UV-crosslinked. For the determination of aP2-mRNA abundance, Northern-blot analysis was conducted on total RNAs as described by Amri et al. [26]. RNA loading was controlled with a R45 cDNA probe corresponding to a fragment of 18 S rRNA (a gift from J. P. Hugnot, Institut Cochin de Génétique Moléculaire, Paris, France).

Membrane preparation and Western-blot analysis

Cells were washed with PBS and homogenized in 20 mM Hepes, 250 mM sucrose, 1 mM EDTA, 1 unit/ml aprotinin, 100 µM 4-(2-aminoethyl)benzenesulphonyl fluoride, 1 µM leupeptin and 1 µM pepstatin A (pH 7.4). Homogenates were centrifuged at 1400 g for 5 min at 4 °C, and the pellet was discarded. The supernatant was subsequently centrifuged at 200 000 g for 90 min at 4 °C. Pellets containing total membranes were resuspended in 30 mM Hepes (pH 7.4) and frozen at -80 °C. SDS/PAGE was carried out using 11% (for GLUT4 and GLUT1) or 7.5% (for SSAO) polyacrylamide gels; protein samples were subjected to reducing conditions. Proteins were then transferred on to nitrocellulose membranes, which were blocked in PBS containing 5% (w/v) non-fat dry milk (for GLUT4 and GLUT1) or 5% (w/v) fish gelatin (for SSAO) and incubated overnight at 4 °C with rabbit antisera against GLUT4 (1:800 dilution), GLUT1 (1:1300 dilution) or SSAO (1:2000 dilution). Primary antibodies were detected with horseradish-peroxidase conjugated monoclonal anti-rabbit IgG (1:20 000 dilution; Sigma) and visualized using enhanced chemiluminescence detection (Amersham). Blots were quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA, U.S.A.) or Phoretix (Phoretix International Ltd, Newcastle upon Tyne, U.K.) software.

Amine oxidase assay

Amine oxidase activity was measured according to the method of Yu [22], using [^{14}C]tyramine or [^{14}C]benzylamine as substrates. MAO activity was defined as the part of oxidation sensitive to preincubation with 0.1 mM pargyline, whereas SSAO activity was sensitive to (inhibited by) 1 mM semicarbazide. Briefly, cells were washed with PBS and homogenized in 200 mM phosphate buffer (pH 7.4). Homogenates were incubated for 30 min at 37 °C in 200 µl of phosphate buffer (approx. 100 µg of protein) in the presence of tyramine (at a final concentration of 0.5 mM; 0.05 µCi of [^{14}C]tyramine/assay) or 0.1 mM benzylamine (0.1 µCi of [^{14}C]benzylamine/assay). Assays were stopped by adding 50 µl of 4 M HCl. Reaction products were extracted by the subsequent addition of 1 ml of solvent (toluene/ethylacetate, 1:1, v/v). Aliquots of the organic phase (0.7 ml) were transferred into scintillation vials and counted for radioactivity. For K_m and V_{max} determinations, homogenates ($\approx$ 100 µg of protein) were incubated with six different concentrations of tyramine or benzylamine (0.01–1 mM) as previously described [1].

Hexose transport

Cells were starved of serum overnight and were preincubated for 30 min at 37 °C in 0.5 ml of Krebs–Ringer buffer (120 mM NaCl, 6 mM KCl, 1 mM CaCl₂, 1.2 mM MgSO₄, 1 mM NaH₂PO₄ and 1 mM Na₂HPO₄) supplemented with 12.5 mM Hepes, 3.5 % (w/v) BSA and 2 mM pyruvate (pH 7.4), and gassed with O₂/CO₂ (19:1). After this period, fresh buffer containing various concentrations of insulin (1–1000 nM) or other tested compounds were incubated with the cells for 45 min. A glucose transport assay was then started by addition of 100 µl of a 2-deoxyglucose isotopic dilution (containing 0.2 µCi of 2-deoxy[³H]glucose) at a final concentration of 140 µM. The assay was carried out for 10 min, after which the radioactive solution was aspirated and cells were rinsed with ice-cold PBS containing 10 mM glucose. Cells were rinsed several times with PBS, treated with 0.5 ml of 0.05 M NaOH and counted for radioactivity. Non-specific glucose uptake was determined by addition of 40 µM cytochalasin B prior to 2-deoxyglucose administration.

RESULTS

MAO and SSAO activities in 3T3 F442A adipocytes

The pattern of amine oxidation by 3T3 F442A cells was initially determined on differentiated adipocytes using tyramine, a substrate of both MAO and SSAO, and benzylamine, an SSAO substrate [27]. In homogenates of differentiated 3T3 F442A adipocytes, the K_m of [¹⁴C]tyramine oxidation by MAO was 125 ± 16 µM and the V_{max} was 1.54 ± 0.27 nmol/min per mg of protein ($n = 4$). The oxidation of tyramine (0.5 mM) was almost totally blocked by 0.01 mM pargyline (an irreversible MAO inhibitor) [28], whereas 1 mM semicarbazide (an SSAO inhibitor) was weakly inhibitory (Figure 1A). Complete abolition of tyramine oxidation was only achieved in the presence of both pargyline and semicarbazide (results not shown), suggesting that in 3T3 F442A adipocytes, tyramine is mainly oxidized by MAO

Figure 1 Dose-dependent inhibition of tyramine and benzylamine oxidation by MAO or SSAO activities in 3T3 F442A adipocytes

Homogenates were prepared from 3T3 F442A adipocytes differentiated for 8 days in the presence of 50 nM insulin and incubated with increasing concentrations of clorgyline (an MAO-A inhibitor, ■), selegiline (an MAO-B inhibitor, ▲), pargyline (●) or with the SSAO inhibitors semicarbazide (○) or hydralazine (□) 15 min prior to the addition of (A) 0.5 mM [¹⁴C]tyramine or (B) 0.1 mM [¹⁴C]benzylamine. Amine oxidation was conducted in a final volume of 200 µl for 30 min at 37 °C as detailed in the Experimental section. Results are expressed as the percentages of total oxidation in the absence of any inhibitor (equivalent to 39.8 ± 6.1 and 9.1 ± 1.0 nmol/30 min per mg of protein for tyramine and benzylamine respectively), and are presented as means $\pm$ S.E.M. of three to four experiments.

Figure 2 MAO and SSAO activities in 3T3 F442A cells during adipose differentiation

Homogenates from 3T3 F442A cells at different times from confluence (day 0) were incubated for 30 min with 0.5 mM [¹⁴C]tyramine (A) or 0.1 mM [¹⁴C]benzylamine (B) in a final volume of 200 µl (50–70 µg of protein). SSAO- or MAO-dependent oxidation was determined by inhibition with 0.1 mM pargyline (an MAO inhibitor) or 1 mM semicarbazide (an SSAO inhibitor). Results are expressed as nmol of oxidized substrate/30 min per mg of protein, and are presented as means $\pm$ S.E.M. of four to thirteen different determinations.

and, to a lesser extent, by SSAO. Studies conducted with increasing concentrations of clorgyline (a selective MAO-A inhibitor) or selegiline (an MAO-B inhibitor) indicated that tyramine oxidation was predominantly due to MAO-A (Figure 1A).

The K_m of SSAO towards [¹⁴C]benzylamine was 18 ± 1 µM and the V_{max} was 0.54 ± 0.04 nmol/min per mg of protein ($n = 4$). Oxidation of benzylamine (0.1 mM) by 3T3 F442A cells was of a lower magnitude than that of tyramine, but was an exclusively SSAO-dependent phenomenon since it was completely inhibited by semicarbazide and another SSAO blocker, hydralazine [14], whereas it was resistant to MAO inhibitors (Figure 1B). For both tyramine and benzylamine, no difference in maximal oxidation rate was detected between intact cells and freshly prepared homogenates, suggesting that substrate accessibility was not a limiting factor whatever the subcellular location of the amine oxidase; MAO is mitochondrial and SSAO is located at the cell surface (results not shown).

MAO and SSAO activities during adipose conversion of 3T3 F442A cells

MAO and SSAO activities markedly differed throughout the adipose conversion process in 3T3 F442A cells cultured under standard conditions [50 nM insulin and 5 % (v/v) FCS]. On one hand, MAO activity was already present at the preconfluent stage and rapidly reached a plateau after a 2-fold increase, 2 days after confluence (Figure 2). On the other hand, SSAO activity, which was practically absent at the preadipocyte stage (at day -2 SSAO-dependent oxidation of benzylamine was 0.5 ± 0.2 nmol/30 min per mg of protein), whereas MAO-dependent oxidation of tyramine was 17.4 ± 4.7 nmol/30 min per mg of protein, dramatically increased during the differentiation process, reaching an oxidation level of 12.5 ± 1.1 nmol/30 min per mg of protein 14 days after confluence.

Table 1 Stimulation of 2-deoxy[³H]glucose uptake by tyramine and benzylamine in 3T3-F442A adipocytes

Differentiated cells were incubated for 45 min with 1 mM tyramine or benzylamine alone or in combination with 100 nM insulin, in the absence (control) or in the presence of 0.1 mM vanadate. Results are expressed as the percentages of the maximal response to insulin, which was 4.4 ± 1.4 nmol of 2-deoxyglucose/10 min per mg of protein, and represent means $\pm$ S.E.M. of six experiments. Significance of differences: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with basal uptake (1.5 ± 0.3 nmol of 2-deoxyglucose/10 min per mg of protein), in the absence or presence of vanadate.

Addition to the medium	Uptake of 2-deoxyglucose (% of maximal insulin effect)	
	—Vanadate	+ Vanadate (0.1 mM)
Basal	34.2 ± 5.2	32.9 ± 4.0
Tyramine (1 mM)	$74.7 \pm 10.7^{**}$	$83.5 \pm 10.8^{**}$
Benzylamine (1 mM)	$60.6 \pm 14.2^*$	$60.0 \pm 12.6^*$
Insulin (100 nM)	100	$102.9 \pm 1.9^{***}$
Insulin + tyramine	$131.7 \pm 23.3^{***}$	$127.6 \pm 18.1^{***}$
Insulin + benzylamine	$128.3 \pm 22.6^{***}$	$122.3 \pm 11.1^{***}$

Table 2 Effects of chronic treatment with tyramine, benzylamine or insulin on cell number, and protein and triacylglycerol content in 3T3 F442A cells

Preadipocytes were seeded at 15 000 cells/well in 12-well dishes ($3.8 \text{ cm}^2/\text{well}$) and grown until confluence (day 0). Cells were then kept until day 8 with DMEM plus 5% FCS alone (control) or in the presence of the indicated final concentrations of insulin, tyramine or benzylamine. Results are presented as means $\pm$ S.E.M. of 12–14 experiments. Significance of differences: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the control group (day 8).

Chronic treatment	Cell number (10^6 cells/well)	Protein content ($\mu\text{g}/10^6$ cells)	Triacylglycerol content (nmol/ 10^6 cells)
Before (day 0)			
Confluent preadipocyte	$0.108 \pm 0.036^{**}$	425 ± 14	$15 \pm 2^{***}$
After (day 8)			
Control	0.356 ± 0.021	461 ± 26	44 ± 5
Tyramine (0.75 mM)	0.353 ± 0.019	$685 \pm 42^{**}$	$85 \pm 9^{***}$
Benzylamine (0.75mM)	0.351 ± 0.027	$735 \pm 73^{**}$	$97 \pm 10^{***}$
Insulin (50 nM)	$0.577 \pm 0.042^*$	$810 \pm 73^{***}$	$164 \pm 22^{***}$
Insulin + tyramine	$0.568 \pm 0.046^*$	$1002 \pm 74^{***}$	$255 \pm 38^{***}$
Insulin + benzylamine	$0.504 \pm 0.039^*$	$1058 \pm 99^{***}$	$298 \pm 37^{***}$

Figure 3 Effects of amine treatment on 3T3 F442A cell morphology

Microphotographs of cultured 3T3 F442A cells after 8 days of post-confluent treatment with: (B) 50 nM insulin; (C) 0.75 mM tyramine; (D), insulin + tyramine; (E) 0.75 mM benzylamine; (F) insulin + benzylamine. Control cells are shown in (A). Bar = $50 \mu\text{m}$.

Figure 4 Effects of pargyline, clorgyline, semicarbazide and hydralazine on triacylglycerol content in 3T3 F442A cells

Confluent cells were kept for 8 days in the presence of 5% (v/v) FCS only (control), with 0.75 mM benzylamine or tyramine alone or in combination with 50 nM insulin. The same chronic treatments were also carried out in the presence of 0.1 mM pargyline (A), 10 μ M clorgyline (B), 1 mM semicarbazide (C) or 10 μ M hydralazine (D). The effects of the inhibitors (added daily from day 0 to day 8) were compared with their respective controls (n = number of paired experiments). *Significant difference from the corresponding chronic treatment without inhibitor ($P < 0.05$).

MAO and SSAO substrates stimulate glucose transport in 3T3 F442A adipocytes

We verified whether the stimulation of glucose transport by tyramine or benzylamine, previously observed in rat adipocytes [1] and 3T3-L1 cells [3], could be reproduced in 3T3 F442A cells. Short-term incubation with 1 mM tyramine or benzylamine was able to partially mimic the effect of insulin on 2-deoxy[3 H]glucose uptake into differentiated 3T3 F442A cells (8 days after confluence) (Table 1). Indeed, 1 mM benzylamine stimulated glucose transport up to 61%, and 1 mM tyramine up to 75% compared with maximal insulin-stimulated glucose uptake, which was equivalent to a 3-fold increase in basal uptake. The presence of 0.1 mM vanadate, which was necessary to achieve this effect in isolated rat adipocytes [1,2], was not required in 3T3 F442A cells. Benzylamine or tyramine did not hamper the effect of insulin on glucose transport; they even exhibited a tendency to increase maximal 2-deoxyglucose uptake when combined with 100 nM insulin (Table 1). Completely different results were obtained with undifferentiated cells (2 days before confluence): 100 nM insulin only stimulated glucose uptake by 1.4-fold, and amines were not stimulatory (results not shown).

Chronic treatment with benzylamine or tyramine induces protein anabolism, lipid accumulation and acquisition of adipocyte morphology in 3T3 F442A cells

We next investigated whether amine oxidase substrates could also mimic long-term effects of insulin, such as the stimulation of adipogenesis. To this aim, a chronic treatment approach was designed: confluent 3T3 F442A preadipocytes were cultured in DMEM containing 5% (v/v) FCS over 8 days and were treated daily with 50 nM insulin, 0.75 mM tyramine, 0.75 mM benzylamine, insulin + tyramine or insulin + benzylamine. Cells kept in DMEM containing 5% (v/v) FCS without any addition served as a control. Adipocyte differentiation was initially assessed by evaluating cell morphology. As expected, control cells were poorly differentiated. Treatment with either ben-

zylamine or tyramine alone mimicked the effect of insulin on the acquisition of typical adipocyte morphology. Moreover, an even larger increase in the number and/or size of the intracellular lipid droplets was detected in cells treated with the combination of amine plus insulin (Figure 3).

We next evaluated whether amines could mimic the effects of insulin on cell proliferation, protein anabolism and lipid accumulation. Cell number determinations showed that long-term treatment with amines did not affect cell viability (Table 2). In fact, both control and treated groups underwent an increase in cell number, as a consequence of the post-confluent clonal expansion phenomenon. A larger increase in cell number was observed in the insulin-treated group, and the addition of amines did not alter this effect. Tyramine- and benzylamine-treated groups exhibited a 2-fold increase in triacylglycerol content while insulin-treated cells accumulated 3-fold more lipids than the control cells. The combined treatment with both insulin and amine oxidase substrates had an additive effect on lipid accumulation, which increased 6-fold compared with the control (Table 2). Chronic treatment with tyramine or benzylamine also mimicked the anabolic effect of insulin on total cellular protein content and, again, the combined treatment with both insulin and amine showed an additive effect on protein content (Table 2). Additional experiments showed that the maximal effect of 8-day treatment with tyramine or benzylamine on adipogenic differentiation was reached at 0.5 mM, did not increase any further at 1 mM and even decreased at 10 mM (results not shown).

Amine-induced adipogenesis in 3T3 F442A cells is prevented by SSAO inhibitors and by the antioxidant *N*-acetylcysteine

In order to assess whether the chronic effects of tyramine and benzylamine on adipogenesis were dependent on their oxidation by MAO and/or SSAO and the subsequent formation of H_2O_2 , we measured the influence of amine oxidase inhibitors and the antioxidant *N*-acetylcysteine on triacylglycerol accumulation. MAO blockade by 100 μ M pargyline or 10 μ M clorgyline failed

Figure 5 Effect of chronic treatment with tyramine or benzylamine on aP2-mRNA expression

Confluent cells were cultured under the different treatments for the indicated periods. Total RNA was extracted and analysed by Northern blotting using 25 μ g of total RNA/lane. To control for equivalency in RNA loading, blots were also hybridized with a cDNA probe for 18 S ribosomal RNA (right-hand panel). An autoradiograph representative of three experiments is shown.

to significantly modify the lipid-accumulating effects in any of the studied chronic treatments (Figures 4A and 4B). Addition of 1 mM semicarbazide or 10 μ M hydralazine over 8 days significantly inhibited the effect of tyramine or benzylamine on adipogenesis, whereas it was without any effect in control or insulin-treated cells. The additive effect of the amines combined with insulin was also abolished by semicarbazide or hydralazine (Figures 4C and 4D). None of the inhibitors tested affected post-confluent cell numbers (results not shown). These data demonstrate that SSAO activity was required for the adipogenic effect of amines, whereas there was no evidence for the involvement of MAO.

N-Acetylcysteine, a scavenger for reactive oxygen species and a precursor for GSH [29], reduced the adipogenic action of the amines. The triacylglycerol content in tyramine- and benzylamine-treated cells fell from 26.6 ± 4.5 and 25.9 ± 4.2 to 16.1 ± 3.2 and 14.2 ± 0.4 nmol/well when 1 mM *N*-acetylcysteine was present in the culture medium ($n = 3$, $P < 0.05$). Therefore treatments designed to reduce H_2O_2 reactivity, either by blocking its formation or by favouring its degradation, inhibited lipid accumulation in 3T3 F442A cells chronically treated with amine oxidase substrates.

Chronic treatment with amine oxidase substrates promotes the emergence of adipocyte differentiation markers in 3T3-F442A cells

Our demonstration that amine oxidase substrates enhance triacylglycerol accumulation led us to investigate whether they could also participate in the triggering of the adipocyte differentiation process. We thus tested the emergence of several well-known adipocyte late differentiation markers under amine chronic treatment. aP2 mRNA, coding for the lipid binding protein ALBP, is a well established marker of adipose conversion [12]. As expected, its expression was detectable in adipocytes fully differentiated under insulin, but not in pre-confluent cells (results not shown). aP2 mRNAs were detectable by Northern-blot analysis after 1 or 2 days of treatment of confluent cells with tyramine or benzylamine, whereas no increase was apparent in control cells or cells treated with insulin (Figure 5). At day 4, the aP2-mRNA abundance was clearly increased in amine-treated cells when compared with the control cells, although it remained

Figure 6 Effect of chronic treatment with amines on SSAO expression

Protein extracts (10 μ g) were loaded in each lane, corresponding to post-confluent cells stopped after 4, 6 or 8 days of the indicated treatments. The polyclonal anti-SSAO antibody recognized a band at 96 kDa. A Western blot representative of three experiments is shown.

Figure 7 Effect of chronic treatment with amines on GPDH activity, and on GLUT4 and GLUT1 protein expression

(A) GPDH activity is expressed as nmol of degraded NADH/min per mg of protein. All of the treated groups were significantly different from the control ($P < 0.001$, $n = 6$). The expression of glucose transporters GLUT4 (B) and GLUT1 (C) was determined by immunoblot analysis of total membranes prepared from 3T3 F442A cells after 8-day chronic treatments. Autoradiographic bands at 45 kDa were quantified by densitometry. Results are expressed as the percentages of the density found in insulin-treated cells (set at 100%), and are presented as means $\pm$ S.E.M. of three separate experiments. Only cells grown under control conditions (white columns) were statistically different from insulin-treated cells ($P < 0.001$).

lower than in insulin-treated cells (Figure 5). At day 8 post-confluence, all the treated groups were highly positive for aP2 mRNA (results not shown).

Figure 8 Effect of chronic treatment with tyramine or benzylamine on glucose transport in 3T3 F442A cells

Confluent 3T3-F442A cells were kept for 8 days in 5% (v/v) FCS without addition (control, ○) or in the presence of 50 nM insulin (●) and/or 0.75 mM benzylamine (closed symbols) or tyramine (open symbols). Serum-starved cells were incubated for 45 min without (basal) or with various concentrations of insulin. Results are expressed as nmoles of uptaken 2-deoxy[³H]glucose/10 min per 10⁶ cells and represent means ± S.E.M. of 18 experiments. Abbreviations used: bas, basal; ins, insulin; 2-DG, 2-deoxyglucose.

SSAO itself has been recently proposed as a differentiation marker in the 3T3 adipocyte lineage [15]. The pattern of SSAO protein expression in tyramine- or benzylamine-treated cells was very similar to that of the insulin-treated group: SSAO protein was detectable at day 4, and by day 8 reached the same high level of expression in all of the treated groups. In contrast, SSAO expression was limited in control cells (reaching only 37 ± 10% of that seen in insulin-treated cells at day 8, according to the densitometric analysis of three blots) (Figure 6). GPDH activity, a well known marker of adipose conversion [11], was also increased in tyramine- and benzylamine-treated groups, reaching one-half of the insulin-promoted increase (Figure 7A). The expression of the insulin-regulatable glucose transporter GLUT4, another marker of adipogenic differentiation [8,9], was also enhanced in cells treated with tyramine or benzylamine [reaching 77 ± 14 and 58 ± 4%, respectively, of the maximal level found in insulin-treated cells (set at 100%), n = 3] (Figure 7B). Again, in tyramine- and benzylamine-treated cells, the expression of the glucose transporter GLUT1 attained intermediate levels between control and insulin-treated cells (Figure 7C). Combined treatment with insulin plus either tyramine or benzylamine failed to significantly improve the induction of GLUT4 expression compared with insulin alone (110 and 90% respectively; results not shown), whereas there was an almost additive effect between insulin and either tyramine or benzylamine on GLUT1 expression compared with insulin alone (165 and 145% respectively; results not shown).

Chronic treatment with amine oxidase substrates increased glucose uptake in 3T3-F442A cells

In accordance with the observed changes in GLUT4 glucose transporter expression, the maximal insulin-stimulated glucose transport of tyramine- or benzylamine-treated cells was similar to that of insulin-treated cells, whereas control cells were poorly responsive to insulin. Furthermore, the combined chronic treatment with insulin and amines further enhanced maximal insulin-dependent glucose transport (Figure 8). Analysis of the dose-response curves did not reveal any significant change in

the sensitivity to insulin: -log EC₅₀ values ranged between 8.1 and 8.5 for the different treatments. Basal glucose transport was significantly higher in all of the treated groups than in the control group, with the greatest increase found in insulin + amine-treated groups (Figure 8). These differences in basal glucose transport were in accordance with the above mentioned changes in GLUT1 protein levels.

DISCUSSION

The present findings demonstrate that tyramine and benzylamine induce adipogenesis in 3T3 F442A cells via their oxidation by amine oxidases, in particular SSAO. The amines are able to mimic several insulin actions: they acutely stimulate glucose uptake and they also induce long-term responses, such as increases in GPDH activity, protein synthesis and lipid accumulation, as well as enhanced expression of ALBP, GLUT1, GLUT4 and SSAO.

We have previously shown in isolated rat adipocytes that both tyramine and benzylamine, in combination with a concentration of vanadate ineffective by itself, are able to partially mimic the stimulatory effect of insulin on glucose transport, via their degradation by amine oxidases and the subsequent translocation of glucose transporters to the cell surface [1,2]. In the present work, we report that this acute effect of amines is also achieved in differentiated 3T3 F442A cells, but not in undifferentiated preadipocytes. Since undifferentiated 3T3 F442A cells lack SSAO and GLUT4 ([15] and present results), it may be that both of these proteins are key elements in the amine action on glucose transport. When tested at 0.1 mM, vanadate did not modify the acute effects of tyramine or benzylamine on glucose uptake into 3T3 F442A adipocytes, whereas it was necessary to potentiate the effect of amines on glucose transport into rat adipocytes [1] or 3T3 L1 cells [3]. However, it is important to note that, without vanadate, amines alone were also able, via their oxidation, to stimulate glucose transport in rat cardiomyocytes [23]. Therefore 3T3 F442A cells, which express both MAO and SSAO and represent a useful *in vitro* model to study adipocyte differentiation, were used to test whether amines reproduce long-term actions of insulin on adipogenesis in experiments conducted without added vanadate.

It is widely accepted that, during adipocyte differentiation of 3T3 F442A cells, the accumulation of cytoplasmic triacylglycerols is closely correlated with the co-ordinate expression of virtually every enzyme involved in *de novo* fatty acid and triacylglycerol biosynthesis [30]. In this regard, tyramine and benzylamine mimicked the effects of insulin on the appearance of intracellular lipid droplets, triacylglycerol accumulation, and induction of GPDH activity. In addition, the amines induced the expression of adipose differentiation markers, such as aP2 mRNA and GLUT4 protein, and allowed the cells to be responsive to the insulin stimulation of glucose transport. The inhibition by semicarbazide and hydralazine of tyramine- and benzylamine-induced accumulation of triacylglycerols, associated with the lack of effect of MAO inhibitors (tested at doses which totally block the enzyme in homogenates as well as in intact cells), suggested that the long-term effects of the tested amines are more dependent on SSAO activity than on MAO activity. This could explain why, although maximal tyramine oxidation was 3-fold higher than that of benzylamine, both amines induced approximately the same level of adipocyte differentiation. In fact, tyramine is oxidized mainly by MAO and to a lesser extent by SSAO, whereas benzylamine is a selective SSAO substrate. Thus it can be postulated that only the SSAO-dependent oxidation of tyramine accounted for its adipogenic activity. Due to its

subcellular location at the cell surface, SSAO may produce H_2O_2 that is able to react with lipids or proteins distinct from those surrounding the mitochondrial MAO. Although the primary cellular targets of the amine oxidation products are still unknown, it can be proposed that only the SSAO-mediated oxidation of substrates affects adipocyte differentiation.

Long-term effects of amines were additive to those of insulin when the following biological events were considered: protein anabolism, triacylglycerol accumulation, GLUT1 expression and glucose transport in the basal and insulin-stimulated states. This, together with the observation that only insulin intensifies the number of post-confluent mitoses, suggests that insulin and amines induce adipogenesis by somewhat independent mechanisms. Nonetheless, the involvement of a tyrosine kinase-mediated signalling pathway has been demonstrated in the differentiation process [30], and it can be postulated that this pathway is involved in the differentiation signal activated by amine oxidases. One common product generated during deaminative oxidation of the diverse substrates of amine oxidases is H_2O_2 [13]. This reactive oxygen species has been shown to increase the amount of membrane and intracellular tyrosine-phosphorylated proteins in many cell types [17], and to stimulate glucose transport in adipocytes [18]. H_2O_2 may therefore constitute a key element in the differentiating effects of amines. In keeping with this, *N*-acetylcysteine, a scavenger for reactive oxygen intermediates that is able to counteract diverse actions of H_2O_2 [29,31], hampered the adipose conversion of cells treated with benzylamine or tyramine. Whether SSAO activity is involved in the redox control of adipocyte differentiation already reported in 3T3 cells in response to other differentiating agents remains to be determined [31]. Similarly, the exact role of the increasing capacity of differentiating adipocytes to oxidize biogenic or exogenous amines deserves further investigation. The recent finding that SSAO is an adhesion protein in endothelial cells may be of valuable interest for determining the role(s) of this enzyme in adipose tissue physiology [32]. In this context, our observations indicate that 3T3 F442A is a potentially useful model to study the link between SSAO activity and adipocyte differentiation.

Various studies have documented the existence of MAO and SSAO activities in rodent fat cells [19,33,34]. We report for the first time that MAO activity is moderately regulated during the adipocyte differentiation process, while our observation that SSAO activity increases during adipogenesis has already been reported for rat preadipocytes in primary culture [35]. More recently, a dramatic increase in SSAO mRNA levels has been shown during adipose differentiation in the 3T3 F442A and 3T3 L1 adipocyte lineages [15]. The increase in SSAO mRNA, initially detected by a cDNA differential display technique, was correlated with a strong induction of amine oxidase activity [15]. The protein encoded by this cDNA is the murine homologue of the human placental amine oxidase [36], and of the partially sequenced plasma membrane amine oxidase present in rat adipocytes [16]. The present study provides the first visualization of the expression of the SSAO protein during adipocyte differentiation, by using a polyclonal antibody directed against SSAO purified from bovine lung [25]. The band recognized at approx. 100 kDa, the typical apparent molecular mass of SSAO under denaturing conditions [25], is practically absent at the pre-adipocyte stage, whereas it is abundant in differentiated 3T3 F442A cells. Our observations confirm that SSAO can be considered as a late marker of adipocyte differentiation, although its exact biological role is not completely elucidated; they also indicate that exposure to amine substrates prompts its appearance.

The insulin-sensitive glucose transporter GLUT4 is a well known adipocyte differentiation marker and a very important element in insulin-regulated glucose metabolism [8]. Expression of GLUT4 was increased in insulin-treated cells and also in benzylamine- or tyramine-treated cells. A higher amount of GLUT1 protein was detected in differentiated 3T3 F442A cells, and was probably involved in the increased basal glucose transport of the cells treated by amine oxidase substrates or by insulin. Studies aimed at analysing the effect of oxidative stress on cultured adipocytes have shown that the continuous presence of exogenous H_2O_2 provoked an enhanced expression of GLUT1 and a reduced expression of GLUT4, resulting in increased basal glucose uptake with no effect on the maximal capacity of the adipocyte to transport glucose. In fact, cells exposed to approx. $10 \mu M$ H_2O_2 for 18 h were less sensitive to insulin stimulation [37]. In the present study, treatment with benzylamine or tyramine increased GLUT1 expression, like exogenous H_2O_2 [37], but also increased GLUT4 expression and, most importantly, allowed the cells to be responsive to insulin (as demonstrated by the same $-\log EC_{50}$ values and the 4-fold increase over basal glucose transport in all the treated groups). In this regard, amine oxidation by amine oxidases, although generating H_2O_2 , cannot be considered as a mere source of deleterious oxygen species in adipocytes and is very different from experimental oxidative stress, which impairs insulin responsiveness [37]. It is worth noting that the cells treated with the combination of insulin and amines expressed the highest levels of glucose carriers and had the greatest glucose transport capacity. This observation can be considered for the development of new therapeutic approaches to treat obesity and/or hyperinsulinaemic/insulin-resistant diseases. For this purpose, it is important to identify the physiological substrates that are able to influence the expression and activity of adipose SSAO.

In conclusion, we have shown that amines induce *in vitro* adipogenesis, based on morphological changes, lipid and protein accumulation, and increases in adipose markers, such as aP2, GPDH, GLUT4 and SSAO. Amines can mimic or reinforce several of the insulin effects on adipocyte differentiation in 3T3 F442A cells mainly via their oxidation by SSAO. This implies a possible physiological function for SSAO in triggering adipogenesis of preadipocytes or in maintaining the differentiated state of adipocytes.

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