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Tyramine and benzylamine partially but selectively mimic insulin action on adipose differentiation in 3T3-L1 cells

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Biogenic amines like tyramine, methylamine and the non-naturally occuring amine, benzylamine, have been described to promote adipose conversion of murine 3T3 preadipocytes. To further investigate these novel effects of amines, we studied whether they selectively mimic the long-term adipogenic action of insulin. To this aim, we decided to use the 3T3-L1 cell line since this model needs a complex combination of inducers to trigger the differentiation programme: insulin, isobutylmethylxanthine (IBMX, an activator of cAMP-signal transduction pathway) and the synthetic glucocorticoid, dexamethasone. A cell culture protocol was designed, by which each component of the differentiation cocktail was replaced with either benzylamine or tyramine, in order to determine whether these amine oxidase substrates could substitute any of the differentiation inducers in 3T3-L1 cells. The incomplete lipid accumulation found in cells grown under IBMX- or dexamethasone-free conditions was not improved by the daily addition of amines to the culture medium. Insulin was the only component of adipose differentiation cocktail of 3T3-L1 that could be replaced, although partially, by tyramine or benzylamine. When used at 0.5 mM, these amines resulted in a significant increase of triacylglycerol accumulated eight days after confluence, when compared to cells kept without insulin. This partial insulin replacement was totally abolished by SSAO-inhibitors, while MAO-blockade did not reduce lipid accumulation. As previously reported for other insulin-sensitive processes, such as stimulation of glucose transport or lipolysis inhibition in mature adipocytes, the stimulation of adipogenesis by tyramine and benzylamine was an SSAO-dependent mechanism that apparently shared common signaling pathways with insulin.

Key words: Adipocyte differentiation, Preadipocytes, Triacylglycerol, Insulin, Semicarbazide-sensitive amine oxidase.

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Recent studies have evidenced that the function of amine oxidases present in adipose tissue is not limited to the degradation of circulating amines (5). In fact, the enzymes that oxidize biogenic or exogenous amines are known to generate hydrogen peroxide and were found to exhibit insulin-mimicking properties (1). The H_2O_2 produced during the oxidative deamination catalyzed by either monoamine oxidase (MAO) or semicarbazide-sensitive amine oxidase (SSAO) was demonstrated to substantially stimulate glucose transport in isolated rat cardiomyocytes (9), adipocytes (3, 5), and vascular smooth muscle cells (2). In fat cells, the stimulation of glucose transport by tyramine (a MAO and SSAO substrate) or benzylamine (a SSAO substrate) was the result of PI3-kinase activation and of GLUT4 translocation and was potentiated in the presence of vanadate (3). These short-term insulin-like effects of SSAO substrates were also extended to human adipocytes where benzylamine or methylamine not only stimulated glucose transport, but also inhibited lipolysis (8). In addition, different protocols of chronic treatment of preadipocyte 3T3 cell lineages with tyramine, benzylamine (4) or methylamine (6) resulted in a stimulation of the adipose conversion. In these studies, SSAO was found to increase considerably during adipogenesis, and was proposed as a novel marker of differentiation (4, 7).

However, differentiation of preadipose cells into adipocytes is a complex process that is, *in vivo*, not only regulated by insulin but also by many agents such as glucocorticoids, prostaglandins, cytokins, and even by factors produced by mature adipocytes themselves. To determine whether the effects of SSAO activity in adipose conversion were of an insulin-like nature, the present work aimed at studying *in vitro* the effect of amines substrates of MAO and SSAO on 3T3-L1 cells. Contrarily to preadipocytes of 3T3 F442A lineage, that only require insulin to achieve their adipogenic programme, full differentiation of 3T3-L1 cells is triggered in the presence of a cocktail consisting of insulin, isobutylmethylxanthine (IBMX, a cAMP phosphodiesterase inhibitor also able to block glucose transporters and adenosine receptors) and dexamethasone (a synthetic glucocorticoid agonist) (11). Our results show that insulin was the only component of the differentiation cocktail of 3T3 L1 that could be partially replaced by chronic treatment with tyramine or benzylamine. The adipogenic action of the amines was depending on SSAO activity, confirming that the activity of this ectoenzyme can positively modulate several steps of the insulin signaling pathways.

Materials and Methods

Cell culture.– 3T3-L1 cells were grown until confluence in Dulbecco's modified Eagle's medium (DMEM) in the presence of 5 % (v/v) calf serum, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin (Gibco BRL, Life Technologies Inc). Cells were kept at 37°C in sterile plasticware (Falcon, Becton Dickinson Labware, NJ.) under 7 % CO_2 atmosphere and medium was changed every two days. To trigger the differentiation programme of 3T3-L1 cells, confluent cultures were changed to DMEM + 10 % (v/v) fœtal calf serum (FCS) and treated during 48 h with a cocktail consisting of 175 nM insulin, 0.5 mM IBMX and 1 µM dexamethasone. After 48 h, the medium was replaced with fresh DMEM containing 10 % FCS and

175 nM insulin for an additional 48 h. After that, medium was removed and cells were kept in DMEM + 10 % FCS until 8 days after confluence.

Determination of triacyglycerol accumulation.– The content in intracellular triglycerides was used as an index of adipose differentiation and was measured by colorimetric reaction using Triglyceride Enzymatic Trinder kit (Biotrol Diagnostic, Chennevieres, France), which is based on the triacylglycerol breakdown by a lipase and the detection of glycerol by glycerol kinase and glycerol-3-phosphate oxidase, coupled with peroxidase. The final product of this series of reactions is a coloured compound, quinoneine, colorimetrically detected at 500 nm. Briefly, cells were washed twice with PBS, then scraped, resuspended in 50 mM Tris/HCl, 1 mM EDTA, pH 7.5 and homogenized. 20 µl aliquots were then mixed with 180 µl of the reaction medium and read after 10 min incubation at room temperature.

Measurement of other biochemical parameters.– Cell number and protein content and amine oxidase activities were determined as previously described (4). MAO activity was measured as pargyline-sensitive oxidation of 0.5 mM labeled tyramine, while SSAO activity was measured as semicarbazide-sensitive oxidation of 0.1 mM benzylamine according to the radiochemical method already detailed (4, 8).

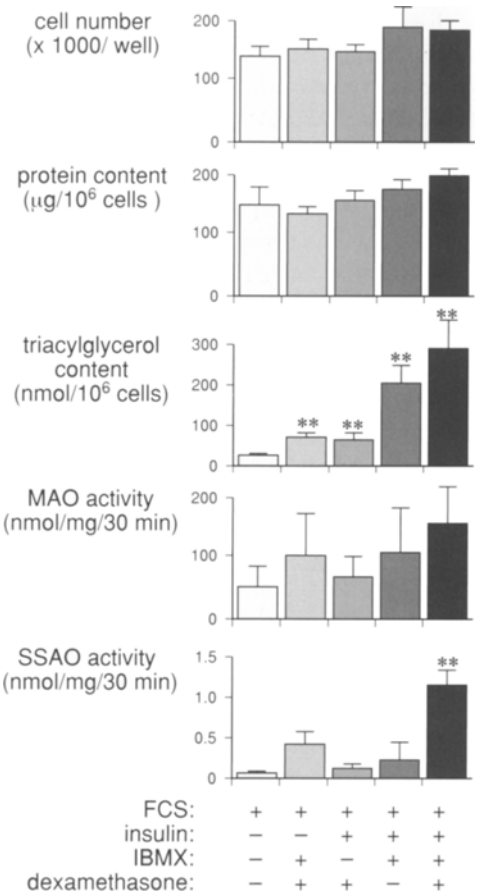

Fig. 1. *Influence of the individual deprivation of insulin, IBMX and dexamethasone on adipose differentiation in 3T3-L1 cells.*
Confluent cells were then kept during 8 days in 10 % fœtal calf serum (FCS) without addition of any differentiation inducer (white columns), in the presence of the full differentiation cocktail for 48 h (black columns), or omitting IBMX, dexamethasone or insulin as indicated. All measurements were done 8 days after confluence. Mean ± S.E.M. of 3 experiments. ** $p < 0.01$ *vs.* only FCS.

Results

3T3-L1 cells acquired typical adipocyte morphological characteristics within 8 days when grown for 48 h post-confluence in the presence of a differentiation cocktail which includes insulin, IBMX and dexamethasone, followed by an additional 48 h in the presence of insulin. Figure 1 shows the effects of removing one of the components of the differentiation cocktail on cell number, protein content, triglyceryde content, MAO and SSAO

activities. In the absence of the differentiation cocktail, 3T3-L1 cells were unable to increase their triacylglycerol content and their SSAO activity, while cell number, protein content and MAO activity were less affected. Likewise, only triacylglycerol content and SSAO activity were affected by the absence of one of the inducers of the cocktail. Removal of insulin or IBMX drastically reduced lipid accumulation, whereas dexamethasone seemed to be less necessary. SSAO activity only reached maximal levels in the presence of the complete differentiation cocktail. As previously reported for preadipocyte 3T3 F442A lineage (4), the MAO activity was present even in non-differentiated cells (Fig. 1).

Replacement by amines of the components of differentiation cocktail.– Although the presence of only two components of the differentiation cocktail was capable of inducing a limited degree of differentiation, maximal differentiation rate required the presence of all three inducers. This prompted us to test whether replacing each one of the components of the differentiation cocktail by amines could allow to further study the adipogenic action of amines. A cell culture protocol was then designed, by which each component of the 3T3-L1 differentiation cocktail was replaced with either benzylamine (0.5 mM) or tyramine (0.5 mM), in order to determine whether these substrates of amine oxidases could substitute any of the differentiation inducers in 3T3-L1 cells. The effect of replacing each one of the components of the differentiation cocktail by amines was evaluated in terms of triacylglycerol accumulation. Addition of amines to the full differentiation cocktail did not increase further the

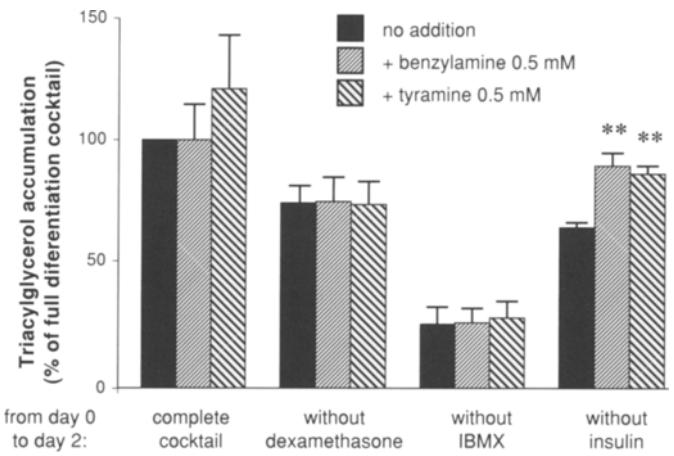

Fig. 2. *Replacement of 3T3-L1 cell differentiation inducers by amines.* Confluent cells were treated for 48 h with the complete cocktail for differentiation or with a mix that had dexamethasone, IBMX or insulin removed (no addition) or replaced by 0.5 mM tyramine or benzylamine. All groups were treated with 175 nM insulin for another additional 48 h after which all groups were kept in 10 % FCS until day 8 after confluence. Mean ± S.E.M. of 5-8 experiments. ** $p < 0.01$ *vs* corresponding control without amine.

triacylglycerol accumulation of the cells differentiated under optimal conditions (Fig. 2). Amines did not increase triglyceride content in the groups lacking IBMX or dexamethasone. Only the absence of insulin could be significantly, albeit partially, compensated by either tyramine or benzylamine. This effect of amines was dose-dependent, hardly detectable at 0.1 mM and maximal at 1 mM (not shown).

Insulin-free conditions.– Since insulin was not only present in the differentiation cocktail during the first post-confluent 48 h, but also alone during an additionnal period of 48 h, as detailed in Methods, the total replacement of the hormone by amines was tested in another set of experiments. When kept under insulin-free conditions, 3T3 L1 hardly accumulated lipid (Fig. 3). Under these conditions, both amines were able to somewhat mimic insulin influence and improved slightly but significantly the lipid storage in 3T3 L1. It was estimated that tyramine reached 25 ± 5 % and benzylamine 38 ± 14 % of the adipogenic efficiency of insulin.

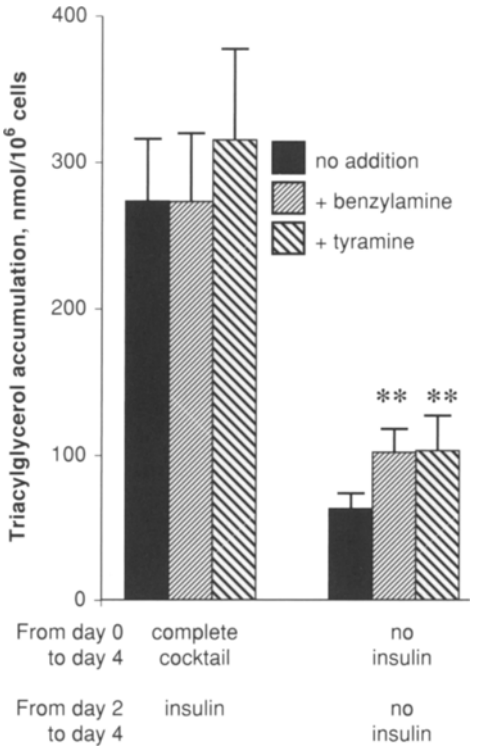

Fig. 3. *Replacement of insulin by 0.5 mM tyramine or benzylamine in differentiating 3T3-L1 cells.* Confluent 3T3-L1 cells were subjected to standard differentiation protocol or under insulin-free conditions. Tyramine or benzylamine was daily added when indicated. Mean ± S.E.M. of 8 experiments. ** $p < 0.01$ amine vs insulin-free.

Effect of amine oxidase inhibitors.– To determine whether the adipogenic effect of tyramine and benzylamine in 3T3-L1 cells was due to their oxidation by MAO and/or SSAO, we used specific inhibitors of each amine oxidase. Treatment with the SSAO inhibitors, semicarbazide (1 mM) and hydralazine (0.01 mM) abolished the triglyceride accumulation prompted by both tyramine and benzylamine in 3T3-L1 cells kept in total absence of insulin (Fig. 4). On the other hand, neither pargyline (0.1 mM) nor clorgyline (0.01 mM) altered the adipogenic effect of amines. In addition, the inhibitors tested did not modify lipid content in neither the cells grown in the absence of IBMX nor in dexamethasone-free conditions, excepted clorgyline which exhibited a tendency to improve lipid deposition (not shown).

Discussion

Taken together, the results on 3T3-L1 cells strengthen the recent proposition aiming at consider SSAO as a marker of adipocyte differentiation, as they confirm previous observations of the appearence

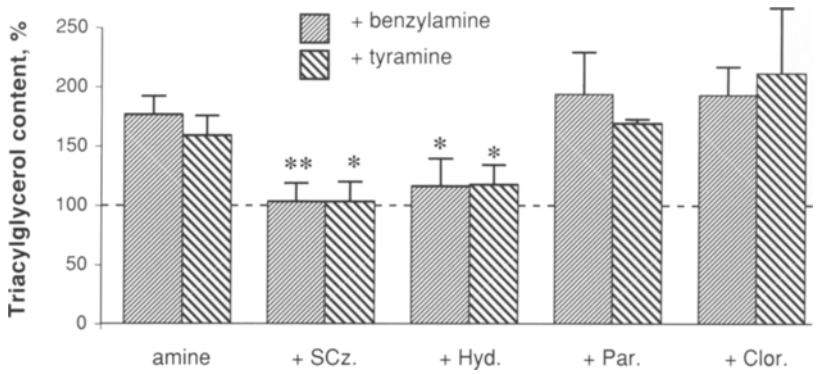

Fig. 4. *Inhibition of the adipogenic effect of amines by MAO and SSAO inhibitors.* 3T3-L1 cells, subjected to the different treatments specified in Fig. 3, were additionally treated with benzylamine or tyramine without (amine alone) or with 1 mM semicarbazide (Sc_Z.), 0.01 mM hydralazine (Hyd.), 0.1 mM pargyline (Par.) and 0.01 mM clorgyline (Clor.), from day 0 to day 8 post-confluence. Results are expressed as percentage of triacylglycerol accumulation relative to insulin-free condition. Mean ± S.E.M. of 3-8 experiments. * $p < 0.05$, **, $p < 0.02$ with inhibitor *vs* amine alone.

of SSAO during adipognenesis in 3T3 L1 (7) and 3T3-F442A (4) cell lineages, as well as in rat preadipocytes (10). In addition, the insulin-like nature of the effect of amines on adipocyte differentiation (4) is further demonstrated, since amines were capable of partially replacing insulin only. Noteworthy, amines were ineffective when IBMX or dexamethasone was lacking in the differentiation cocktail. This is in agreement with the fact that, in all, IBMX is a lipolytic agent while amines have been reported to be antilipolytic (8, 12). The results obtained with specific inhibitors seemed to indicate that, as it was also the case for 3T3-F442A cells (4), amine oxidation by SSAO activity was responsible for the adipogenic effect of amines in 3T3-L1 while MAO activity seemed less involved. This apparent divergence in the role of both amine oxidases expressed in adipocyte is still unclear and deserves further studies.

Although SSAO has been proposed as a late marker of adipogenesis (7), the administration of amines from the first days of post-confluent differentiation induced a significant increase of lipid storage into 3T3-L1 cells. This may indicate that the MAO already present at this early stage could be involved in the observed adipogenic effect. However this hypothesis appears unlikely regarding to the lack of blockade by MAO inhibitors. Before the completion of this work, the only described adipogenic effect of a SSAO substrate into 3T3-L1 was that of methylamine, which was administred at least 4 days after confluence, when SSAO is abundantly expressed, as well as other key elements of the differentiation programme (6). In this context, SSAO was suspected to only accelerate lipogenesis and to lead by an accelerated metabolism to lipid-laden cells. Accordingly, a net increase in the glycerol-3-phosphate dehydrogenase activity was found in response to amine treatment in both 3T3 F442A and 3T3-L1 lines (4, 6). However, these observations and the present results do not answer to the following question: are the adipogenic properties of amines resulting from a

stimulation of terminal adipocyte differentiation, by improving lipid storage, or from an activation of early events of the adipogenic programme, including changes in the expression of transcription factors? Since tyramine and benzylamine replaced partially insulin actions at all the steps of the routinely protocol used for optimal differentiation of 3T3 L1, it cannot be excluded that these amines could alter expression of key adipogenic transcription factors. Nevertheless, the major concern of these *in vitro* observations is to establish how they are predictive for physiological situations. In other words, further investigations on the adipocyte recruitment in animal models challenged with different amount of amines are required to determine the physiological implication of amine oxidases in the development of fat stores and/or in insulin sensitivity.

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C. SUBRA, E. FONTANA, V. VISENTIN, X. TESTAR y C. CARPÉNÉ. *Tiramina y benzilamina mimetizan la acción de la insulina sobre la diferenciación en adipocitos de las células 3T3-L1.* J. Physiol. Biochem., **59** (3), 209-216, 2003.

Se ha descrito que las aminas naturales, como tiramina o metilamina, y las aminas sintéticas, como benzilamina, estimulan la conversión en adipocitos de las células 3T3. En el presente trabajo, se estudia si esas aminas pueden mimetizar de forma selectiva la acción estimulante de la insulina sobre la adipogénesis. Se han utilizado células 3T3-L1, ya que, para la inducción del programa de diferenciación en adipocitos, requieren una mezcla compleja de insulina, isobutilmetilxantina (IBMX) y dexametasona. De acuerdo con el protocolo de cultivo celular escogido, cada componente de la mezcla de diferenciación se reemplazaba por benzilamina o por tiramina, a fin de determinar si esos sustratos de las amino-oxidasas pueden sustituir alguno de los factores de diferenciación necesarios para la transformación de las celulas 3T3-L1 en adipocitos. La acumulación incompleta de lípidos en células 3T3-L1 cultivadas en ausencia de IBMX o dexametasona no se corrigió por la administración diaria de aminas en el medio de cultivo. La insulina resultó ser el único componente del medio de diferenciación que se podía reemplazar, aunque de manera parcial, por tiramina o benzilamina. En comparación con las células incubadas en ausencia de insulina, dichas aminas, a concentración 0,5 mM, aumentaron significativamente la acumulación de triacilglicéridos 8 días después de alcanzada la confluencia. Este efecto de las aminas, reemplazando parcialmente el de la insulina, se bloqueó totalmente por la adición al medio de cultivo de inhibidores de la SSAO, mientras que la adición de inhibidores de la MAO no redujo el efecto de la acumulación de lípidos. Como ha sido previamente descrito para otras respuestas dependientes de la insulina, los efectos adipogénicos de la tiramina y benzilamina son dependientes de la actividad SSAO y parecen estar relacionados con las vías de señalización de la insulina.

Palabras clave: Adipogénesis, Preadipocitos, Triacilglicéridos, Insulina, Amino-oxidasas.

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