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ORIGINAL ARTICLE

Effects of the amino acid derivatives, β-hydroxy-β-methylbutyrate, taurine, and N-methyltyramine, on triacylglycerol breakdown in fat cells

Mélanie Leroux[1,2] · Tristan Lemery[1,2] · Nathalie Boulet[1,2] · Anaïs Briot[1,2] · Alexia Zakaroff[1,2] · Anne Bouloumié[1,2] · Fernando Andrade[3] · Patricia Pérez-Matute[4] · Jose M. Arbones-Mainar[5] · Christian Carpéné[1,2]

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Abstract

Various amino acid (AA) metabolites are used as supplements to facilitate metabolic control and enhance responsiveness of insulin-sensitive tissues. β-hydroxy-β-methylbutyrate (HMB) is a leucine metabolite proposed to prevent muscle wasting and to mitigate insulin resistance. Taurine, commonly added to energizing drinks, is a metabolite of methionine and cysteine present in bile juice, and proposed to be involved in lipid digestion and to be pro-lipolytic in adipocytes. N-methyltyramine (NMT) is a phenylalanine metabolite found in orange juices at 0.1–3 ppm while its effects on lipid mobilization remain controversial. Here, the putative lipolytic effects of these AA metabolites were studied and it was tested whether they could enhance insulin antilipolytic response in adipocytes. Release of glycerol and non-esterified fatty acids (NEFAs) was measured after a 2-h incubation of adipocytes obtained from control and diet-induced obese mice or from obese patients. In mouse, none of the tested AA derivatives was lipolytic from 1 μM to 1 mM. These compounds did not improve insulin antilipolytic effect or isoprenaline lipolytic action, except for 1 mM NMT that impaired triacylglycerol breakdown in obese mice. In human adipocytes, HMB and taurine were not lipolytic, while NMT weakly activated glycerol and NEFA release at 1 mM. However, 100 μM NMT impaired isoprenaline-stimulated lipolysis in a manner that was hardly added to insulin antilipolytic effect. Since none of these AA derivatives acutely helped or replaced insulin antilipolytic effect in adipocytes, the present in vitro observations do not support their proposed insulin-sensitizing properties. Moreover, NMT, HMB, and taurine were not notably lipolytic.

Keywords Adipose tissue · Human · Insulin resistance · Lipolysis · Branched-chain amino acids

Introduction

Various amino acids (AA) and their derivatives are used as ergogenic supplements by trained athletes, not only to enhance protein synthesis but also to favor metabolic control especially after periods of intense physical exercise, which are accompanied by glycogen utilization, muscular triglyceride lipolysis, increased blood glucose, and increased production of lactate and reactive oxygen species (ROS). In this regard, strenuous exercise is leading to an endocrinometabolic situation resembling to an insulin-resistant state. Since various AA derivatives are supposed to facilitate recovery of such imbalance, and are widely consumed by athletes [15] or frail elderly subjects [29] under more or less controlled conditions, the goal of this work was to assess whether such supplements could be of interest for the treatment of obesity-associated insulin resistance. A special attention was focused on testing several of these AA derivatives directly on native mature adipocytes. The antilipolytic

✉ Christian Carpéné
Christian.carpene@inserm.fr

1 Institute of Metabolic and Cardiovascular Diseases, INSERM, UMR1048, Team 1, I2MC, CHU Rangueil, BP84225, 1 avenue Jean Poulhès, 31432 Toulouse cedex 4, France

2 University of Toulouse, Paul Sabatier University, UMR1048, Toulouse, France

3 Metabolomics Platform, BioCruces Bizkaia Health Research Institute, linked clinical group of Rare Diseases CIBER (CIBERER), Barakaldo, Spain

4 Infectious Diseases Department, Center for Biomedical Research of La Rioja (CIBIR), Logroño, Spain

5 Adipocyte and Fat Biology Laboratory, Instituto Aragonés de Ciencias de la Salud (IACS), Instituto de Investigación Sanitaria (IIS) Aragón. Zaragoza, Spain. CIBER Fisiopatología Obesidad y Nutrición (CIBERObn), Instituto Salud Carlos III, Madrid, Spain

effect of insulin is one of the pleiotropic effects of the pancreatic hormone that is impaired in insulin resistance. Consequently, the antilipolytic response of fat cells was used as an index to evaluate the putative insulin-like or insulin-sensitizer actions of these micronutrients. Additionally, since several of the AA metabolites are structurally close to neurotransmitters or neuromodulators (e.g., biogenic monoamines) and are considered as stimulants, it was also of interest to test their lipolytic effect. Since the regulation of adipocyte lipolysis exhibits interspecies differences [4], a large part of the investigations performed in mice were translated to human adipocytes. In fact, a previous approach indicated that octopamine, one of the metabolites of phenylalanine and tyrosine, closely related to noradrenaline, stimulates lipolysis in rodent adipocytes while being almost inactive in human fat cells [5]. Such interspecies differences described for endogenous molecules that exert both stimulation and inhibition of lipolysis (e.g., epinephrine acting on β- and α_2-adrenoceptors) likely apply also for potential nutritional supplements. Thus, three molecules retained attention for such comparative studies: β-hydroxy-β-methylbutyrate (HMB, derived from leucine), taurine (derived from cysteine), and N-methyltyramine (NMT, derived from phenylalanine).

β-Hydroxy β-methylbutyrate ($C_5H_{10}O_3$, HMB) is a metabolite of the branched-chain amino acid (BCAA) leucine that is supposed to prevent skeletal muscle decline and to mitigate insulin resistance (as reviews, see [12] for the enhancement of growth, and [23] for impaired AA metabolism in obesity). BCAAs have been described to activate adipocyte lipolysis but to increase hepatic lipotoxicity [36], and to act differently from their metabolites, such as HMB [2]. However, the effects of HMB in obesity remain controversial [3, 29]. Lastly, since BCAA catabolism has been reported to play a crucial role in adipocyte differentiation [17], this prompted us to verify HMB influence on lipid handling by mature fat cells.

N-methyltyramine ($C_9H_{13}NO$, NMT) is a phenolic amine naturally occurring in foods. It was originally discovered in barley, malt, and derived products (e.g., beer, at approx. 5 mg/L), then found in various fruits (e.g., *Citrus*). It is a metabolite of phenylalanine, but it is present in the plasma in small amounts, belonging to the family of trace amines. However, its use as a supplement is currently banned by the World Anti-Doping Agency (WADA, https://www.wada-ama.org) since considered as one of the prohibited derivatives of phenethylamine. NMT is recognized to promote the release of noradrenaline as does tyramine. It is considered as a stimulant since it is believed to stimulate gastrin release and to help the organism to burn fat. Indeed, NMT is a partial agonist/antagonist at adrenergic receptors [32] but the descriptions of its actions on adipose tissue functions remain scarce.

Taurine ($C_2H_7NSO_3$) is one of the cysteine metabolites alongside H_2S and glutathione. It is present up to millimolar doses in tissues, and its plasma level oscillates around 50 μmol/L. The metabolism of cysteine appears to be altered with obesity [14] and adipocytes have been proposed as one of the multiple sources of taurine, owing to their capacity to metabolize cysteine [33]. When supplemented at 2% in a high-fat diet, taurine has been reported to reduce body weight gain and adiposity in diet-induced obese rodents, likely by limiting adipogenesis [20]. Long-term treatment with taurine is also able to act in vitro on differentiated preadipocytes by promoting autophagy [18] and in vivo on streptozotocin-induced diabetic rats by preventing the loss of insulin stimulation of glucose uptake in mature adipocytes [9]. Taurine has been proposed to improve insulin sensitivity in a rat model of type 2 diabetes [26] whereas it inhibited insulin stimulation of hydrogen production in rat adipocytes [27]. To decipher its effects on human adipocytes, it was decided to study its modulatory action in parallel with the two above-mentioned AA derivatives.

The following comparative data will show that both mouse and human adipocytes exhibit antilipolytic responses to high doses of NMT only, while the two other AA derivatives did not cause any noticeable alteration of lipolytic activity, at least in the range of treatment duration tested.

Materials and methods

Animals

Mice on C57BL/6 background (Charles River, L'Arbresle, France) were housed at up to four animals per cage with free access to water under a 12/12-h light/dark cycle with controlled temperature (22 ± 2 °C) and humidity (50–60%). All animal procedures complied with the principles established by the Institut National de la Santé et de la Recherche Médicale (INSERM, France) according to the Protocol Permission Number 12-1048-03-15 (on the 20/03/2012) and were approved by the local Ethics Committee of US006 CREFRE (Centre Régional d'Exploration Fonctionnelle et Ressources Expérimentales, Toulouse, France). Seven-week-old mice of both genders were fed with either a standard chow or a high-fat diet having 60% of calories as fat (Ssniff, Soest, Germany) for 16 weeks. At the end of treatment, mice were sacrificed by cervical dislocation after overnight fasting. Adipose tissue samples were removed from perigonadal and subcutaneous areas and pooled to obtain adipocyte preparations for immediate determination of lipolytic activities as described below.

Adipose tissue sampling from human subjects

For lipolysis studies, the human adipose tissue samples were obtained from eight obese patients (6 women, 2 men, age range 31–53 years) undergoing plastic surgery at the Rangueil hospital, Toulouse (France). Their mean body mass index (BMI) was 30.4 ± 0.7 kg/m^2. Supplemental studies on

NMT antilipolytic responses were performed on another group of eight overweight women (BMI 27.8 ± 0.4). All individuals gave their informed consent for their participation to the study as validated by the local ethic committee for the protection of individuals under the reference Comité de Protection des Personnes Sud Ouest et Outre Mer II, DC-2008-452. After surgical removal, pieces of subcutaneous abdominal human adipose depot were transported to the laboratory in less than half an hour. Then, adipose tissue (AT) was minced with scissors and digested by collagenase for adipocyte preparation (final concentration 250 IU/mL, from Sigma-Aldrich, Saint-Quentin-Fallavier, France).

Adipocyte preparation

The medium used for collagenase digestion (at 37 °C under agitation, as described in [16]) was Krebs-Ringer containing 15 mM sodium bicarbonate, 10 mM HEPES, 3.5% bovine serum albumin, and 5.5 mM glucose (pH 7.4). After digestion, the buoyant adipocytes were separated from the stromavascular fraction by filtration through a 250-μm nylon mesh-screen and two washes in the same buffer without collagenase. For both species, fat cell suspensions were prepared, diluted, and handled at 37 °C until the lipolysis assays within 3 h from AT removal.

Assays of adipocyte lipolysis

The end products of triacylglycerol breakdown—non-esterified fatty acids (NEFA) and glycerol—were determined after 2 h of incubation of isolated fat cells distributed in multiwell plates and incubated in a final volume of 200 μL/well with the tested molecules and/or with reference agents for lipolysis activation (isoprenaline) or inhibition (insulin). After 2 h under gentle agitation, incubation was stopped by placing the plates on ice. The glycerol released by adipocytes was assayed in the incubation medium, using 20 μL aliquotes and 200 μL of Free Glycerol Reagent (ref F6428, Sigma-Aldrich, St Louis, MO). Then, the determination of optical density at 540 nm of unknowns and glycerol standard was performed as recommended by the supplier. This already described simplified assay [16] based on glycerol kinase/glycerol phosphate oxidase/peroxidase gave the same results than the previous method using ATP, NAD, and glycerol kinase/glycerol phosphate dehydrogenase [7]. The free fatty acids released by the adipocytes during the same conditions were assessed with Wako NEFA-HR2 kit (Wako Chemicals GmbH, Neuss, D) by spectrophotometric evaluation at 540 nm with a multiplate reader (Labsystems-iEMS® Reader MF) as indicated by the manufacturer. Both indexes of lipolysis were normalized as μmoles released per 100 mg of cellular lipids, or expressed as percentage of maximal lipolytic stimulation in response to 10 μM isoprenaline as previously reported [8].

Cell viability assay

Cell viability was assayed with the Cell Counting Kit-8 (# 96992 from Sigma-Aldrich), based on the reduction of a highly water-soluble tetrazolium salt by cell dehydrogenases, and measuring at 450 nm the formazan generated in 96-well microplates after 2 h incubation at 37 °C with adipocytes under control conditions and in the presence of tested agents.

Chemicals

Isoprenaline (equivalent to isoproterenol), N-methyltyramine, HMB (also called β-hydroxyisovaleric acid), taurine, bovine insulin, and human insulin (I 9278), as well as most of the reagents, were from Sigma-Aldrich-Merck. BTT 2052 was a generous gift from M. Salmi and D. Smith (Turku, Finland). Bromoxidine and RX821002 were a gift from late Dr. H. Paris.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). One-way ANOVA followed by Tukey's post hoc tests and Student's *t* tests were performed to determine differences between treatments. All values in figures and tables are presented as mean ± SEM. Statistical significance was set at $p < 0.05$.

Results

Lipolytic test of the influence of AA metabolites

It was first tested whether HMB, taurine, and NMT exhibited dose-dependent effects on lipolysis in mouse adipocytes. In contrast to well-recognized lipolytic agents, isoprenaline and forskolin that were fully stimulatory, not one of the three metabolites of AA induced glycerol release (Fig. 1a) or NEFA release (Fig. 1b) at doses increasing from the micromolar to the millimolar range. These data support that the tested AA derivatives did not stimulate lipolytic activity on their own. However, in the same conditions, both end products of lipolysis were released either by low dose of adrenocorticotropin hormone (1 μM ACTH) or by high dose of a nonhydrolyzable cAMP analog (1 mM dibutyryl-cAMP), which exceeded isoprenaline-stimulated lipolysis, taken as reference (Fig. 1). All the positive controls of lipolytic stimulation were characterized by a NEFA release that was approximately threefold larger than the glycerol release (see legend of Fig. 1).

However, a lack of direct lipolytic effect of the AA derivatives is not sufficient to conclude that adipocytes are totally insensitive to these metabolites. Before making such assessment, it was necessary to test prestimulated states of lipolysis or to use other conditions to unmask

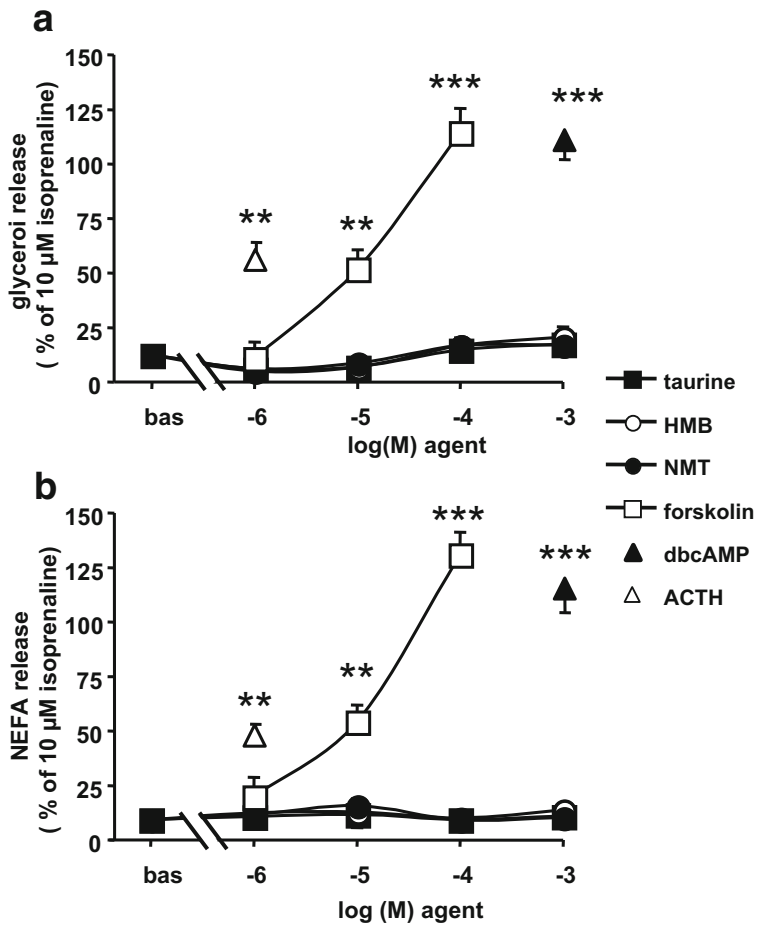

Fig. 1 Dose-dependent lipolytic effects of AA derivatives on mouse adipocyte with reference to forskolin, IBMX, ACTH, and dibutyryl-cAMP. Adipocyte suspensions (final suspension, 8.5 ± 0.7 mg cell lipids/200 µL) were incubated in the absence (basal, bas) and in the presence of the tested derivatives, taurine (closed squares), β-hydroxy-β-methylbutyrate (HMB, open circles) or N-methyltyramine (NMT, closed circles), or with lipolytic agents of reference: forskolin (open squares), dibutyryl-cAMP (dbcAMP, closed triangle), or ACTH (open triangle). Glycerol (**a**) and NEFA release (**b**) was measured in parallel after 2 h incubation and expressed as percentage of 10 µM isoprenaline-stimulated release that reached 1.06 ± 0.20 µmol of glycerol and 2.77 ± 0.57 µmol of NEFAs per 100 mg of cell lipids. Response to 10 µM isoprenaline was used as reference for maximal activity, but could be overpassed in several conditions, e.g., in response to forskolin or dbcAMP stimulation. Each point is the mean ± SEM of 14 cases. Different from baseline at $^{**}p < 0.01$, $^{***}p < 0.001$

modulatory capacities of the AA derivatives, or even to verify whether the putative adipocyte sensitivity to such agents was altered in pathological states.

Effects of AA metabolites on basal, stimulated, or repressed lipolysis in adipocytes from healthy or obese mice

HMB, taurine, and NMT were further tested under conditions of submaximal stimulation by isoprenaline—or inhibition by insulin—in fat cells from non-obese and from insulin-resistant obese mice. Adipocyte preparations obtained from diet-induced obese mice weakly responded to 100 nM isoprenaline while adipocytes from chow-fed non-obese mice strongly responded to the β-adrenergic agonist (Fig. 2). The effects of HMB, taurine, and NMT did not noticeably modify basal lipolysis in both models, at least when incubated for 2 h at 0.1 mM with fat cells.

In adipocytes from non-obese mice, the increase of lipolysis in response to 100 nM isoprenaline was near from maximal activity and was partially impaired by 100 nM insulin (glycerol release lowered from 78.4 ± 4.8 to 46.5 ± 5.3% of maximal lipolysis, $p < 0.001$), whereas it remained unaltered in the presence of AA derivatives (Fig. 2a). None of the AA derivatives tested at 0.1 mM enhanced the insulin-induced antilipolysis. In contrast, an impairment of the insulin antilipolytic effect was observed in the presence of 0.1 mM taurine (Fig. 2a).

Adipocytes from obese mice exhibited a reduced lipolytic response to 100 nM isoprenaline and signs of insulin resistance (Fig. 2b), since the pancreatic hormone was unable to inhibit isoprenaline-stimulated lipolysis (respective glycerol release were 26.6 ± 3.5 and 20.8 ± 2.5% of maximal lipolysis, NS).

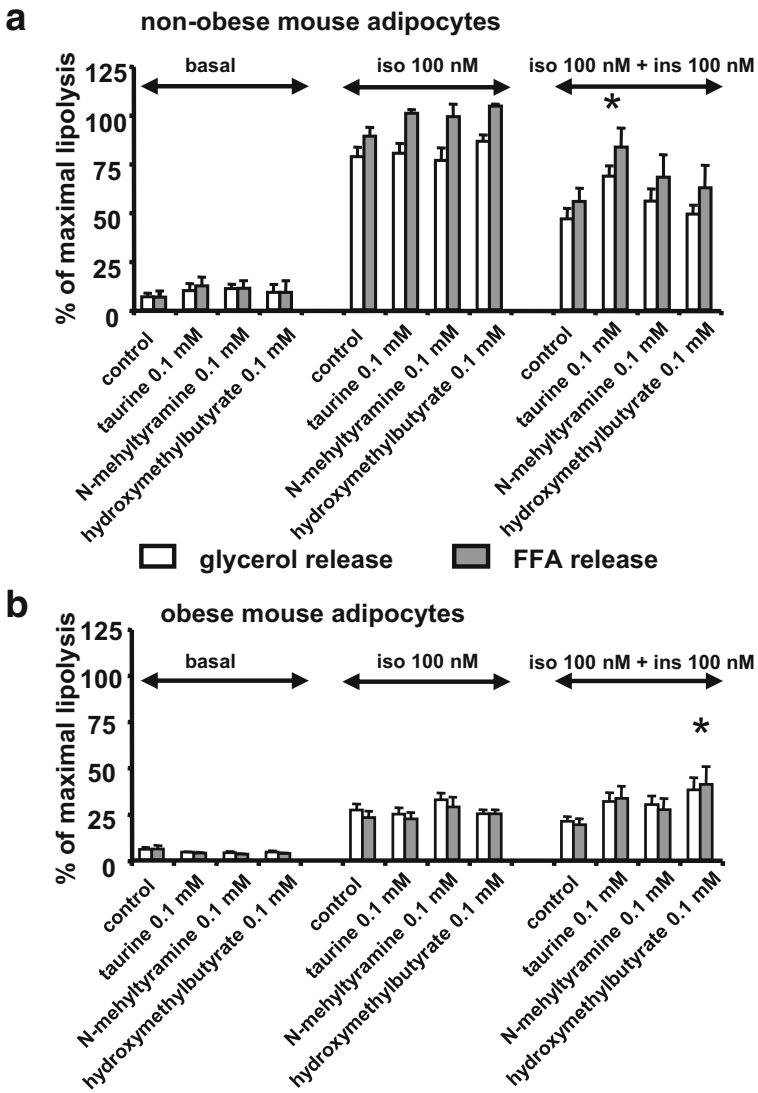

Fig. 2 Comparison of the antilipolytic effects of taurine, HMB, and NMT to that of insulin in adipocytes from **a** non-obese and **b** HFD-fed mice. Adipocytes were incubated 2 h with 100 μM of the AA derivatives alone (basal, left part of the graph), with 100 nM isoprenaline (iso, middle panel), or with isoprenaline plus insulin (iso + ins; right panel). Glycerol (open columns) or NEFA (shaded columns) release is expressed as percentage of 10 μM isoprenaline-stimulated release. Mean ± SEM of 10 (non-obese) to 16–22 (obese) adipocyte preparations. Difference for both glycerol and NEFA from respective control condition at $^{*}p < 0.05$

The presence of 0.1 mM taurine or NMT did not improve these blunted responses to isoprenaline. The highest lipolytic response was observed when isoprenaline, insulin, and HMB were combined. Therefore, short-term incubation of insulin-resistant adipocytes with 0.1 mM AA derivatives was not sufficient to restore insulin or β-adrenergic responsiveness. Similarly, when lipolysis was stimulated by 10 μM forskolin in adipocytes from obese mice, none of the AA derivatives exhibited acute pro-lipolytic or pro-antilipolytic activities (not shown).

Influence of AA derivatives on the isoprenaline dose-dependent stimulation of lipolysis

To further explore their putative regulatory effects on lipolysis, HMB, taurine, and NMT were tested at 1 μM or 1 mM in the presence of increasing doses of isoprenaline. Figure 3a shows that, in adipocytes from obese mice, the altered sensitivity to β-adrenergic activation of glycerol release—evidenced by a blunted response to 10^{-7} M isoprenaline—was not modified by micromolar dose of HMB, taurine, or NMT. When AA metabolites were tested at 1 mM, only NMT impaired the β-adrenergic stimulation of triacylglycerol breakdown (Fig. 3b).

This approach indicated that among the AA metabolites tested, only NMT exhibits a short-term regulatory effect in pathological adipocytes.

Test of the AA derivatives on human adipocytes

Because interspecies differences exist in the control of lipolysis, it was decided to study the acute influence of HMB, taurine, and NMT on human fat cells. Viability tests were performed on a subset of three preparations of human adipocytes. Even when present at 1 mM for 2 h with adipocytes, none of the AA metabolites was more cytotoxic than the vehicle used for their solubilization (DMSO at 4% *v*/*v* final, which left 84 ±

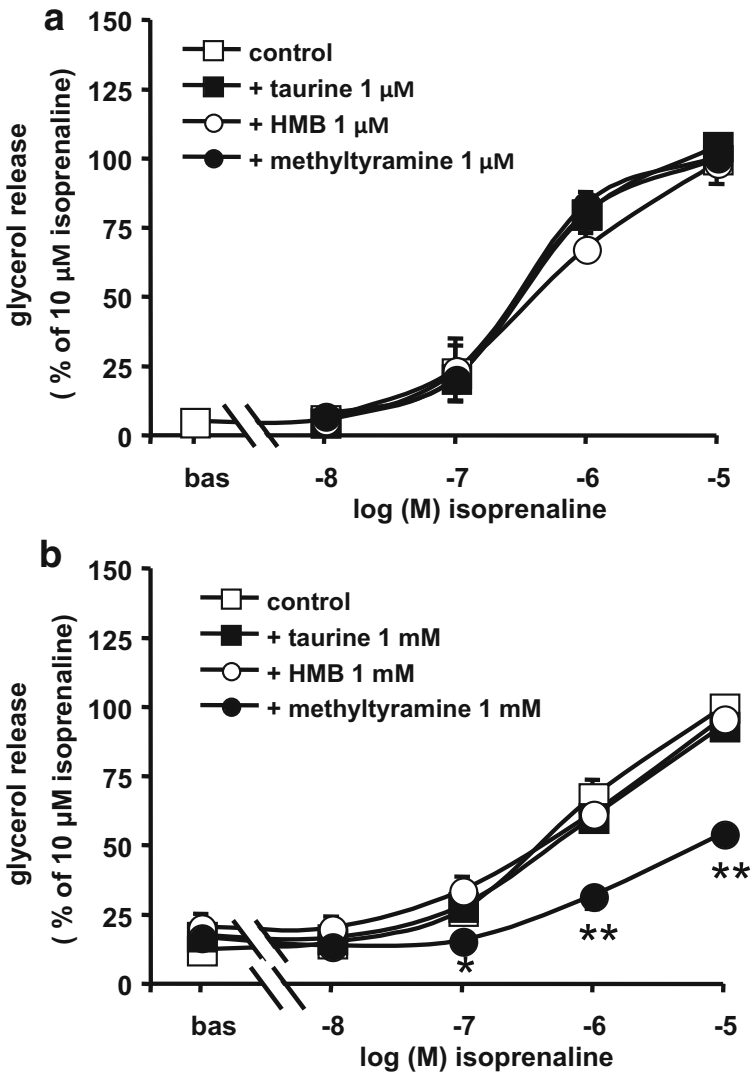

Fig. 3 Influence of **a** micromolar or **b** millimolar doses of AA derivatives on increasing lipolytic responses to isoprenaline. Adipocytes from obese mice were incubated 2 h without (bas) or with the indicated concentrations of isoprenaline alone (control, open squares) or together with taurine (closed squares), HMB (open circles), or NMT (closed circles) at 1 μM (upper panel) or 1 mM (lower panel). Mean ± SEM of 8 to 14 cases. Different from respective control at $^{*}p < 0.05$, $^{**}p < 0.01$

7% of cells unaffected, not shown). Similarly, the reference agents for lipolysis and antilipolysis did not alter cell viability: isoprenaline 100 nM and insulin 100 nM resulting in 107 and 108% of control, respectively. NMT and taurine were then tested at 100 μM and 1 mM in the presence of human adipocytes. A larger concentration range (10^{-8}–10^{-3} M) was used for HMB since it acts on other cellular models at doses as low as 6 μM [30] while its parent AA leucine is effective at millimolar doses [31].

The basal lipolytic activity of freshly isolated human adipocytes incubated without any added pharmacological agent was 0.10 ± 0.03 and 0.12 ± 0.04 μmol/100 mg lipids/2 h for glycerol and for NEFA release, respectively. Such spontaneous triacylglycerol breakdown was activated by a fivefold factor with 10 μM isoprenaline (see legend of Fig. 4). Among the AA derivatives tested, only 1 mM NMT increased basal lipolysis (Fig. 4). Although modest when compared to the isoprenaline lipolytic action, such increase was not due to the DMSO vehicle used to solubilize the derivatives.

Exploring pro- or antilipolytic short-term effects of AA derivatives in human adipocytes

The influence of the AA derivatives on glycerol release in the presence of isoprenaline alone is shown in Fig. 5a. Only NMT was limiting at 100 μM the isoprenaline stimulation of glycerol release. The AA derivatives were also tested in the presence of isoprenaline plus insulin. As expected, 100 nM human insulin shifted the β-adrenergic dose-response curve to the right (Fig. 5b). The glycerol release promoted by 0.1 μM isoprenaline was partially impaired by insulin. However, this insulin antilipolytic action was not clearly enhanced in the presence of NMT (Fig. 5b). The same pattern was observed for NEFA release (not shown). Thus, as observed in mouse adipocytes, no acute treatment with HMB, taurine, or NMT succeeded in enhancing the insulin antilipolytic responses of human adipocytes.

Lastly, the mechanisms underlying the NMT antilipolytic action were explored. Since it has been reported that tyramine

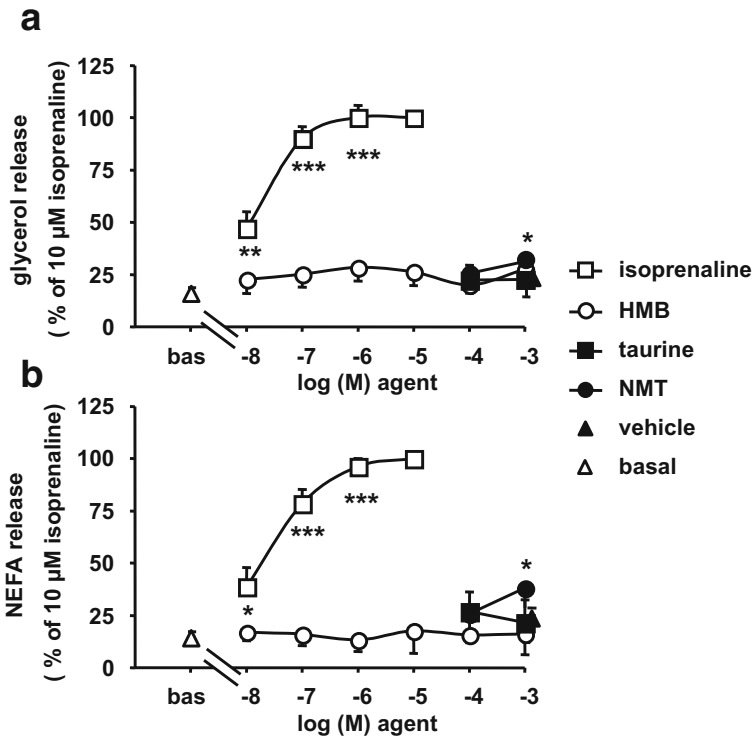

Fig. 4 Effects of increasing doses of AA derivatives on human adipocyte lipolysis with reference to isoprenaline. Human subcutaneous adipocytes were distributed at a density of 9.6 ± 1.2 mg cell lipids/200 µL and incubated 2 h at 37 °C without (bas, open triangle) and with the indicated concentrations of the tested derivatives, taurine (closed squares), HMB (open circles), NMT (closed circles), or with the vehicle used for dissolution of their millimolar dose (DMSO 4% *v*/*v* final, closed triangle). Glycerol (**a**) and NEFA release (**b**) is expressed as percentage of 10 µM isoprenaline-stimulated release, which reached 0.56 ± 0.15 and 0.97 + 0.30 µmol/100 mg lipids, respectively. Each point is the mean ± SEM of 8 individual cases. Different from basal release at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

antilipolytic effects are mediated in human fat cells by hydrogen peroxide released during amine oxidation rather than via Gi-coupled membrane receptors [8], and since the antilipolytic action of another amine, benzylamine, is dependent on its oxidation by amine oxidase activity [25], it was tested whether such mechanism also applies to NMT. In an additional set of adipocyte preparations, the monoamine oxidase (MAO) inhibitor pargyline, or the semicarbazide-sensitive amine oxidase (SSAO) inhibitors—semicarbazide and BTT 2052—was unable to prevent the NMT-induced antilipolysis (Table 1). In contrast, the two latter inhibitors blocked the benzylamine-induced antilipolysis, confirming its mediation by a SSAO-dependent mechanism in human fat cells [25]. All these data did not argue in favor for a clear

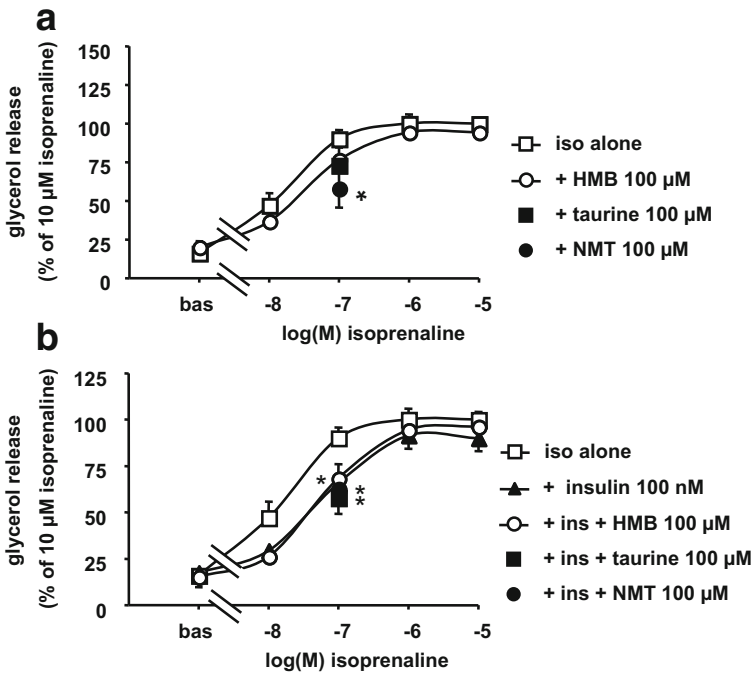

Fig. 5 Test of the antilipolytic properties of AA derivatives on their own or in combination with insulin on isoprenaline-stimulated lipolysis in human adipocytes. **a** Effect of taurine, HMB, and NMT (same symbols as in Fig. 4) on glycerol release stimulation by isoprenaline alone (open squares). **b** Effect on isoprenaline plus 100 nM insulin (closed triangles). Mean ± SEM of 8 human adipocyte preparations. Different from respective control (iso alone) at $*p < 0.05$

Table 1 Antilipolytic effects of NMT and benzylamine do not share the same sensitivity to amine oxidase inhibitors

	N-methyl-tyramine		benzylamine
% of stimulated lipolysis	0.1 mM	1 mM	0.1 mM
Iso 100 nM	65.1 ± 10.1	40.3 ± 4.8	64.0 ± 7.6
Iso + parg 0.1 mM	68.4 ± 11.3	49.2 ± 4.6	65.9 ± 13.3
Iso + semi 1 mM	69.7 ± 4.3	39.0 ± 8.2	85.9 ± 3.5*
Iso + BTT 10 μM	62.5 ± 6.5	40.4 ± 3.7	90.1 ± 4.0*

Glycerol release was measured after 2 h incubation and expressed as percentage of the lipolysis obtained in the presence of isoprenaline alone (Iso), which reached 0.97 ± 0.11 μmol of glycerol per 100 mg of cell lipids. Influence of the indicated dose of pargyline (parg), semicarbazide (semi) or BTT 2052 (BTT) on the NMT or benzylamine impairment of isoprenaline is measured via the percentages of glycerol release, set at 100% in response to 100 nM isoprenaline alone. Difference from the corresponding % of isoprenaline stimulation detected in the presence of N-methyltyramine or benzylamine (first line) at $*p < 0.05$

dependency of NMT-induced antilipolysis on amine oxidation.

It was finally verified how NMT effect could resemble to that of α_2-adrenergic agents. As expected, IBMX-induced lipolysis was inhibited by bromoxidine (agonist of antilipolytic α_2-ARs abundant in human fat cells) but not by the α_2-AR antagonist RX821002: 1 mM IBMX represented 87.9 ± 7.2% of maximal lipolysis ($n = 5$), while IBMX + 10 μM bromoxidine was lowered to 20.6 ± 15.0% ($p < 0.05$), and IBMX + 10 μM RX821002 remained at 87.2 ± 7.4%. NMT reproduced part of the antilipolytic effect of the bromoxidine (IBMX + 50 μM NMT, 58.1 ± 10.1% of maximal lipolysis; $n = 5$), suggesting that the methylated tyramine can interplay with α_2-ARs, as previously proposed in other cell models [6, 21].

Discussion

Taken together, the results indicate that HMB and taurine are not endowed with rapid insulin-like effects, and that they are unable to promptly sensitize fat cells to one of the regulatory actions of the pancreatic hormone—the inhibition of triacylglycerol breakdown. Only the methylated tyramine derived from phenylalanine can induce antilipolytic responses in human and murine fat cells, at least within 2-h in vitro conditions. However, for unclear reasons, the antilipolytic effect of NMT was not fully accumulative with the one of insulin. Before commenting how these results reconcile with the literature, it is important to mention that such observations do not exclude that longer exposure to AA metabolites might reveal insulin-sensitizer properties. Indeed, one of the limitations of the present approach is that only short-term exposure to the three natural AA derivatives was tested to investigate the influence on triacylglycerol breakdown in a relevant in vitro model. It would be interesting to study the influence of AA metabolites on adipose tissue physiology after more prolonged treatments in order to identify putative genomic effects modulating insulin signaling or lipid metabolism pathways. However, for such nutrigenomic approach, it would likely be more appropriate to perform long-term assays with adipose tissue explants [11] rather than with preparations of primary adipocytes exhibiting limited survival. Alternatively, studies on cultured preadipocytes could bring insights into the long-term modulatory actions of these metabolites (as it is the case for the sirtuin-dependent beneficial outcomes of HMB in 3T3L1 [3]), but could not explore in deep the acute lipolytic or antilipolytic actions analyzed here.

In the scarce previous reports about the effects of HMB, taurine, or NMT on adipocytes, none has described that these molecules were efficient on their own in activating triacylglycerol breakdown [1, 24, 27]. Noteworthy, the lack of lipolysis stimulation by NMT evidenced here in mouse adipocytes has been previously reported for rat fat cells [24]. However, such lack of effect in murine cells remains somewhat controversial with the faint lipolytic effect of NMT already reported in adipocytes from non-obese individuals [24] and confirmed here in obese subjects. Thus, the moderate lipolytic effect of NMT detected only at high concentrations in human fat cells clearly illustrates the interspecies variations that were supposed to occur in the present comparative approach. Nevertheless, the pattern of interspecies differences of NMT is opposite to that of octopamine, another biogenic amine found in *Citrus* fruit juices, which is much more lipolytic in rodent than in human adipocytes [6]. Indeed, octopamine is endowed with β_3-adrenergic agonist lipolytic properties (more efficient in rodents than in humans), while this is apparently not the case for NMT. Though probably related to members of the of Gs-coupled receptors family, the exact nature of the receptors activated by high concentration of NMT and leading to lipolysis stimulation in human adipocytes remains to be determined. Considering that NMT is a trace amine found in human plasma at levels lower than the micromolar range, it appeared more pertinent to unravel the mechanism of NMT antilipolytic action, which was detected at lower doses than the lipolytic component.

In spite of the α_2-AR antagonist properties attributed to NMT in other models [21], it is proposed here that NMT acts rather as a partial agonist than as a pure antagonist of human α_2-ARs in adipocytes. Since a partial agonist can enter in competition with a full agonist, our current interpretation is in complete agreement with previous observations showing that NMT was apparently competitive with pure α_2-AR agonists [24]. In a previous work, we showed that NMT did not enhance adrenaline-induced lipolysis, contrarily to the selective α_2-AR antagonist RX 821002, which blocked the inhibitory α_2-component and unmasked the lipolytic β-component of adrenaline in human adipocytes [6]. All these converging

observations led to propose an α_2-adrenergic-mediated antilipolytic action for NMT, which readily applies for human adipocytes, having a developed α_2-adrenergic receptivity [22]. However, this interpretation hardly explains how murine adipocytes, having lower α_2-AR equipment than human fat cells [28], also exhibit antilipolytic response to 1 mM NMT. Since the antilipolytic effect of tyramine in human adipocytes is hydrogen peroxide-dependent and mediated via oxidation by amine oxidases while not due to Gi-coupled receptor activation [8], it was hypothesized that a similar signaling occurs with NMT. This was tested but not validated in human fat cells, since NMT antilipolysis resisted to MAO and SSAO blockade, whereas it was confirmed that benzylamine, another dietary amine, was antilipolytic in a SSAO-dependent manner [25]. Unfortunately, such verifications were not performed in mouse adipocytes. Considering that interspecific differences exist in the substrate selectivity of amine oxidases as well as in the adipocyte adrenoreceptor equipment, the exact signaling involved in the antilipolytic effect of NMT in murine adipocytes remains to be determined. Providing further insights into this mechanism would be useful to explain why there was not a clear addition or even potentiation between the antilipolytic effects of NMT and insulin.

Irrespective of the mechanisms underlying NMT antilipolytic effect, another important issue is to determine whether this modulatory property is nutritionally relevant, upon ingestion of the AA derivative, especially when considering that NMT is present in widely consumed beverages, including beer and orange juices. Since NMT concentration is grossly in the micromolar range in orange juice, it can be barely envisaged that NMT accumulates up to millimolar levels in the adipose tissue environment after dietary intake. In fact, NMT is found in commercial orange juices at levels averaging 0.3 mg/kg, thereby less abundant than another protoalkaloid, synephrine, present at approximately 20 mg/kg [19]. Thus, when considering the stronger lipolytic effect of synephrine in human adipocytes [24] and its higher levels in fruits, the relative role of NMT appears minor in any attempt to modulate adipocyte lipolysis with *Citrus* extracts. Since these alkaloids are more concentrated in the peel than in the pulp of *Citrus* fruits (e.g., more than 1000 mg/kg of synephrine and 4 mg/kg of NMT in mandarin peel), it cannot be excluded that the sustained consumption of enriched supplements might lead to sufficiently elevated dose around the adipocytes. Since NMT is less abundant than other alkaloids in *Citrus* fruits and less lipolytic on adipocytes, our interpretation totally agrees with the conclusions published by Stohs and Hartman in a review of the pharmacology of NMT, from which we quote hereafter and approve the following sentences: "NMT is not an ingredient that should be used in dietary supplements designed to promote weight loss. It may result in an increase in perceived energy by promoting appetite and the digestion and absorption of nutrients while inhibiting the breakdown to fats to energy." [32].

In contrast to NMT, HMB did not influence lipolytic activity in the present tests. Of note, this metabolite of the BCAA leucine should not be confused with β-hydroxybutyrate, a short chain fatty acid that binds and activates the Gi-coupled receptor GPR109, thereby promoting antilipolysis in adipocytes [34]. Nevertheless, since BCAAs have been reported to reduce the effect of HFD on body weight gain, partly by increasing adipocyte lipolysis at the expense of liver injury [36], some influence of one of their metabolites, namely HMB, was expected to be detectable on fat cells. In fact, studies performed with HMB challenge on adipocytes are scarce, and among them, those from Bruckbauer and co-workers were indicative of only a moderate effect that needed to be potentiated with antidiabetic agents to enhance sirtuin-dependent beneficial outcomes [1, 3]. In the present study, HMB was devoid of immediate modulatory properties on lipolytic activity save when it enhanced the glycerol and NEFA release in response to a combination of isoprenaline and insulin in adipocytes from obese mice. Only this latter observation agrees with a lipid mobilizing action involved in the limitation of fattening observed with HMB when chronically administered in pigs [13]. Otherwise, the present observations remain in line with previous reports indicating that HMB treatment in rodents does not support any rapid insulin-like effect on carbohydrate handling [35]. HMB is probably an insulin-sensitizer when considering protein anabolism, especially in muscle [30], but this metabolite does not seem to mitigate insulin resistance in adipocytes, even from obese mice.

The lack of acute effect of taurine on lipolysis was also a little disappointing. Taurine is a metabolite of sulfur AA methionine and cysteine naturally abundant in tissues, which has been reported to exhibit beneficial effects in obese and diabetic animal models [20, 26], and to increase blood glycerol levels in humans after physical exercise [10]. However, the fact that taurine did not acutely modify the baseline lipolytic activity of mouse or human adipocytes is in agreement with data reported in rats [27]. Nonetheless, and in contradiction with the same previous report [27], taurine did not improve the isoprenaline lipolytic effect in primary adipocytes. As for NMT, the discrepancies between the present study and previous observations might be explained by differences in the duration of the exposure rather than by the concentrations at which the metabolites were added to the adipocyte preparations. Save for the conditions used by Pina-Zentella et al. (30 min) [27], the 2-h treatment of fat cells used here was shorter than in other recent approaches [18, 20]. Thereby, as it appears that taurine is active only after a long lag upon its addition, it deserves the term "pro-lipolytic agent" rather than "lipolytic agent," since the latter qualify factors capable of increasing triacylglycerol breakdown, immediately and on

their own, by modulating the activity of the enzymes belonging to the lipolysis pathway.

In summary, this study demonstrates that in human adipocytes, none of the AA derivatives tested was able to acutely enhance the antilipolytic responses to insulin. This does not exclude that such metabolites known to readily cross the intestinal barrier can facilitate other anabolic actions of insulin on other target cells, as it has been proposed for HMB in muscles. The purported capacity of these AA derivatives to ameliorate diabetic complications therefore does not seem to require a rapid and substantial modulation of adipocyte lipolytic activity.

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Compliance with ethical standards

All animal procedures complied with the principles established by the Institut National de la Santé et de la Recherche Médicale (INSERM, France) according to the Protocol Permission Number 12-1048-03-15 (on the 20/03/2012) and were approved by the local Ethics Committee of US006 CREFRE (Centre Régional d'Exploration Fonctionnelle et Ressources Expérimentales, Toulouse, France). All individuals gave their informed consent for their participation to the study as validated by the local ethic committee for the protection of individuals under the reference Comité de Protection des Personnes Sud Ouest et Outre Mer II, DC-2008-452.

Conflict of interest The authors declare that they have no conflict of interest.

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