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Potential involvement of adipocyte insulin resistance in obesity-associated up-regulation of adipocyte lysophospholipase D/autotaxin expression

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Abstract *Aims/hypothesis:* Autotaxin is a lysophospholipase D that is secreted by adipocytes and whose expression is substantially up-regulated in obese, diabetic *db/db* mice. The aim of the present study was to depict the physiological and cellular mechanisms involved in regulation of adipocyte autotaxin expression. *Methods:* Autotaxin mRNAs were quantified in adipose tissue from *db/db* mice (obese and highly diabetic type 2), gold-thioglucose-treated (GTG) mice (highly obese and moderately diabetic type 2), high-fat diet-fed (HFD) mice (obese and moderately diabetic type 2), streptozotocin-treated mice (thin and diabetic type 1), and massively obese humans with glucose intolerance. *Results:* When compared to non-obese controls, autotaxin expression in *db/db* mice was significantly increased, but not in GTG, HFD, or streptozotocin-treated mice. During *db/db* mice development, up-regulation of autotaxin occurred only 3 weeks after the emergence of hyperinsulinaemia, and simultaneously with the emergence of hyperglycaemia. Adipocytes from *db/db* mice exhibited a stronger impairment of insulin-stimulated glucose uptake than non-obese and HFD-induced obese mice. Autotaxin expression was up-regulated by treatment with TNF α (insulin resistance-promoting cytokine), and down-regulated by rosiglitazone treatment (insulin-sensitising compound) in 3T3F442A adipocytes. Finally, adipose tissue

autotaxin expression was significantly up-regulated in patients exhibiting both insulin resistance and impaired glucose tolerance. *Conclusions/interpretation:* The present work demonstrates the existence of a *db/db*-specific up-regulation of adipocyte autotaxin expression, which could be related to the severe type 2 diabetes phenotype and adipocyte insulin resistance, rather than excess adiposity in itself. It also showed that type 2 diabetes in humans is also associated with up-regulation of adipocyte autotaxin expression.

Keywords Adipocyte · Adipose tissue · Autotaxin · *db/db* · Diabetes · High-fat diet · Humans · Mouse · Obesity

Abbreviations COS-7: African green monkey · 2-DG: 2-deoxy-glucose · ENPP: ectonucleotide-pyrophosphate pyrophosphatase phosphodiesterase · GTG: gold thioglucose · Hb: haemoglobin · HFD: high-fat diet · HOMA: Homeostasis model assessment · IGT-D: impaired glucose tolerance, diabetic · LPA: lysophosphatidic acid · LPC: lysophosphatidylcholine · NGT: normal glucose tolerance · PPAR: peroxisome proliferator-activated receptor

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Introduction

In parallel to its implication in regulating energy balance via its capacity to store excess lipid energy, adipose tissue is known to possess an important secretory function. It secretes several factors (leptin, adiponectin, TNF α , angiotensinogen, eicosanoids, lysophosphatidic acid, etc.), also called adipokines, that are involved in the development of morbid complications of obesity such as cardiovascular diseases, hypertension, diabetes and cancer [1].

Our group has described the existence of a new adipokine: autotaxin [2], which is a soluble protein of 120 M_r belonging to the ectonucleotide-pyrophosphate pyrophosphatase phosphodiesterase (ENPP) family [3]. Autotaxin catalyses a lysophospholipase D-activity [2, 4, 5] leading to

the hydrolysis of lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA), a bioactive phospholipid known to control several cellular events via activation of specific G-protein-coupled receptors [6].

We demonstrated that the expression of autotaxin was substantially up-regulated during adipocyte differentiation in culture, as well as in adipocytes from obese, diabetic *db/db* mice when compared to non-obese mice [2]. These observations suggested a possible involvement of autotaxin in the normal or pathological development of adipose tissue and/or pathologies associated with obesity.

In the present work, we analysed autotaxin expression in adipose tissue from different mouse models exhibiting various degrees of obesity and/or diabetes, as well as from massively obese human subjects. We concluded that adipocyte autotaxin expression is not directly influenced by excess adiposity, but is rather linked to impaired glucose homeostasis and insulin resistance in adipocytes.

Subjects, materials and methods

Animals

Mice were obtained from Charles River Laboratories (l'Arbresle, France) and were handled in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM). They were housed conventionally in an animal room with constant temperature (20–22°C), humidity (50–60%) and with a 12-h light/dark cycle (lights on at 8.00 h). All mice had free access to food and water throughout the experiments. Obese *db/db* mice were 5- to 12-week-old C57BlKS/J *db/db* females fed daily with standard rodent chow. Non-obese controls of *db/db* mice were C57BlKS/J *+/+* mice of the same age and were fed with the same diet. Obese gold thioglucose (GTG) mice were 13-week-old FVB/n females which had received a single intraperitoneal injection (0.5 g/kg) of gold thioglucose (GTG, Sigma, St. Louis, MO, USA) at 8 weeks of age and been fed with a regular diet. Non-obese controls of GTG mice were 13-week-old FVB/n female mice fed with the same diet. Obese high-fat diet (HFD) mice were 13-week-old C57Bl6/J females fed daily with a high-fat diet (energy content distribution in %: 20% protein, 35% carbohydrate and 45% fat) from 5 weeks of age. Non-obese controls of HFD mice were 13-week-old C57Bl6/J mice fed daily with a low-fat diet (energy content distribution in %: 20% protein, 70% carbohydrate and 15% fat). Standardised diets were from UAR, France.

For streptozotocin treatment, 8-week-old C57Bl6/J mice received four daily intra-peritoneal injections of 70 mg/kg streptozotocin and were killed 4 weeks later.

Animals were killed in the non-fasting state. The blood was collected on heparin and glucose was immediately measured with a glucose meter. Plasma concentrations of insulin were determined with an RIA kit (Diagnostics Pasteur, Paris, France). Adipose tissue was dissected out and weighed before separating the adipocytes from the stroma-vascular fraction (see below).

Isolation of adipocytes and stroma-vascular cells of adipose tissue

Adipose tissue was minced and incubated while shaking in 5 ml of DMEM (Gibco, Invitrogen, Paisley, UK) supplemented with 1 mg/ml collagenase and 1% BSA. This was done for 30 min at 37°C. Digested tissue was filtered through a 150-µm screen and centrifuged for 5 min at 900×g in order to get adipocytes in the floating fraction, and stroma-vascular cells (preadipocytes, endothelial cells and macrophages) in the pellet.

Deoxyglucose transport in isolated adipocytes

Deoxyglucose transport was measured as previously described [7]. Isolated adipocytes were incubated in the presence or not of 100 nmol/l insulin for 45 min at 37°C in a final volume of 400 µl. Then, 2-deoxy-D-[³H]-glucose (2-DG, 14.8 kBq) was added at a final concentration of 0.1 mmol/l for 10 min. Assays were stopped with 100 µl of 100 µmol/l cytochalasin B and aliquots of the cell suspension were centrifuged in microtubes containing di-isononyl phthalate (density 0.974 g/ml), to separate adipocytes from the buffer and count the intracellular [³H]2-DG.

Human samples

Subcutaneous adipose tissue Human subcutaneous adipose tissue samples were obtained from patients undergoing abdominal dermolipectomy for plastic surgery. No clinical data from these patients were available.

Intra-abdominal adipose tissue We investigated 23 patients before bariatric surgery. In order to limit any confounding effect, we excluded patients with evidence of chronic or inflammatory diseases. We obtained 20 cm³ of intra-abdominal adipose tissue perioperatively at the level of the omentum. Tissue samples were immediately frozen in liquid nitrogen.

Blood glucose was measured by the glucose oxidase method (Beckman Instruments, Fullerton, CA, USA). Plasma insulin was determined by immunoassay (Insulin IMX, Abbott Laboratories, Tokyo, Japan). The intra-assay coefficient of variation was 4% and the inter-assay coefficient was 6%. Cross reactivity with proinsulin was 0.05%. HDL-cholesterol was measured after precipitation of apo B-containing lipoproteins using sodium phosphotungstate/magnesium chloride (Boehringer Mannheim, Mannheim, Germany). Total cholesterol and triglycerides were measured with commercial kits (Boehringer) adapted to a Hitachi 911 analyser (Japan). LDL-cholesterol was calculated according to Friedewald et al. [8]. Homeostasis model assessment (HOMA) was used to evaluate insulin sensitivity, according to the following formula: $\text{HOMA} = \text{fasting insulin (mU/ml)} \times \text{fasting glucose (mmol/l)} / 22.5$.

HbA_{1c}, determined in patients with known diabetes, was measured as a percentage of the total haemoglobin following high-performance liquid chromatography separation (Diamat, BioRad Laboratories, Hercules, CA, USA) (normal range 4.5–6%).

Some patients were given an OGTT. Subjects, having fasted overnight, received 75 g glucose in water solution, and plasma was sampled before intake and 30, 60, 90, and 120 min afterwards, to determine glucose and insulin.

The subjects of our study gave informed consent and investigations were carried out in accordance with the Declaration of Helsinki as revised in 2000 (<http://www.wma.net/e/policy/b3.htm>).

Cell lines

We obtained 3T3F442A adipocytes as previously described [2]. Briefly, confluent 3T3F442A preadipocytes were cultured in a differentiating medium consisting of DMEM supplemented with 10% fetal calf serum plus 50 nmol/l insulin. 3T3F442A adipocytes were used after 7 days of culture. African green monkey (COS-7) cells were grown in DMEM supplemented with 10% FCS and transfected with pcDNA3 (Invitrogen) or with pcDNA-m autotaxin-FLAG [2], using DEAE dextran as previously reported [2].

RNA extraction and real-time RT-PCR analysis

Total RNAs were extracted from adipose tissue, adipocytes or stroma-vascular cells using the RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA (1 µg) was reverse-transcribed for 60 min at 37°C using Superscript II reverse transcriptase (Life Technology, Invitrogen SARL, Cergy Pontoise, France) in the presence of random hexamers. A minus RT reaction was performed in parallel to ensure the absence of genomic DNA contamination. Real-time PCR was performed starting with 25 ng cDNA and a 900-nmol/l concentration of both sense and antisense oligonucleotides (mouse autotaxin: sense 5'-GACCCTAAAGCCATTATTGCTAA-3', antisense 5'-GGGAAGGTGCTGTTTCATGT-3'; human autotaxin: sense 5'-GGACCAACATCTCCGGATCTT-3', antisense 5'-GGAGGTCCAGCCTCTTGAAG-3') in a final volume of 25 µl using the SYBR green TaqMan Universal PCR Master Mix (Applied Biosystems, Applera, Courtaboeuf, France). Fluorescence was monitored and analysed in a GeneAmp 7000 detection system instrument (Applied Biosystems). Analysis of 18S ribosomal RNA was performed in parallel using the Ribosomal RNA control Taqman Assay Kit (Applied Biosystem) in order to normalise gene expression. Results are expressed as follows: $2^{-(Ct_{18S} - Ct_{gene})}$ where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold. Oligonucleotide primers were designed using Primer Express software (Perkin-Elmer Life Sciences, Courtaboeuf, France).

Preparation of conditioned media

Adipose tissue explants were prepared from subcutaneous adipose tissue as previously described [9]. Conditioned media were prepared by incubating 7 g of explants in 10 ml of serum-free DMEM for 7 h at 37°C in a humidified atmosphere containing 7% CO₂. Conditioned media from 3T3F442A adipocytes and from COS-7-cells were obtained by incubating the cells in 5 ml (for a 10-cm-diameter plate) in serum-free DMEM for 7 h at 37°C in a humidified atmosphere containing 7% CO₂. Conditioned media were concentrated (about 50-fold) using an Amicon Ultra 10,000 (Millipore, SAS, St-Quentin en Yvelines, France) and the presence of autotaxin protein was studied by Western blot analysis as well as by measuring its lysophospholipase D activity.

Measurement of lysophospholipase D activity

Lysophospholipase D activity of autotaxin was measured by converting radiolabelled LPC into radiolabelled LPA as previously described [2]. First, a solution of [¹⁴C]palmitoyl-lysophosphatidylcholine (NEN 2035 MBq/mmol) at 0.092 kBq/µl in DMEM supplemented with 1% non-esterified fatty acid BSA was prepared, and 20 µl of this solution was incubated with 500 µl thawed conditioned medium plus 1 µl of sodium orthovanadate 0.5 mmol/l for 90 min at 37°C. At the end of the incubation period, phospholipids were extracted with 500 µl of 1-butanol, evaporated, spotted on a silica gel 60 TLC glass plate (Merck International SAS, Strasbourg, France), and separated using CHCl₃/MeOH/NH₄OH (60:35:8) as a migration solvent. The thin layers were autoradiographed overnight at -80°C using a Biomax-MS film (Kodak) in order to localise radiolabelled LPA spots, which were scraped and counted with 3 ml of scintillation cocktail.

Western blot analysis

We separated 30 µg of protein from concentrated conditioned medium on a Gel Nu-PAGE 4–12% (Invitrogen) and transferred it on to nitrocellulose membrane. The blot was pre-incubated for 1 h at room temperature in TBS/Tween 0.05% containing 3% dried milk, and incubated overnight at 4°C in the same solution supplemented with 0.7 µg/ml autotaxin-antibody. After washing in PBS/Tween 0.05%, autotaxin was visualised by enhanced chemiluminescence detection (ECL, Amersham Biosciences) using an anti rabbit-HRP antibody (Sigma). The autotaxin-antibody (AGRO-BIO, La Ferté Saint Aubin, France) was a rabbit polyclonal antibody directed against the peptide ESCNS-SEDESKWVE present in both human (position 881–894) and mouse (position 803–816) autotaxin.

Fig. 1 Expression of adipocyte autotaxin in different mouse models of obesity. Perigonadic fat pad weight (a), adipocyte autotaxin mRNA levels (b) plasma concentrations of glucose (c) and plasma concentrations of insulin (d) were measured in 12-week-old *db/db*- ($n=4$), 13-week-old high-fat diet (HFD)-fed mice ($n=3$), and 13-week-old gold thioglucose (GTG)-treated mice ($n=4$). Values obtained with these obese mice (black bars) were compared with those obtained from their non-obese counterparts (white bars) respectively: +/+ mice ($n=4$), low-fat diet (LFD)-fed mice ($n=3$) and untreated mice ($n=3$). Values are means $\pm$ SE. Comparisons between obese and non-obese mice were performed using a Student's *t*-test. * $p<0.05$; ** $p<0.01$

Statistical analysis

Results are means $\pm$ SE. Student's *t*-test was used to compare two groups of data. Correlations were analysed using the non-parametric Spearman rank test. A *p* value of less than 0.05 was considered significant.

Results

Adipocyte autotaxin expression is not up-regulated in mouse models of obesity other than db/db mice In order to determine whether up-regulation of adipocyte autotaxin expression in *db/db* mice could also occur in other models of obesity, autotaxin mRNA levels were measured in adipocytes from GTG and HFD mice (see “Subjects, materials and methods”). The obesity of these mice was attested by a significantly higher fat pad weight than that in lean counterparts (Fig. 1a). Mean body weights were 40, 39 and 29 g for *db/db*, GTG, and HFD mice, respectively. The highest fat pad weight values were found in GTG mice (Fig. 1a) ($p<0.05$ when compared with *db/db* mice; $P<0.05$ when compared with HFD mice). The fat pad weight from *db/db* mice was not statistically different from that of HFD mice (Fig. 1a). As previously described [2], adipocyte autotaxin mRNA levels were increased by about fourfold in *db/db* mice when compared to lean counterparts (Fig. 1b). In contrast, no change in adipocyte autotaxin mRNA levels was observed in GTG and HFD mice when compared to their respective non-obese controls (Fig. 1b). A similar absence of autotaxin up-regulation was observed in adipose tissue from *ob/ob* mice (data not shown). In the three mouse models, obesity was associated with a significant

Fig. 2 Influence of streptozotocin treatment on regulation of adipose tissue autotaxin expression. Plasma concentrations of glucose (a), perigonadic fat pad weight (b), and adipose tissue autotaxin mRNA levels (c) were measured in 12-week-old C57Bl6J mice treated (Strepto) ($n=4$) or not (Cont) ($n=4$) with streptozotocin as described above. Values are means $\pm$ SE. Comparisons between control and streptozotocin-treated mice were performed using a Student's *t*-test. ** $p<0.01$

increase in plasma concentrations of glucose (Fig. 1c) and insulin (Fig. 1d). Hyperglycaemia was significantly higher in *db/db* mice than in GTG mice ($p<0.05$) and HFD mice ($p<0.05$). Hyperinsulinaemia was significantly higher in *db/db* mice than in GTG mice ($p<0.01$) and HFD mice ($p<0.01$). These data show that: (1) despite an equivalent adiposity to *db/db* mice, GTG and HFD mice did not exhibit up-regulation of adipocyte autotaxin expression; (2) *db/db* mice exhibited more pronounced diabetic symptoms (hyperglycaemia and hyperinsulinaemia) than GTG and HFD mice. We concluded that the adiposity of *db/db* mice was not sufficient to regulate adipocyte autotaxin. Consequently, up-regulation of adipocyte autotaxin expression is more likely to be linked to the diabetic status of *db/db* mice.

Autotaxin is not up-regulated in streptozotocin-induced hyperglycaemic mice In order to test whether hyperglycaemia in itself could influence autotaxin expression, C57Bl6/J mice were treated with streptozotocin, a toxin that induces hypoinsulinaemia by destroying the pancreatic beta cells. As shown in Fig. 2a, streptozotocin treatment led to a strong and significant increase in plasma glucose concentration close to that observed in *db/db* mice (see Fig. 1b). Streptozotocin treatment was also associated with a significant reduction in fat pad weight (Fig. 2b). In contrast, no modification of adipose tissue autotaxin mRNA levels was observed (Fig. 2c).

These results show that streptozotocin-induced hyperglycaemia had no influence on adipocyte autotaxin expression, suggesting that hyperglycaemia in itself was not sufficient to explain the up-regulation of adipocyte autotaxin expression in *db/db* mice.

Association between hyperglycaemia and up-regulation of adipocyte autotaxin expression during the development of *db/db* mice In order to get better insight into the mechanisms involved in up-regulation of adipocyte autotaxin expression in *db/db* mice, adipocyte autotaxin mRNA levels, the fat pad weight, and non-fasting plasma concentrations of glucose and insulin were examined in 5- to 12-week-old *db/db* and control mice (Fig. 3). Whereas fat pad weight and insulin concentrations were already significantly higher in 5-week-old *db/db* mice than in control mice of the same age (Fig. 3a, b), autotaxin mRNA levels and glucose concentrations became significantly elevated only at week 8 (Fig. 3c, d).

These observations reveal that up-regulation of adipocyte autotaxin expression occurred rather late in the onset of obesity in *db/db* mice, and was more closely associated with the emergence of hyperglycaemia than with fat-store accretion or hyperinsulinaemia.

Up-regulation of autotaxin in adipose tissue of *db/db* mice is restricted to adipocytes Adipose tissue contains adipocytes and stroma-vascular cells (mainly preadipocytes, endothelial cells, macrophages). Autotaxin was previously found to be expressed in both adipocytes and stroma-vascular cells [2]. Adipocyte expression of autotaxin was found to be substantially increased in obese *db/db* mice when compared to adipocytes from non-obese mice [2]. To determine whether such up-regulation of autotaxin-expression also occurred in stroma-vascular cells, inguinal and perigonadic adipose tissue from control and *db/db* mice were dissociated with collagenase and adipocytes were separated from the stroma-vascular cells. In control mice, and regardless of the fat depot, autotaxin mRNA levels were not significantly different between adipocytes (Fig. 4a) and

Fig. 3 Expression of adipocyte autotaxin during the growth of *db/db* mice. Perigonadic fat pad weight (a), plasma insulin concentrations (b), plasma glucose concentrations (c), and adipocyte autotaxin mRNA levels (d) were measured in *db/db* (squares) and control (triangles) mice killed at different stages of their development (5 to 12 weeks). Values are means $\pm$ SE. Comparisons between *db/db* and *+/+* mice of the same age (except at age 8 weeks when *db/db* mice were compared with 7-week-old *+/+* mice) were performed using a Student's *t*-test. ** $p<0.01$

Fig. 4 Expression of autotaxin in adipocytes and stroma-vascular cells from *db/db* mice adipose tissue. Adipocytes and stroma-vascular cells were isolated from inguinal (ING) and perigonadic (PG) adipose tissue from 12-week-old *db/db* ($n=4$) (black bars) or

+/+ mice ($n=4$) (white bars), and autotaxin mRNA levels (/18 S RNA $\times 10^4$) were analysed by real-time RT-PCR. Values are means $\pm$ SE. Comparisons between +/+ and *db/db* mice were performed using a Student's *t*-test. ** $p<0.01$

stroma-vascular cells (Fig. 4b). In *db/db* mice, however, adipocyte autotaxin mRNA levels were significantly increased when compared with control mice (Fig. 4a), but no significant modification was observed in stroma-vascular cells, either in inguinal or in the perigonadic fat depots (Fig. 4b).

These observations reveal that the up-regulation of autotaxin expression in adipose tissue of *db/db* mice was restricted to adipocytes. We concluded that there could be an adipocyte-specific mechanism of regulation of autotaxin gene expression.

Adipocytes from db/db mice exhibit severe impairment of glucose uptake and insulin sensitivity *Db/db* mice are known to exhibit profound impairment of glucose homeostasis, accompanied by a reduction in insulin responses in muscles, liver and adipose tissue [10]. Glucose uptake was measured in adipocytes from obese *db/db* and HFD mice and compared with non-obese control mice of the same age. As shown in Fig. 5, insulin treatment (100 nmol/l) of adipocytes from non-obese and obese HFD mice led to a strong increase (about fivefold) in basal glucose uptake. In contrast, in adipocytes from *db/db* mice, insulin only led to a 2.1-fold increase in basal glucose uptake. This corresponds to a 2.4-fold reduction of the insulin response, compared to control.

These results indicate that *db/db* adipocytes exhibited severe impairment of glucose uptake and insulin sensitivity.

TNF α and rosiglitazone regulate autotaxin expression in 3T3F442A adipocytes In order to test whether autotaxin expression could be directly dependent on adipocyte glucose homeostasis and insulin sensitivity, mouse 3T3F442A adipocytes were treated with TNF α (a diabetogenic cytokine known to impair glucose utilisation and insulin sensitivity in adipocytes) or rosiglitazone (a peroxisome proliferator-activated receptor [PPAR]- γ agonist known to improve adipocyte glucose utilisation and insulin sensitivity in adipocytes) [11]. Compared with untreated cells, 24 h of treatment with 10 ng/ml TNF α led to a significant increase (1.5-fold) in autotaxin mRNA levels, whereas

treatment with 1 μ mol/l rosiglitazone led to a strong and significant reduction (80% reduction) (Fig. 6). Co-treatment with TNF α and rosiglitazone resulted in a smaller reduction of autotaxin mRNA levels than treatment with rosiglitazone alone (Fig. 6).

These results indicate that factors altering glucose utilisation and insulin sensitivity in adipocytes were also able to regulate adipocyte autotaxin expression, suggesting a possible involvement of adipocyte insulin sensitivity in adipocyte autotaxin expression.

Expression and secretion of autotaxin expression by human adipose tissue In contrast to mouse adipose tissue [2], the expression and secretion of autotaxin by human adipose tissue had not yet been investigated. Secretion and expression of autotaxin were first investigated on fresh subcutaneous abdominal adipose tissue samples obtained from

Fig. 5 Stimulation of glucose uptake by insulin in adipocytes from *db/db* mice adipocytes. Adipocytes were isolated from non-obese (control) ($n=4$), *db/db* mice ($n=4$), or obese high-fat-diet (HFD) mice ($n=4$) and exposed (black bars) or not (white bars) to 100 nmol/l insulin before measuring glucose uptake (see "Subjects, materials and methods"). Values are means $\pm$ SE. Comparison between basal (white bars) and insulin-stimulated (black bars) glucose uptake was performed using a Student's *t*-test. * $p<0.05$; *** $p<0.001$

Fig. 6 Influence of $\text{TNF}\alpha$ and rosiglitazone on autotaxin expression in 3T3F442A adipocytes. Seven-day post-confluent 3T3F442A adipocytes were placed in serum-free DMEM and treated or not (Cont) with $\text{TNF}\alpha$ (20 ng/ml) (TNF), or rosiglitazone (1 $\mu\text{mol/l}$) (rosi), or both (TNF+rosi). After 24-h treatment autotaxin mRNA levels were measured. Values are means $\pm$ SE of three separate experiments. Comparisons with control were performed using a Student's *t*-test. * $p<0.05$; ** $p<0.01$

plastic surgery (see “Subjects, materials and methods”). Autotaxin mRNA levels were found to be slightly, but significantly higher in adipocytes than in stroma-vascular cells: autotaxin mRNA/18S RNA 69 ± 13 for adipocytes ($n=10$); 35 ± 6 for stroma-vascular cells ($n=9$); $p<0.03$.

The capacity of human adipose tissue to secrete autotaxin was tested by looking for the presence of the autotaxin protein and activity in conditioned media from small explants of subcutaneous adipose tissue (see “Subjects, materials and methods”). As a positive control for the presence of autotaxin, conditioned media prepared from 3T3F442A adipocytes and from Cos-7 cells transfected with an expression vector encoding mouse autotaxin [2] were used. As shown in Fig. 7a, incubation of [^{14}C]lysophosphatidylcholine in conditioned media from human adipose tissue led to the formation of [^{14}C]lysophosphatidic acid. This is characteristic of the lysophospholipase D activity of autotaxin [2]. Autotaxin protein itself was also detected in the conditioned media by Western blot analysis

using a rabbit polyclonal antibody (see “Subjects, materials and methods”) directed against autotaxin protein (Fig. 7b).

These results show that human adipose tissue expresses and secretes autotaxin.

Expression of autotaxin in adipose tissue from insulin-resistant patients Autotaxin mRNA levels were quantified in intra-abdominal adipose tissue from 23 women exhibiting massive obesity (mean BMI 48 ± 1) justifying abdominal surgery (gastroplasty) (see “Subjects, materials and methods”). All the patients were hyperinsulinaemic (mean plasma insulin concentration 21.7 ± 2 mU/l, expected normal values 5–6 mU/l) and insulin-resistant (mean HOMA values 5.26 ± 0.58 , expected normal values 1–1.2).

Since adipose tissue samples were frozen immediately after surgery, isolation of adipocytes could not be performed and autotaxin expression was analysed on whole-tissue samples from each patient. Autotaxin mRNAs were detected in all samples with autotaxin mRNA/18S RNA ratio values ranging from 12 to 45.

According to the OMS criteria for diabetes diagnosis [12], three groups of patients could be distinguished: 11 subjects [normal glucose tolerance (NGT) group] had no diabetes mellitus (mean fasting glycaemia= 5.33 ± 0.11 mmol/l) and NGT (mean glycaemia= 7.92 ± 0.55 mmol/l at 120 min of the OGTT); four subjects (IGT group) had no diabetes mellitus (mean fasting glycaemia= 5.33 ± 0.11 mmol/l) but exhibited a statistically significant IGT (mean glycaemia= 7.7 ± 5.5 mmol/l at 120 min of the OGTT) when compared with NGT group ($p<0.001$); eight subjects (Diabetes group) had type 2 diabetes mellitus (mean fasting glycaemia= 9.35 ± 1.1 mmol/l and mean HbA_{1c} = $7.4\pm0.6\%$) and exhibited IGT (mean glycaemia= 11.44 ± 0.22 mmol/l at 120 min of the OGTT). Because of its relative glucose intolerance and its limited number of subjects, the IGT group was combined with the Diabetes group (IGT-Diabetes group). NGT- and IGT-Diabetes subjects exhibited no significant differences in BMI, plasma

Fig. 7 Secretion of autotaxin by human adipose tissue. Conditioned media were prepared from Cos-7 cells transfected with empty pcDNA vector (1), cos-7 cells transfected with pcDNA-m autotaxin (2), human adipose tissue (3), and 3T3F442A adipocytes (4). Lane 0 corresponds to culture medium background. (a) The presence of a lysophospholipase D activity was analysed by the formation of [^{14}C]

LPA after incubation of conditioned media with [^{14}C]lysophosphatidylcholine as described in “Subjects, materials and methods”. (b) The presence of autotaxin protein in conditioned media was visualised by Western blot using a polyclonal autotaxin-antibody (see “Subjects, materials and methods”). Autoradiograms are representative of at least three separate experiments

Table 1 Clinical parameters of obese women either with NGT or with IGT and diabetes

Clinical parameters	NGT		IGT-D		<i>p</i> <
	Means±SE	Number	Means±SE	Number	
Age (years)	39±4	11	43±3	12	NS
Body weight (kg)	124±5	11	124±4	12	NS
Body mass index (kg/m ²)	47±2	11	49±2	12	NS
Plasma glucose (mmol/l)	5.17±0.16	11	7.92±0.99	12	0.008
Plasma insulin (mU/l)	21.3±2.5	5	22.1±2.8	6	NS
HOMA	5.14±0.68	5	5.83±1.23	6	NS
HbA _{1c} (%)	ND		7.4±0.6	8	
Plasma triglycerides (mmol/l)	2.24±0.26	11	1.31±0.15	12	0.0049
Plasma cholesterol (mmol/l)	5.07±0.20	11	4.71±0.23	12	NS
Plasma HDL (mmol/l)	1.07±0.07	8	1.28±0.10	12	0.0368
Plasma LDL (mmol/l)	3.20±0.17	8	2.87±0.15	12	NS
Oral glucose tolerance test					
AUC insulinaemia	15774±2099	7	12288±2345	6	NS
Glucose after 120 min	1.2±0.1	7	1.8±0.1	6	0.001

Comparisons between the two groups were performed using a Student's *t*-test
 ND not determined

insulin concentrations, HOMA values and AUC values of insulinaemia (Table 1). The IGT Diabetes group exhibited lower concentrations of plasma triglycerides and HDL than the NGT group (Table 1).

Mean autotaxin mRNA values were significantly higher in the IGT Diabetes group than in the NGT group (Fig. 8). No significant correlation could be established between autotaxin mRNA levels and the different clinical parameters available, except plasma concentrations of LDL ($p<0.041$, $r=0.62$) and the age of the subjects ($p<0.048$, $r=0.59$) in the IGT Diabetes group (not in the NGT group).

These results suggest a possible link between alterations in glucose homeostasis and autotaxin expression in human adipose tissue.

Fig. 8 Expression of autotaxin in intra-abdominal adipose tissue from subjects with normal glucose tolerance (NGT) and subjects with IGT and diabetes (IGT-D). Autotaxin mRNA levels were measured in whole adipose tissue from massively obese patients undergoing gastroplasty, and exhibiting non-diabetic or diabetic phenotypes (see Table 1). The figure represents individual values as well as means±SE (horizontal bars). Comparisons between the two groups were performed using a Student's *t*-test

Discussion

We have previously demonstrated that autotaxin is a lysophospholipase D secreted by adipocytes and is responsible for the synthesis of LPA [2]. We also reported that autotaxin expression is up-regulated in obese-diabetic *db/db* mice when compared with non-obese mice [2], suggesting the involvement of adipocyte autotaxin in obesity and/or diabetes.

In the present study, up-regulation of adipocyte autotaxin expression in *db/db* mice was not observed in other mice models of obesity (GTG, HFD), suggesting the existence of a *db/db*-specific mechanism involved in regulation of adipocyte autotaxin expression.

Since GTG and HFD mice exhibited equivalent (HFD mice) or higher (GTG mice) adiposity than *db/db* mice, we conclude that adiposity was not sufficient, in *db/db* mice, to explain up-regulation of adipocyte autotaxin expression. The same conclusion could be drawn from the analysis of *db/db* mouse development, showing that young *db/db* mice (5 weeks of age) already had excess adiposity when compared to control mice, but did not yet exhibit changes in autotaxin expression.

Obese *db/db* mice had higher hyperinsulinaemia and higher hyperglycaemia than obese GTG or HFD mice. Therefore, *db/db* mice obviously exhibited a strong impairment of glucose homeostasis. Consequently, *db/db*-specific up-regulation of adipocyte autotaxin expression could be linked to the type 2 diabetes phenotype of *db/db* mice. However, analysis of *db/db* mouse development showed that young (5 weeks) *db/db* mice already had maximal hyperinsulinaemia, whereas up-regulation of autotaxin expression occurred only at week 8. In addition, in streptozotocin-treated mice, hyperglycaemia reached a level comparable to that observed in *db/db* mice, but no change in adipocyte autotaxin expression was observed. We can therefore conclude that hyperinsulinaemia or hyperglycaemia in themselves are not sufficient to up-regulate auto-

taxin expression, suggesting the involvement of an additional regulating factor.

As previously described [2] and confirmed in the present study, autotaxin was not only expressed in adipocytes, but also in stroma-vascular cells of adipose tissue. However, conversely to adipocytes, autotaxin-expression in stroma-vascular cells from *db/db* mice was not modified when compared with control mice. This suggests the existence of an adipocyte-specific mechanism involved in the regulation of autotaxin expression in the *db/db* mouse.

The present work shows that adipocytes from *db/db* mice exhibited a strong impairment in insulin sensitivity as demonstrated by the reduction in insulin-stimulated glucose uptake when compared with non-obese and HFD-induced obese mice. Since insulin resistance is known to greatly influence gene expression in adipocytes [11], it is likely that insulin resistance of *db/db* adipocytes could play a role in the up-regulation of autotaxin gene expression. This hypothesis was supported by the direct positive effect of TNF α (a cytokine that promotes insulin resistance), and the negative regulatory effect of rosiglitazone (a PPAR γ agonist well known for its ability to improve insulin sensitivity) on autotaxin expression in 3T3F442A adipocytes.

Finally, the present study shows that autotaxin expression is higher in patients with reduced tolerance to glucose or diabetes mellitus, when compared to patients with NGT. This observation suggests that, as in *db/db* mice, human autotaxin expression could be influenced by alterations in glucose homeostasis. Human adipose tissue autotaxin expression was not correlated with BMI, plasma glucose, or plasma insulin, indicating that these parameters, as in *db/db* mice, are not sufficient to explain the up-regulation of autotaxin expression in IGT Diabetes subjects.

Based upon plasma insulin concentrations and HOMA values, there was no apparent difference in insulin sensitivity between NGT and IGT Diabetes subjects. This could indicate that, conversely to *db/db* mice, insulin resistance was not responsible for regulating autotaxin in human adipose tissue. However, these clinical parameters characterise only global insulin sensitivity and say nothing about insulin sensitivity of adipocytes. Complementary experiments will be necessary to evaluate adipocyte insulin sensitivity in NGT and IGT Diabetes subjects. In parallel, other factors could also be involved in the up-regulation of autotaxin in IGT Diabetes patients.

Indeed, we observed a significant correlation between autotaxin expression and age as well as plasma concentrations of LDL cholesterol. In addition, IGT Diabetes subjects exhibited lower plasma triglyceride and HDL cholesterol. These observations suggest a possible involvement of circulating cholesterol and/or duration of type 2 diabetes in the regulation of autotaxin expression. This hypothesis is supported by a previous report showing an increase of

lysophospholipase D-activity in hypercholesterolaemic rabbits [13].

In conclusion, the present work revealed the existence of a *db/db*-specific up-regulation of adipocyte autotaxin expression that is independent of excessive adiposity in itself, but could instead be linked to the severe type 2 diabetes phenotype and adipocyte insulin resistance. In parallel, the up-regulation of autotaxin expression in adipose tissue of patients exhibiting type 2 diabetes suggests the possible implication of autotaxin in the human form of the disease.

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