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Adipose Tissue Macrophages (ATM) of obese patients are releasing increased levels of prolactin during an inflammatory challenge: A role for prolactin in diabesity?☆

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ABSTRACT

Background: Obesity, characterized by low grade inflammation, induces adipose tissue macrophage (ATM) infiltration in white adipose tissue (AT) in both humans and rodents, thus contributing to insulin resistance. Previous studies have shown altered prolactin secretion in obesity, however, studies linking ATM infiltration and prolactin (PRL) secretion to the pathogenesis of the metabolic syndrome, obesity and diabetes are lacking.
Methods/Results: In vivo, qPCR and Western blot analysis demonstrated that prolactin expression was increased in AT of obese rats and also in human AT from obese, obese pre-diabetic and obese diabetic compared to lean counterparts. Immunohistochemistry of obese rat and human AT sections demonstrated a specific expression of prolactin in macrophages. In vitro, we demonstrated that hyperglycemia and inflammation stimulated macrophages (human THP-1 cell line and sorted rat ATM) to express PRL, when challenged with different glucose concentrations with or without IL1β. In in vivo and in vitro experiments, we assessed the expression of Pit-1 (PRL-specific transcription factor) and found that its expression was parallel to PRL expression.
Conclusions: In this study, we show that rodent and human macrophages synthesize prolactin in response to inflammation and high glucose concentrations.
General significance: Our data shed new light on the potential role of macrophages in the physiopathology of diabesity via the PRL expression and on its expression mechanism and regulation.

1. Introduction

Obesity is a major health problem with increasing prevalence worldwide. Since 1980, obesity has more than doubled. Estimations report 2.8 million deaths as a result of being overweight or obese. The development of type 2 diabetes in obese patients led to the term diabesity. Obesity is predisposing to a variety of age-related inflammatory diseases including insulin resistance, type 2 diabetes, atherosclerosis, osteoarthritis, fatty liver diseases and cancer [1]. One key feature of obesity is a chronic low-grade inflammation induced by various inflammatory mediators [2]. This inflammation occurs in the expanding adipose tissue and is associated with infiltration of immune cells like macrophages, lymphocytes and mast cells.

In mammals, adipose tissue includes both white adipose tissue (WAT) and Brown adipose tissue (BAT) implicated in lipid storage and thermogenesis respectively. Since 1993, WAT is no longer considered just as a storage tissue but as an endocrine organ controlling numerous physiological and pathological processes. Adipose tissue is composed of mature adipocytes and other cell types (i.e. cells of the haematopoietic lineage like macrophages, endothelial cells, fibroblasts and preadipocytes) present in the stroma vascular fraction (SVF) [3–5]. Tissue macrophages are composed of different subpopulations exerting different physiological properties. Two well-known subtypes: M1 (classically activated macrophages) with pro-inflammatory properties, and M2 (alternatively activated macrophages) with anti-inflammatory

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properties have been identified [6,7]. These macrophages are capable of dynamic inter-conversion depending on the encountered environment [8]. Obesity increases tissue infiltration of macrophage [9] and polarization towards the M1 phenotype [10–12].

Adipose tissue is able to release hundreds of biologically active molecules grouped under the term "adipokines" that can be cytokines, chemokines or hormones. These adipokines, released also by the cells of the SVF, affect appetite and satiety (leptin), glucose and lipid metabolism (adiponectin, visfatin, resistin), blood pressure (angiotensinogen), inflammation (TNFα, IL1β, IL6, CCL2) and immune functions (prolactin).

Prolactin (PRL) is a polypeptide hormone mainly secreted by the pituitary gland and by the placenta in rodents, whereas in humans, its expression is observed in many extra-pituitary sites like mammary gland, skin, decidua, prostate, immune cells (B- and T-lymphocytes, natural killers and macrophages) and brain [13,14]. This wide-spread expression partially explains its involvement in various processes like reproduction, behavior, immune modulation and metabolism. Aside from gestation where PRL favors the development of the mammary gland, PRL is implicated in the modulation of the innate and adaptative immune system by stimulating the secretion of cytokines and the expression of cytokine receptors [15,16]. PRL expression is under the control of the pituitary transcriptional factor-1 (Pit-1 also known as Pou1f1) which is of paramount importance for the expression of PRL in the pituitary gland [17]. Pit-1 is one of the members of the POU homeodomain family [18,19]. The action of Pit-1 on PRL transcription has been extensively studied in pituitary cells [20]. In extra-pituitary tissues, Pit-1 is binding to an alternative promoter to induce the PRL expression. The stimuli inducing Pit-1 expression and action on PRL expression are still unclear and need to be clarified.

Prolactin regulates also energy balance. Recently, a prolactinoma has been associated with obesity and its associated perturbations like metabolic syndrome and insulin resistance [21,22]. Studies demonstrated that patients [23–25] and animals [26,27] with hyperprolactinemia were more prone to gain weight. Prolactin seems to be released proportionally to the quantity of fat mass in obese patients [28]. Weight loss in these patients resulted in a decrease in the 24 h-prolactin release [29].

The mechanisms implicated in this phenomenon are still poorly understood and need further investigations. The normalization of PRL levels by dopamine agonists like bromocriptine was associated with weight loss [23,25,30]. The reduction of prolactinemia, via the use of dopamine-D2 receptor agonists like bromocriptine or cabergoline, surprisingly ameliorated the glycemia of type 2 diabetic patients without increasing insulin release [31–33]. Prolactin expression/secretion is regulated by different stimuli provided by the environment. In the immune system, PRL is thought to act as a locally produced cytokine with relevance for immune regulation and modulation of T- and B-cell function. Nevertheless, the molecular mechanisms regulating PRL expression in the immune system and the factors implicated are still not fully understood.

We demonstrate in this paper, that human and rat macrophages synthesize prolactin in response to inflammation and high glucose concentrations as can be encountered during obesity and diabetes. Moreover, we observed that PRL transcription is mediated, in human and rat macrophages, by Pit-1 which expression is also dependent on inflammation and glucose concentrations. Our data are bringing new light on the potential role of resident adipose tissue macrophages in maintaining/worsening inflammation and obesity and on the mechanism leading to prolactin secretion in response to hyperglycemia and inflammation.

2. Materials and methods

2.1. Animals

Wistar rats (Janvier SAS (Le Genest Saint Isle, France)) received food and water ad libitum and were maintained at 25 °C with 10–14 h dark–light cycle. Experimental procedures were performed after approval by the ethics committee of the Université de Lille 2.

Animals of the obese group (OB group) were fed an obesogenic diet (Western RD Diet, Special Diet Service, Argenteuil, France) composed of 50% of carbohydrate (mainly sucrose), 21.4% of crude fat, 17.5% of proteins, 4.1% of ash and 3.5% of fibers for 4 months to induce obesity. During the feeding period, animals were weighed twice a week to follow weight gain. At the end of the period, animals were sacrificed under anesthesia and visceral adipose tissue was collected. Tissues were conserved in RNA later (Sigma Aldrich) for subsequent RNA extraction and pulverized in protein lysis buffer (RIPA) for protein analysis before being stored at −80 °C.

For the diabetic group (STZ group), the animals received a single dose of Streptozotocin (STZ) (Sigma-Aldrich Chimie, France) (65 mg/kg dissolved in 0.1 M citrate buffer pH4.5) by intraperitoneal injection. The control group received a single injection of an equivalent volume 0.1 M citrate buffer pH4.5.

For all groups, trunk blood samples were collected in heparinized tubes and centrifuged to extract the plasma. Before use, samples were stored at −20 °C.

2.2. Rat and human primary adipose tissue macrophages (ATM) isolation

Adipose tissues coming from control, STZ and OB animals were processed. Briefly, perigenital and perirenal adipose tissues were collected, washed by a rapid immersion in 70%, ethanol minced with sterile scissors, centrifuged to remove red blood cells and digested by a collagenase solution (1 mg/ml of collagenase P (Sigma) in Hank's Balanced Sodium Salt (HBSS) (Life Technologies, Saint Aubin, France)) at 37 °C waterbath. After 15 min, digestion was stopped by adding HBSS supplemented with 5% Bovine Serum Albumin (BSA) (HBSS-BSA) (Sigma Aldrich). Samples were passed through a sterile strainer (70 μm) to remove undigested tissue. Three consecutive washes were done to remove the floating adipocytes and to collect the stromal fraction constituted mainly of preadipocytes and red and white blood cells. The SVF was treated with red blood cell lysis solution (155 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$ and 90 μmol/l EDTA) for 3 min on ice and washed two times in HBSS. To collect all ATM, the CD68 antibody (AbDSerotec, Colmar, France), a pan macrophage marker was used. Dissociated cells were stained for 1 h at 4 °C with a mouse CD68 antibody under constant shaking. After 3 washes in PBS-EDTA (1 mmol/l), anti-mouse magnetic beads (Miltenyi Biotec, Paris, France) were added to the cells for another 30 min at 4 °C under constant shaking. Cells were washed again 3 times and sorted with the AutoMACS (Miltenyi Biotec). Following sorting, cells were washed 3 times in HBSS-BSA and splitted in 24-well culture plate for culture and treatment.

For human ATM, the sorting procedure has been described elsewhere [34]. ATM were collected from adipose tissue (visceral fat) coming from obese women; all patients were non diabetic (glycemia < 6 mmol; HbA1c < 6%). Patient characteristics are summarized in Table 1.

2.3. Human adipose tissue and serum

Visceral adipose tissue (n = 24) was obtained from diabetic and non-diabetic morbidly obese patients undergoing bariatric surgery at the University Hospital of Lille (Lille, France). This study was approved by the ethics committee of the CHRU of Lille under the Atlas Biologique de l'Obésité Sévère (ABOS) framework. None of the patients had clinical symptoms of systemic inflammation and all gave written informed consent. Patients used in this study were age-matched and 3 male and 3 females were selected per group to evaluate sexual dysmorphism effect. Patient characteristics are summarized in Table 2.

Sera were collected from obese (n = 18) and lean (n = 6) subjects and prolactin concentration was measured by ELISA (Eurobio, Les Ulis, France).

Table 1
Characteristics of visceral fat samples used to collect ATM.

Characteristics	OB1	OB2	OB3	OB4	OB5
Age (years)	24	37	43	19	46
Gender	F	F	F	F	F
BMI (kg/m^2)	54.8	55.8	45.6	45.2	48.7
Weight of AT collected (g)	42	57	38	100	48
ATM in VST					
% ATM	6.3	9.5	11.5	12.5	13.3
% ATM/weight AT	0.15	0.17	0.30	0.13	0.28
Co-morbidities					
Diabetes	–	–	–	–	–
Hypertension	–	–	–	–	–
Hyperlipidemia	–	–	–	+	–
Sleep apnea	–	–	–	–	+
Medication for					
Diabetes	–	–	–	–	–
Hypertension	–	–	–	–	–
Hyperlipidemia	–	–	–	–	–

2.4. Cell Culture

The human monocytic cell line (THP-1), (LGS Standards, Molsheim, France) was grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 30 mM glucose in an air–CO_2 (95:5) atmosphere at 37 °C. Cells were seeded in a 6-well plate until reaching confluence. Confluent cells were treated for 3 days with Phorbol 12-Myristate 13-Acetate ((PMA), Sigma Aldrich) (100 μM) to induce differentiation of floating monocytes into adherent macrophages. Following differentiation, cells were washed two times with PBS and treated for 24 h with medium containing different glucose concentrations (5.5, 11 or 30 mM) supplemented or not with recombinant human interleukin-1beta (IL1β) (100 ng/ml) (Sigma Aldrich).

Rat adipose tissue macrophages were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 5.5 mM glucose in an air–CO_2 (95:5) atmosphere at 37 °C. After 1 day of recovery, ATM were treated with medium containing different glucose concentrations (5.5, 11 or 30 mM) supplemented or not with recombinant rat IL1β (100 ng/ml) (Sigma Aldrich).

2.5. RNA extraction and cDNA synthesis

Total RNA was isolated from rodent and human adipose tissues, adipose tissue macrophages and THP-1 cells with TRIZOL, according to the manufacturer's instructions (Life Technologies). RNA concentrations were evaluated by measuring absorbance at 260 nm with a spectophotometer (NanoDrop, Thermo Fisher Scientific, France). RNA quality was evaluated with bioanalyzer 2100 (Agilent Technologies). Only samples reaching a RNA Integrity Number (RIN) >8 were used for qPCR analyses.

500 ng of total RNA was used for retro-transcription using oligo-d(T) to selectively amplify mRNA. cDNAs were synthesized with First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Illkirch, France) as described by the manufacturer.

2.6. Gene expression analysis

The RT-qPCR experiments and calculations using the $2^{-\Delta\Delta Ct}$ method were performed as previously described [35]. Data were normalized to housekeeping genes (hprt (Hypoxanthine-guanine phosphoribosyltransferase) for rat or β-actin for human). The -RT controls tested for the considered genes assessed the absence of genomic DNA contamination for each sample. Primers (Table 3) have been designed to split an exon–exon junction to avoid genomic DNA amplification.

2.7. Western blotting

Adipose tissue, ATM and THP-1 proteins (40 μg) were resolved in a 15% SDS-PAGE gel under reducing conditions. After separation, proteins were transferred to nitrocellulose membranes (0.2 μm; Millipore, Molsheim, France). The membrane was blocked in TBS-0.1% tween (TBST) and 5% nonfat dry milk, followed by an overnight incubation with primary antibodies in TBST and 5% BSA (goat anti-prolactin (1:1000 (Santa Cruz Biotechnology, Heidelberg, Germany)) and as a loading control mouse anti-beta actin (1:5000 (Sigma Aldrich, France))). The

Table 2
Characteristics of fat samples used for prolactin evaluation.

Characteristics	Lean	OB	OBPreD	OBD
Age (years)	54 ± 2.2	45 ± 1.6	50 ± 2.0	49 ± 4.0
Patients per group	6	6	6	6
Gender, number				
male	3	3	3	3
female	3	3	3	3
BMI (kg/m^2)	24.3 ± 0.6	44.9 ± 2.9*	47.2 ± 3.2*	49.7 ± 3.1*
Fasting Glycemia (mM)	4.3 ± 0.4	5.0 ± 0.4	5.8 ± 0.2**	12.6 ± 1.4**
HbA_{1c} (%)	5.45 ± 0.05	5.25 ± 0.10	5.98 ± 0.08	10.45 ± 0.46
OGTT (glycemia at 2 h (mM))	3.48 ± 0.4	6.08 ± 0.4******	9.38 ± 0.2******	18.8 ± 1.4***
Co-morbidities				
Hyperlipidemia	0/6	2/6	2/6	3/6
Hypertension	0/6	4/6	3/6	3/6
Sleep apnea	0/6	5/6	3/6	4/6
Medication for				
Diabetes				
Metformin	–	–	–	5/6
Insulin	–	–	–	2/6
Sulfonylurea	–	–	–	3/6
Hypertension	–	2/6	3/6	3/6
Hyperlipidemia	–	2/6	2/6	3/6

Each group was composed of 3 males and 3 females. Patients in each group were age-matched. No sexual dysmorphism was observed. No significant differences have been observed for age and HbA_{1c} between all the groups.

* $p < 0.001$ OB, OBPreD, OBD vs lean.
** $p < 0.001$ OB, OBPreD vs OBD.
*** $p < 0.001$ OBPreD and OBD vs lean.
**** $p < 0.05$ OB vs OBPreD.

Table 3
Primers used for quantitative PCR on rat and human samples with Genbank accesion number.

Gene	Genbank	Forward (5′-3′)	Reverse (5′-3′)
h PRL	NM_000948.5	ACCAGGAAAAGGGAAACGAATGCC	CGTTGCAGGAAACACACTTCACCA
h Pit-1	NM_001122757.1	CAACGTTGGGAAACACAGCA	CAAGGGGTTAAACTACCTGCCA
h β-actin	NM_001017992	CATGTACCCAGGCATTGCT	ACTCCTGCTTGCTGATGCA
PRL	NM_012629.1	GGGAGGGGAAGAGGATGCCTG	GTGTCCCTGCTTTCCGGGCTG
Pit-1	NM_013008.3	GAGATCATGCGGATGGCTGA	ATGGCTACCACAGGCAAGTC
hprt	NM_012583.2	ACCTGGGACCGAGACATGTA	GAAGATGGTGTGCTCATTGC

h PRL: human prolactin; h β-actin: human β-actin; h Pit-1: human pituitary-specific transcription factor.
Prl: rat prolactin; Pit-1: Pituitary-specific transcription factor; Hprt: rat Hypoxanthine phosphoribosyltransferase.

membranes were washed in TBST and labeled for 1 h at room temperature with infrared fluorescent-conjugated secondary antibody (1:20,000, Li-Cor, ScienceTec, Courtaboeuf, France) in the dark. The signal was detected with the Odyssey CLx scanner (Li-Cor, ScienceTec) and pixel density quantified using ImageJ.

2.8. Prolactinemia

Concentration of circulating prolactin was measured in sera of control and obese rats using rat prolactin ELISA kit (Cusabio, Wuhan Hubei, China). For human samples, prolactin was assayed by ELISA (Eurobio, Courtaboeuf, France) in lean, obese, obese prediabetic and obese diabetic patients. Each of the experimental groups was constituted of 6 individuals. Samples were processed following the manufacturer's instructions.

2.9. Immunohistochemistry

Tissue samples were fixed in 4% PFA for 24 h and embedded in paraffin. After deparaffination, sections were treated with methanol supplemented with 3% hydrogen peroxide to inhibit endogenous peroxidase. Antigen retrieval was performed by heating the sections for 5 mins at 96 °C in citrate buffer pH 6 supplemented with 0.1% Tween. After a cooling period of 20 mins, sections were incubated with mouse monoclonal anti-rat or anti-human CD68 (1:500; AbDSerotec). All sections were incubated with Alexa 488-conjugated anti-goat and Alexa 594-conjugated anti-mouse antibodies (Life Technologies, France). Nuclei were counterstained with mounting medium containing DAPI (Eurobio, Les Ulis, France). After staining, counting have been realized in lean and obese (for rat) and in lean and obese diabetic patients (for human) to evaluate the quantity of adipose tissue infiltrating macrophages.

2.10. Statistical analysis

Otherwise stated, all results were analyzed with ANOVA followed by Bonferroni post-tests with GraphPad Prism (GraphPad Software, San Diego, CA). The results were reported as means ± sem. For human sample analysis, statistical differences between groups were analyzed by Mann–Whitney rank-sum test. Differences of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Prolactin expression is increased in the adipose tissue of obese rats and humans

We compared the expression of PRL in different tissues in rats and humans (Fig. 1A and B). In the rat, we used the pituitary gland as a positive control for PRL expression. The expression of PRL was higher in the adipose tissue from obese rats compared to pituitary and adipose tissue from control animals ($p < 0.01$ and $p < 0.001$ respectively). As shown in Fig. 1B, in human adipose tissue, the expression of PRL was significantly higher ($p < 0.01$) in obese vs0020lean patients.

3.2. Prolactin is expressed by macrophages in the adipose tissue of obese rats

To identify which cells in adipose tissue express PRL, immunofluorescent staining was performed on rat adipose tissue sections from lean and obese animals. In Fig. 2A, histological analyses of obese rat adipose tissue identified macrophages as cells expressing PRL. With the development of obesity and the subsequent inflammation, macrophages infiltrate adipose tissue (CD68 staining in red and PRL staining in green). In lean animals, few macrophages infiltrated the adipose tissue (Fig. 2B) and corresponding PRL staining was weak.

In the AT of obese rats, most of the macrophages were localized at the junctions of two or more adipocytes (Fig. 2A). Crown structures can be identified only in obese adipose tissue (Fig. 2C). Counting of macrophages on AT sections revealed a significant difference between quantities of infiltrating macrophages in lean compared to obese (2 ± 0.4 vs 34.6 ± 4.3 respectively, $p < 0.01$).

3.3. Prolactin is expressed by ATM following inflammation

Obesity is often accompanied by insulin resistance predisposing to type 2 diabetes which is diagnosed by elevated blood glucose concentration. To identify which stimuli induce the expression of PRL in macrophages, ATM were sorted from adipose tissue of lean animals and treated with different glucose concentrations for 24 h. We observed that increasing glucose concentrations (5.5, 11 mM and 30 mM, $^*p < 0.05$) 5–10 times increased the expression of PRL in the macrophages (Fig. 3A). IL1β, a well-known pro-inflammatory cytokine, also enhanced PRL expression in macrophages incubated with for 5.5 and 11 mM glucose demonstrating an additive effect of inflammation

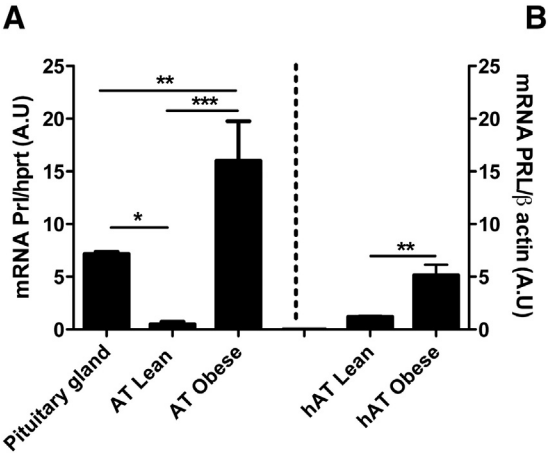

Fig. 1. Evaluation of prolactin expression in the adipose tissue of obese and lean rats and humans. qPCR analysis showed a 12 fold increase in the expression of prolactin mRNA between AT from lean and obese in rodents. The expression in the AT of obese animals was 2 times higher than in the pituitary gland. For humans, the prolactin expression was 2 times higher in adipose tissue from obese (compared to lean control). Results are means ± sem of 5 independent experiments, $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ANOVA with Bonferroni correction.

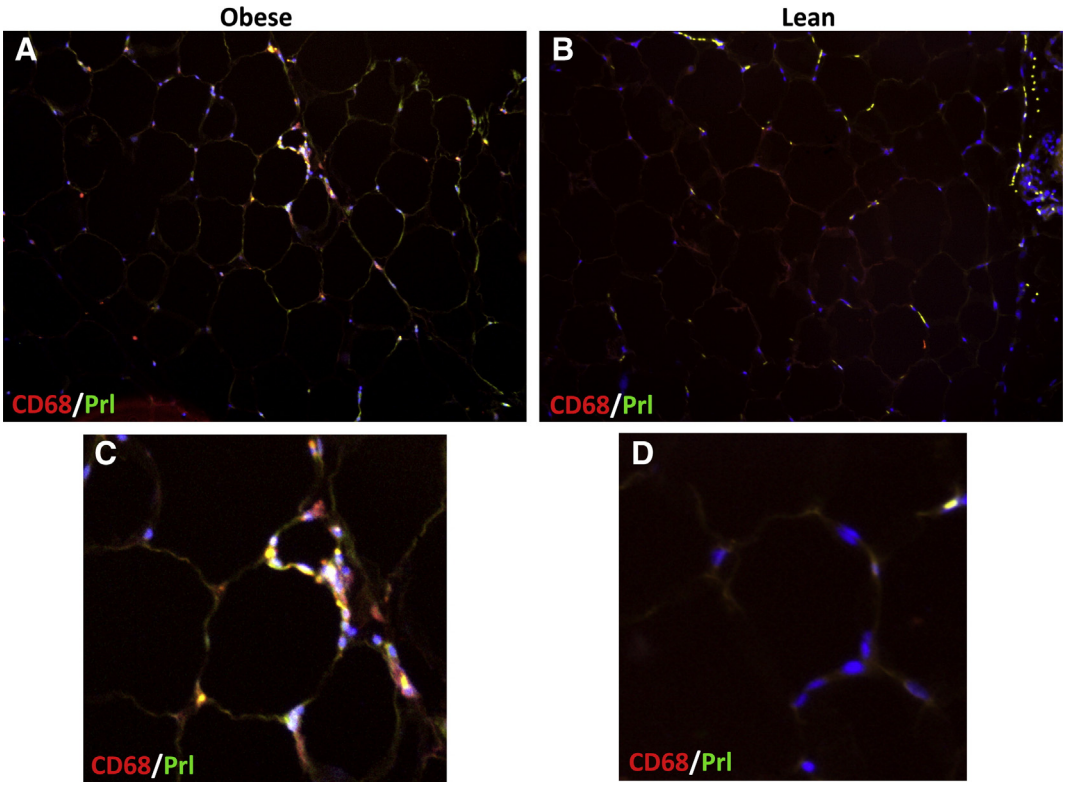

Fig. 2. Adipose Tissue Macrophages (ATM) express prolactin in obese animals. Immunohistochemistry for prolactin (green) and CD68 (red), a pan-macrophage marker performed in the adipose tissue of lean (B, D) vs obese (A, C) animals showed a clear expression of prolactin by the macrophage only in the adipose tissue of obese animals. Crown structures can be observed only in obese animals. Magnification ×200 (A and B); Magnification ×400 (C and D).

(IL1β) on glucose. At high glucose concentration (30 mM), IL1β did not further amplify the response showing a saturation of the system.

Western blot analysis demonstrated that increasing glucose concentrations significantly induced PRL protein levels ($p < 0.01$) (Fig. 3B). Combined treatment with glucose plus IL1β also induced PRL expression significantly at 11 and 30 mM ($p < 0.001$). As previously observed at mRNA level, the combination of 30 mM glucose supplemented or not with IL1β induced a lower PRL expression than 11 mM, highlighting a possible saturation of the system.

3.4. Prolactin is expressed in human macrophages under inflammatory conditions

To confirm the observations made in rodent adipose tissue, analysis of adipose tissue from lean and obese patients and ATM from obese patients were carried out. As previously observed in Fig. 1B, a 4.5 fold higher expression of PRL was observed in tissues from obese diabetic compared to lean individuals. To investigate if hyperglycemia had an additive effect on obesity, we evaluated PRL expression at the mRNA

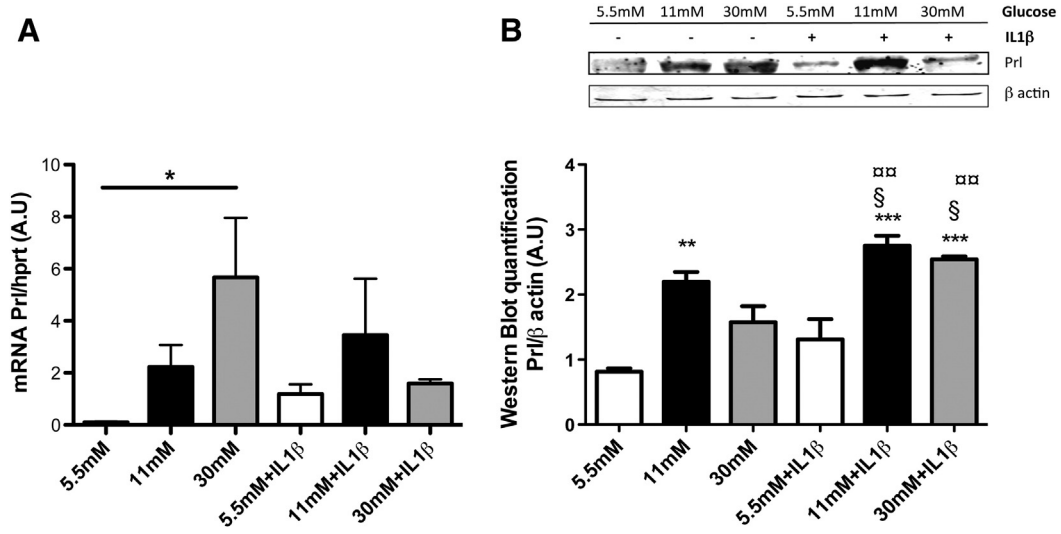

Fig. 3. Prolactin expression by macrophages is activated in vitro in the rat adipose tissue macrophages by hyperglycemia and inflammation. (A) qPCR analysis revealed a significant induction of prolactin expression dependent on glucose concentration. Treatment with IL1β was also capable of inducing prolactin expression by the macrophages demonstrating an additive effect when added to high glucose containing medium. (B) As observed at the mRNA level, Western Blot analysis and quantification revealed prolactin synthesis in response to increasing concentrations of glucose. The additive effect of IL1β and glucose on prolactin synthesis could also be observed. Results are means ± sem of 5 independent experiments; $^{**}p < 0.01$, $^{***}p < 0.001$ vs 5.5 mM; $^{¤¤}p < 0.01$ vs 5.5 + IL1β; ANOVA with Bonferroni correction.

and protein level in adipose tissue from obese pre-diabetic (OBpreD) and obese diabetic patients (OBD). As shown in Fig. 4A, C and D, obesity with diabetes (called Diabesity) resulted in a higher PRL expression. It should be pointed out that obese diabetic patients received daily anti-diabetic treatment (mainly metformin alone or in combination with insulin) potentially weakening the hyperglycemia effects. As PRL was expressed by macrophages, we evaluated the expression of PRL in ATM from morbidly obese patients. As control, we used Monocyte-Derived Macrophages (MDM) coming from the same patients. We observed 2000 times higher expression of PRL in ATM compared to MDM (Fig. 4B).

As previously observed for rat adipose tissue, immunohistochemistry performed on human adipose tissue samples coming from lean (Fig. 5A), obese (Fig. 5B), obese prediabetic (Fig. 5C) and obese diabetic (Fig. 5D) subjects demonstrated increased macrophage infiltration in obese compared to lean patients (44.2 ± 6.8 and 1.8 ± 0.5% for obese diabetic and lean respectively, $p < 0.01$). Prolactin expression (green staining) in macrophages (CD68 positive, red staining) was strongest in adipose tissue from obese prediabetic and obese diabetic (Fig. 5C and D).

To confirm that the same mechanism was occurring in human cells as observed in rat, THP-1 cells were studied. As shown for rat ATM, culturing THP-1 cells, in media with different glucose concentrations resulted in an increase of PRL expression at the mRNA and protein levels, an effect further enhanced by IL1β treatment (Fig. 6A & B). For all glucose concentrations evaluated, adding IL1β increased PRL mRNA levels 2 times.

3.5. Obesity increases prolactinemia

We evaluated the level of serum prolactin (prolactinemia) in obese rats, using as a positive control, sera from rat at the 20th day of gestation (peak of circulating prolactin). We observed a significant increase in obese animal vs lean animal sera ($p < 0.05$) (Fig. 7A). Moreover, PRL concentrations were increased in sera from obese, obese prediabetic and obese diabetic patients indicating that obesity complicated by diabetes exacerbates prolactinemia ($p < 0.05$) (Fig. 7B).

3.6. Pit-1 expression is regulated by inflammation and hyperglycemia

We compared the expression of Pit-1, the PRL transcription factor, in rat ATM (Fig. 8A) and THP-1 cells (Fig. 8B) treated with different glucose concentrations combined or not with IL1β. As previously observed for PRL expression, we observed a significant increase of Pit-1 expression ($^{*}p < 0.05$, $^{**}p < 0.01$) 10–20 times after the glucose treatment (Fig. 8A & B). IL1β enhanced Pit-1 expression in THP-1 cells incubated with all tested glucose concentrations demonstrating an additive effect of inflammation (IL1β) on glucose (Fig. 8B). For rat macrophages, IL1β treatment didn't induce an enhancement of Pit-1 expression (Fig. 8A). In human adipose tissue samples from lean, obese, obese prediabetic and obese diabetic subjects, we observed a significant increase of Pit-1 expression in obese ($p < 0.05$) and obese prediabetic ($p < 0.01$) compared to the lean subjects. For the obese diabetic, we observed a 300 times increased expression compared to the lean counterparts ($p < 0.001$).

4. Discussion

In the present study, we show that macrophages are a source of prolactin expression and that this expression is enhanced in pathophysiological conditions such as obesity and diabetes. We show in vitro that high glucose concentrations and IL1β (mimicking the combination of hyperglycemia and inflammation) enhanced the expression of PRL in macrophages.

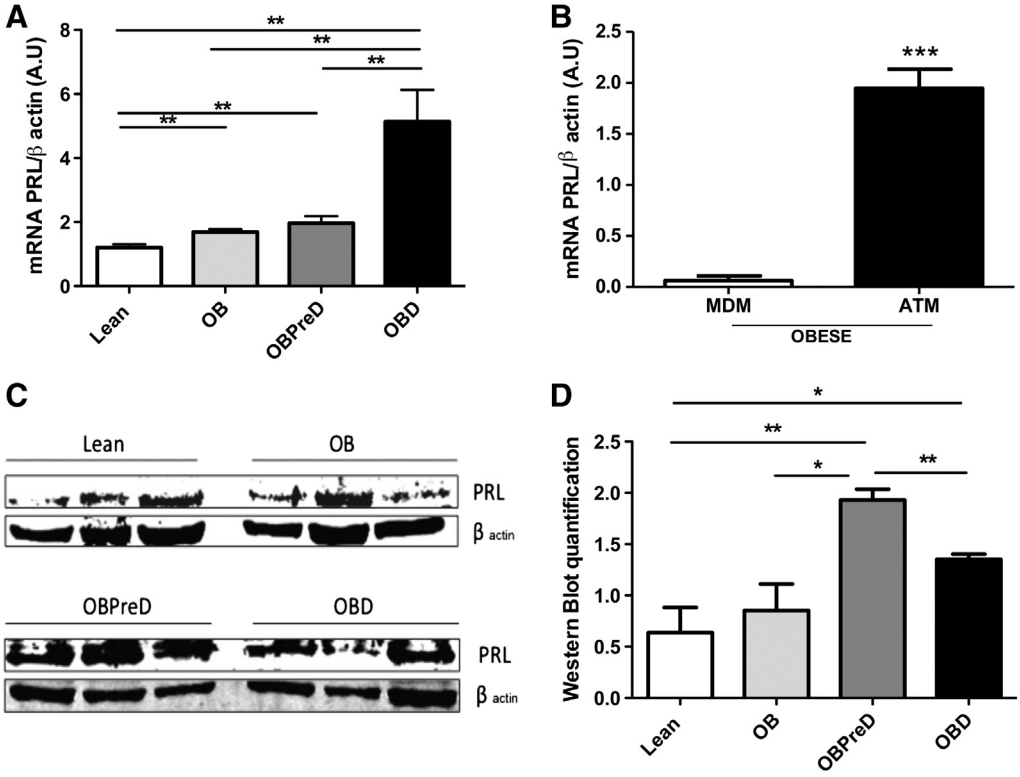

Fig. 4. Evaluation of prolactin expression in human adipose tissue, Adipose Tissue Macrophages (ATM) and Monocyte-Derived Macrophages (MDM) from obese and lean subjects. (A) qPCR analysis of lean ($n = 6$) and obese subjects with different glycemic status (Obese (OB) ($n = 6$), Obese Pre Diabetic (OBPreD) ($n = 6$); Obese Diabetic (OBD) ($n = 6$)) revealed a significative increase in prolactin mRNA for obese (independent of their glycemic status) compared to lean. $^{**}p < 0.01$, $^{***}p < 0.001$. (B) qPCR analysis of prolactin expression in ATM (■) and MDM (□) from 5 different obese patients. For each subject, ATM and MDM were collected for comparison. $^{***}p < 0.001$ vs MDM. (C–D) Western blot analysis and quantification of prolactin expression by the visceral adipose tissue of lean and obese patients with different glycemic status (Obese (OB) ($n = 6$), Obese Pre Diabetic (OBPreD) ($n = 6$); Obese Diabetic (OBD) ($n = 6$)). Prolactin expression is significantly increased in obese patients compared to lean $^{*}p < 0.05$; $^{**}p < 0.01$. β-actin was used to assess equal protein loading. Results are means ± sem of 6 independent experiments for each of the experimental groups considered, ANOVA with Bonferroni correction.

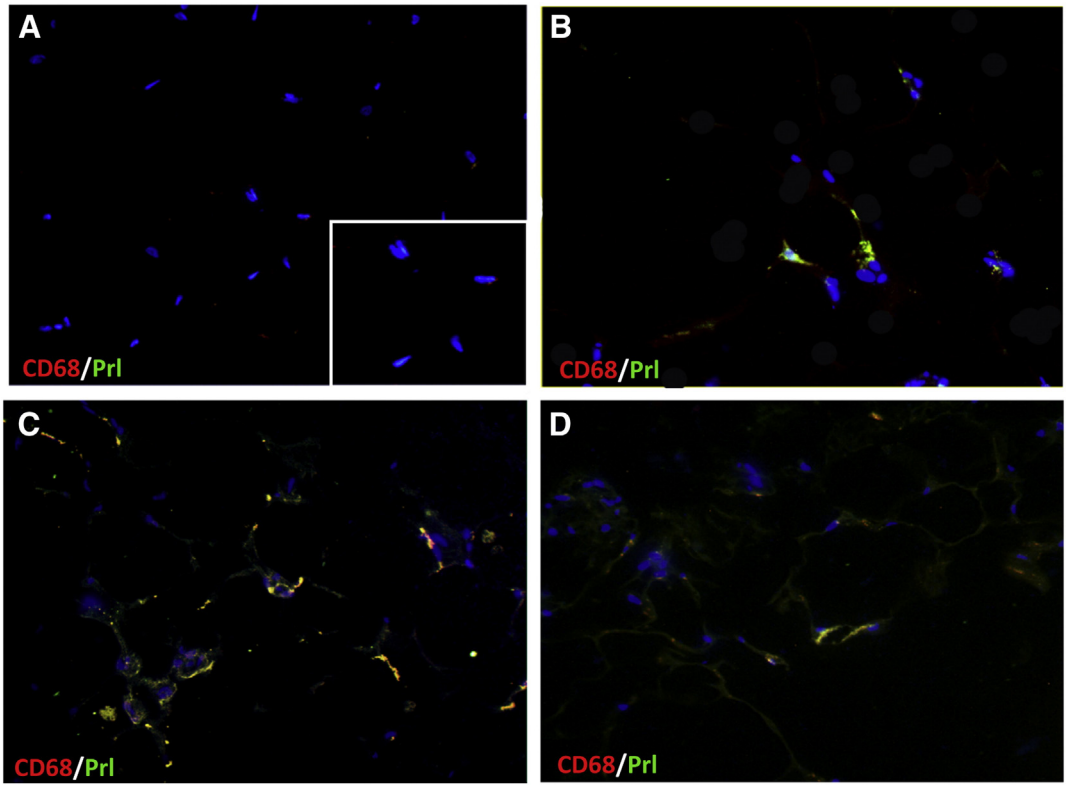

Fig. 5. Prolactin expression by Adipose Tissue Macrophages in Lean, Obese (OB), Obese PreDiabetic (OBPreD) and Obese Diabetic (OBD) humans. Immunohistochemistry for prolactin (green) and CD68 (red), performed in the adipose tissue of lean (A), obese (B), obese prediabetic (C) and obese diabetic (D) patients showed a clear increased expression of prolactin by the macrophage in the adipose tissue of obese individuals. An enhancement of prolactin expression was observed in obese prediabetics (C) and obese diabetics (D). Magnification ×200 (A, B, C, D); magnification ×400 (picture included in picture A).

Hyperglycemia and inflammation, occurring in diabesity, showed an additive effect on prolactin expression by the macrophages both in adipose tissue macrophages in rodents and in monocyte/macrophage human cell line THP-1, human adipose tissue and human ATM.

Obesity is formerly considered as the expansion of the adipose tissue mass associated with the development of a chronic low grade inflammation located in the adipose tissue but spread in the whole organism by the blood flow. This inflammation, linked to insulin resistance but also to other obesity-related morbidities, is promoting macrophage infiltration of the adipose tissue [36,37]. Macrophage density is directly proportional to the level of obesity [9]. In the present study, we confirmed in rats and humans that there are increasing quantities of macrophages in the fat of obese individuals. Infiltrating macrophages are in direct contact with the cytokines/chemokines released by the adipocytes. A recently published study on transgenic rats expressing the human prolactin gene demonstrated that, in a model of experimental inflammation by thioglycollate injection, prolactin expression is highly increased in peritoneal macrophages [38]. We observed, in our

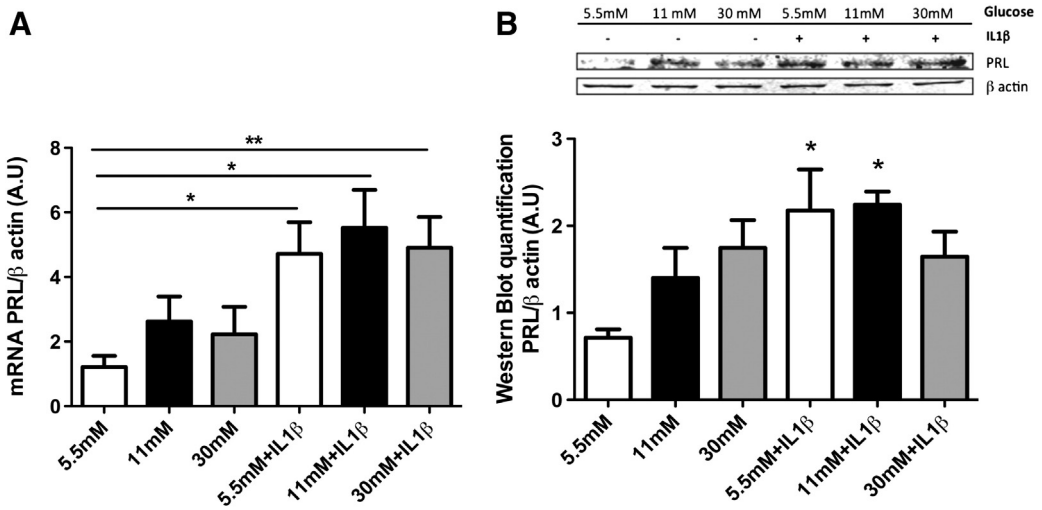

Fig. 6. Human monocyte/macrophage cell line THP-1 expresses prolactin in vivo when treated with different glucose concentrations supplemented or not with IL1β. (A) qPCR analysis of prolactin mRNA expression demonstrated an increased expression after high glucose treatment for 24 h. As previously observed, an additive effect of IL1β can be observed. *$p < 0.05$; **$p < 0.01$. (B) Western blot analysis of prolactin expression and quantification. β-actin was used as a loading control. *$p < 0.05$ vs 5.5 mM. Results are mean ± sem of 6 independent experiments, ANOVA with Bonferroni correction.

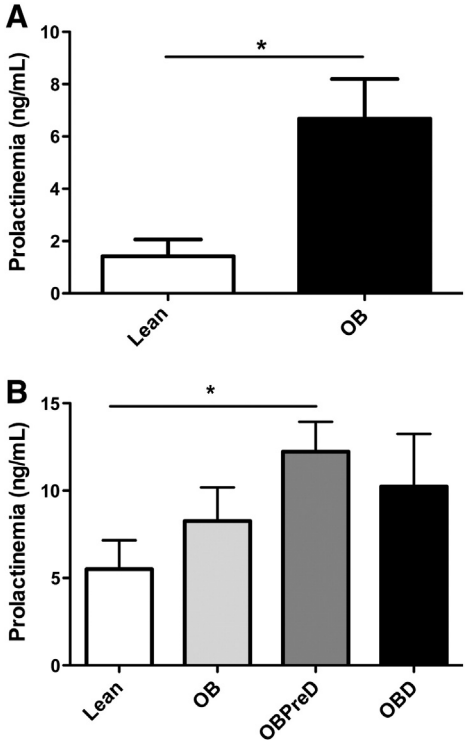

Fig. 7. Increased prolactinemia in obese subjects. (A) prolactinemia, evaluated in lean and obese rats, demonstrated a 3 fold increase in obese animals. *$p < 0.05$ vs control (lean). Results are means ± sem of 5 independent experiments, ANOVA with Bonferroni correction. (B) Prolactinemia, assayed in the serum of lean and obese subject with different glycemic status showed a tendency to increase with obesity. The significant additive effect of hyperglycemia can be observed in OBPreD samples. *$p < 0.05$. In OBD subjects, no statistical significance was achieved probably due to the antidiabetic treatment received by these patients. Results are means ± sem of 6 independent experiments, ANOVA with Bonferroni correction.

experiments, that treatment of THP-1 and adipose tissue macrophages with pro-inflammatory cytokines, such as IL1β, induces a prolactin expression per se, confirming the observations previously made in the transgenic rat model [38]. In our study, Pit-1, the PRL-specific transcription factor has been found expressed in the macrophages of rat and human origin. The kinetic of Pit-1 expression is parallel to the one of PRL showing that, in our model, the PRL expression is due (at least partly) to the increased expression of Pit-1. Some papers reported that Pit-1 expression is induced by cAMP/PKA or PKC action and subsequent phosphorylation of protein implicated in the signaling pathway. Unpublished data generated in our laboratory demonstrated that treatment of purified populations of macrophages in the same culture conditions (with different glucose concentrations; with or without IL1β (an inhibitor of PI3 kinase)) was blocking the increased expression of Pit-1 and as a consequence, the PRL expression (data not shown).

We observed also that rat peritoneal macrophages, non stimulated by thioglycollate injection, but placed in the same culture conditions as the ATM or the THP-1, also respond to elevated concentrations of glucose and inflammation by increasing prolactin expression (unpublished data). It appears that the mechanism of prolactin secretion is occurring in all populations of macrophages regardless of their tissue location.

The effect of elevated glucose on macrophages, as observed during diabetes, has been evaluated in many papers over the recent years [39]. Wen et al. demonstrated that high glucose treatment of mouse peritoneal macrophages induces, in a time- and dose-dependent manner, the expression (>2× compared to control) of IL1β and numerous other pro-inflammatory cytokines [40]. In vivo experiments on diabetic db/db mice confirmed the observations made in vitro on the increased expression of IL1β. Moreover, Dasu et al. [41], using human monocytic THP-1 cells, demonstrated that high-glucose treatment (15 mmol/L for 24 h) induces the expression of the Toll-Like Receptors TLR2 and TLR4 leading to the activation of the MyD88-mediated signaling pathway and the nuclear translocation of NFkB. Data generated by our group on isolated placental macrophages demonstrated that mild (300 mg/dl) or severe (>500 mg/dl) hyperglycemia, during gestation, induced an early activation of the pro-inflammatory genes mediated by the TLR2/TLR4 pathway [39].

Prolactin, as an endocrine hormone, is released in the blood stream. The prolactinemia, evaluated in our rat and human samples follow the same pattern as observed for prolactin mRNA expression. Even through some studies carried out in humans, didn't show any modifications in prolactinemia in obesity [42], our experiments in rats and humans combined with previous published studies of other groups demonstrated a significant increase with obesity. Observations made in mildly obese women detected an enhanced prolactin secretion across the 24 hour cycle (compared to normal-weight women) [28]. After subsequent weight loss (by a low calorie diet or bariatric surgery), the 24 h circadian prolactin release in obese women was reduced clearly demonstrating that increased prolactin levels are a consequence rather than a cause of obesity [29,43].

Prolactin has many effects in animals and humans and is known to regulate seasonal and non-seasonal fat mass in different species, like birds, fish and mammals [44,45]. Based on our observations, we hypothesize that the infiltrating macrophages, in a pro-inflammatory environment, will locally release prolactin which could stimulate immune cells and worsening obesity. This vicious cycle could be responsible to the maintenance of obesity in patients. Genome-wide association studies revealed that a common variant adjacent to the prolactin gene is positively associated with obesity in males [46], reinforcing the potential importance of prolactin on obesity. In adipose tissue samples from obese patients, we observed an increased expression of prolactin at mRNA and protein levels and also at plasmatic level when obesity was complicated with diabetes. Surprisingly, the higher prolactin expression was observed in Obese Pre Diabetic patients and not in Obese Diabetic patients. This could be explained by the fact that only Obese Diabetic patients received antidiabetic drugs (mainly metformin and insulin) reducing their hyperglycemia. As demonstrated in this paper, hyperglycemia, per se, is inducing prolactin expression by the macrophages. We can hypothesize that antidiabetic drugs given to the Obese Diabetic patients will normalize glycemia and then naturally reduce prolactin expression by the macrophages. This observation is reinforcing the additive effect of hyperglycemia and inflammation on prolactin expression by the macrophages observed in isolated ATM and THP-1.

In conclusion, our findings provide new insight into the potential involvement of the macrophages in obesity, diabetes and diabesity. We demonstrated that macrophages, stimulated by high glucose and/or inflammation, are synthesizing prolactin that participates in the increased prolactinemia observed in obese patients. The additive effect of high glucose and inflammation on the prolactin expression is of great interest for the treatment of obese diabetic patients. Combining antidiabetic medications with prolactin-reducing therapies could provide a new approach for obesity treatment.

Author contribution statement

T.B. and G.S. conceived and organized the study, performed the experiments, organized the data, realized statistical analysis, wrote and discussed the manuscript. S.A. performed the experiments and analyzed the data. A.V., B.S., L.S., P.F., G.C.G., J.K.C. and F.P. reviewed the manuscript.

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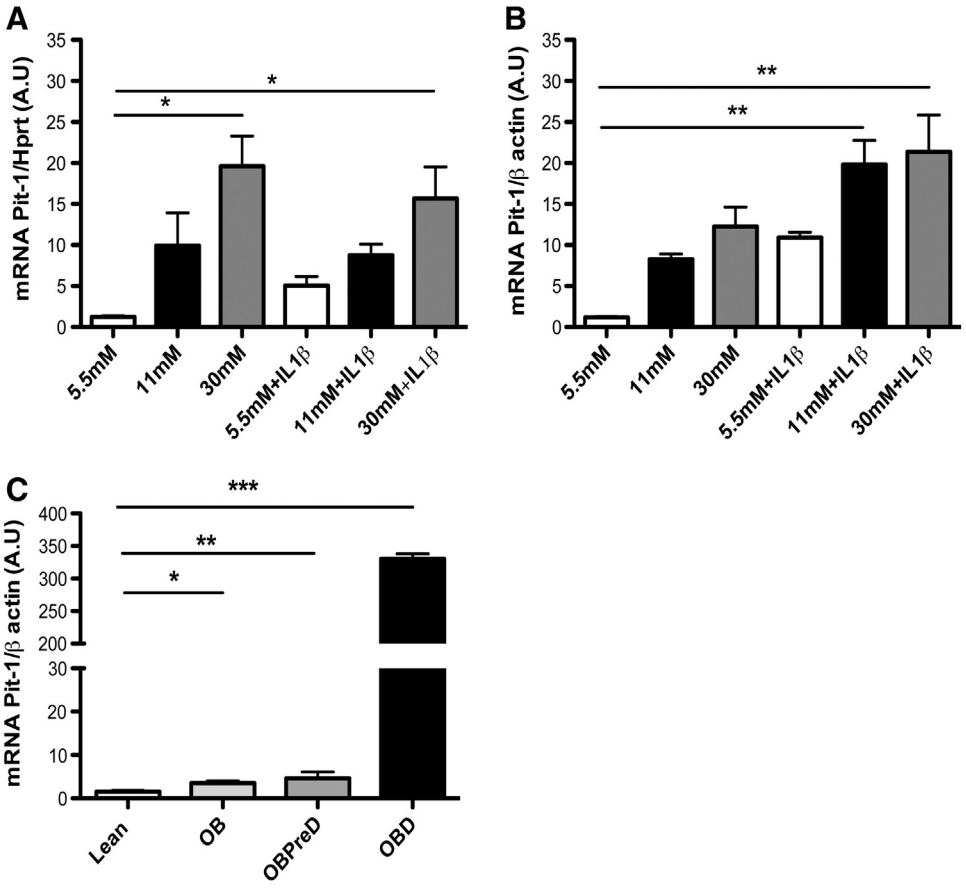

Fig. 8. Pit-1 expression in rat and human macrophages treated with different glucose concentrations supplemented or not with IL1β and in adipose tissue of obese patients. qPCR analysis of Pit-1 mRNA expression demonstrated an increased expression after high glucose treatment for 24 h in rat macrophages (A) and human monocyte/macrophage cell line THP-1 (B). $^{*}p < 0.05$; $^{**}p < 0.01$. As previously observed for PRL expression, an additive effect of IL1β for THP-1 (B)–(C) qPCR analysis of Pit-1 expression by the visceral adipose tissue of lean and obese patients with different glycemic status (Obese (OB), Obese Pre Diabetic (OBPreD); Obese Diabetic (OBD)). Pit-1 expression is significantly increased in all categories of obese patients compared to lean $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Results are means ± sem of 5–6 independent experiments ($n = 6$ for patient adipose tissue and $n = 5$ for rat macrophages and THP-1), ANOVA with Bonferroni correction.

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