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Proadipogenic effects of lactoferrin in human subcutaneous and visceral preadipocytes☆

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Abstract

Lactoferrin has been associated with insulin sensitivity in vivo and in vitro studies. We aimed to test the effects of lactoferrin on human subcutaneous and visceral preadipocytes. Human subcutaneous and visceral preadipocytes were cultured with increasing lactoferrin (hLf, 0.1, 1, 10 μM) under differentiation conditions. The effects of lactoferrin on adipogenesis were studied through the expression of different adipogenic and inflammatory markers, AMPK activation and Retinoblastoma 1 (RB1) activity. The response to insulin was evaluated through ^{Ser473}AKT phosphorylation. In both subcutaneous and visceral preadipocytes, lactoferrin (1 and 10 μM) increased adipogenic gene expressions and protein levels (fatty acid synthase, PPARγ, FABP4, ADIPOQ, ACC and STAMP2) and decreased inflammatory markers (IL8, IL6 and MCP1) dose-dependently in parallel to increased insulin-induced ^{Ser473}AKT phosphorylation. In addition to these adipogenic effects, lactoferrin decreased significantly AMPK activity (reducing ^{pThr172}AMPK and ^{pSer79}ACC) and RB1 activity (increasing the $^{pser807/811}$RB1/RB1 ratio). In conclusion, these results suggest that lactoferrin promotes adipogenesis in human adipocytes by enhancing insulin signaling and inhibiting RB1 and AMPK activities.

Keywords: Lactoferrin; Adipogenesis and insulin action

1. Introduction

Adipose tissue is recognized as an endocrine organ that plays an important role in human diseases such as type 2 diabetes and cancer. Adipogenesis can be divided in two stages: adipocyte commitment of multipotent stem cells and terminal differentiation to mature adipocytes. Adipocyte commitment under tissue culture conditions is achieved by cell confluency. Cells under high confluency can be further induced to become mature adipocytes by various adipogenic inducers. Transcriptional pathways important for the later stages of adipogenesis have been extensively studied. In particular, several key transcription factors such as PPARγ and members of the C/EBP protein family play pivotal roles in dictating cascades of transcriptional activation required for establishing the terminally differentiated state [1,2]. The negative effects of inflammatory mediators on adipocyte differentiation has been extensively reported [3,4], leading to concomitant blunting of insulin action.

Lactoferrin is a pleiotropic glycoprotein (80 kDa) and a prominent component of the first line of mammalian host defense, acting on specific lactoferrin receptors that exist in a variety of cells, like monocytes, lymphocytes and adipocytes [5]. Its expression is up-regulated in response to inflammatory stimuli [5]. Lactoferrin is able to bind and buffer several pathogen-associated molecular patterns such as lipopolysaccharide, viral components and soluble components of the extracellular matrix [6]. This ability is associated with the putative lactoferrin anti-inflammatory activity, as demonstrated in several studies [7-10]. Lactoferrin also participates in the regulation of cellular growth and differentiation [11,12]. In primary osteoblasts, lactoferrin stimulated proliferation and differentiation and acted as a survival factor, activating PI3 kinase-dependent AKT signaling, inhibiting apoptosis induced by serum withdrawal and inhibiting osteoclastogenesis in a murine bone marrow culture [13]. Recently, we have reported that lactoferrin is associated with insulin sensitivity in vivo in humans and in vitro (increasing ^{473Ser}AKT phosphorylation) in HepG2 and 3T3-L1 cell lines. We also showed that lactoferrin inhibited adipogenesis in 3T3-L1 cell line through the increase of AMPK and Retinoblastoma 1 (RB1) activity [14,15].

Lactoferrin is found in considerable concentrations in breast milk (1 mg/ml) and in colostrum (7 mg/ml). LF is a natural component of breast milk which is ingested by infants. Lactoferrin administration in

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mouse and human reduced fasting triglycerides, glucose and visceral obesity [16-20]. This in vitro study on human preadipocytes has been designed to gain insight in the mechanism that underlies these lactoferrin nutritional effects on metabolism. Here, we aimed to investigate the possible effects of lactoferrin on adipogenesis in human preadipocytes. We use both visceral and subcutaneous preadipocytes to explore extensively the role of lactoferrin in preadipocyte differentiation.

2. Methods and materials

2.1. Differentiation of human subcutaneous and visceral preadipocytes

Isolated subcutaneous and visceral preadipocytes from lean (BMI <25) subjects (Zen-Bio Inc., Research Triangle Park, NC) were plated on T-75 cell culture flasks and cultured at 37°C and 5% CO_2 in DMEM/Nutrient Mix F-12 medium (1:1, v/v) supplemented with 10 U/ml P/S, fetal bovine serum (FBS) 10%, HEPES 1% and glutamine 1% (all from GIBCO, Invitrogen S.A., Barcelona, Spain). One week later the isolated and expanded human visceral and subcutaneous preadipocytes were cultured (~40,000 cells/cm^2) in 12-well plates with preadipocytes medium (PM, Zen-Bio Inc.) composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, penicillin and streptomycin in a humidified 37°C incubator with 5% CO_2. Twenty-four hours after plating, cells were checked for complete confluence (Day 0) and differentiation was induced using differentiation medium (DM, Zen-Bio Inc.) composed of PM, human insulin, dexamethasone (DXM), isobutylmethylxanthine and PPARγ agonists (rosiglitazone). After 7 days (Day 7), DM was replaced with fresh adipocyte medium (AM, Zen-Bio Inc.) composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, biotin, panthothenate, human insulin, DXM, penicillin, streptomycin and amphotericin. Negative control (nondifferentiated cell) was performed with preadipocyte medium during all differentiation process. Fourteen days after the initiation of differentiation, cells appeared rounded with large lipid droplets apparent in the cytoplasm. Cells were then considered mature adipocytes, harvested and stored at −80°C for RNA extraction. Nondifferentiated control was performed using proliferation medium during 14 days. Lactoferrin (Sigma, Barcelona, Spain) (0.1, 1 and 10 μM) co-incubations with differentiation medium and lactoferrin (10 μM) with proliferation medium were performed during 14 days. The experiment was performed in triplicate for each sample. We performed four experiments; in each experiment all the treatments have been performed in three independent replicates. First experiment was designed to collect total RNA to evaluate mRNA levels (in RT-PCR each sample run in triplicate). Second experiment was designed to collect total protein to perform Western and enzyme-linked immunosorbent assay (ELISA) determinations. Third experiment was designed to study insulin-induced ^{pSer473}AKT. Finally, fourth experiment was designed to evaluate the cytotoxicity and cell viability.

The differentiation was monitored with the fatty acid synthase (FASN), acetyl-coenzyme A carboxylase alpha (*ACC*), peroxisome proliferator-activated receptor gamma (*PPARγ*), adiponectin (*ADIPOQ*), fatty acid binding protein 4, adipocyte (*FABP4*), six-transmembrane protein of prostate 2 (*STAMP2*), interleukin 6 (*IL6*), interleukin 8 (*IL8*), and monocyte chemoattractant protein-1 (*MCP1*) expression.

2.2. RNA expression

To study gene expressions, RNA was prepared from these samples using RNeasy Lipid Tissue Mini Kit (QIAgen, Izasa SA, Barcelona, Spain). The integrity of each RNA sample was checked by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was quantified by means of a spectrophotometer (GeneQuant, GE Health Care, Piscataway, NJ) reverse transcribed to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems Inc., Madrid, Spain) according to the manufacturer's protocol.

Gene expression was assessed by real-time PCR using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc.), using TaqMan technology suitable for relative genetic expression quantification.

The commercially available and prevalidated TaqMan primer/probe sets used were as follows: endogenous control *PPIA* (4333763, cyclophilin A) and target genes fatty acid synthase (*FASN*, Hs00188012_m1), acetyl-coenzyme A carboxylase alpha (*ACC*, Hs00167385_m1) peroxisome proliferator-activated receptor gamma (*PPARγ*, Hs00234592_m1), fatty acid binding protein 4, adipocyte (*FABP4*, Hs00609791_m1), adiponectin (*ADIPOQ*, Hs00605917_m1), interleukin 6 (*IL6*, Hs00985639_m1), monocyte chemoattractant protein-1 (*MCP1*, Hs00234140_m1), interleukin 8 (*IL8*, Hs00174103_m1) and six-transmembrane protein of prostate 2 (*STAMP2* or *STEAP4*, Hs00226415_m1), all obtained from Applied Biosystems Inc. The RT-PCR TaqMan reaction was performed in a final volume of 25 μl. The cycle program consisted of an initial denaturing of 10 min at 95°C, then 40 cycles of 15 s denaturizing phase at 95°C and 1 min annealing and extension phase at 60°C. A threshold cycle (Ct value) was obtained for each amplification curve and a ΔCt value was first calculated by subtracting the Ct value for human *Cyclophilin A* (*PPIA*) RNA from the Ct value for each sample. Fold changes compared with the endogenous control were then determined by calculating $2^{-\Delta Ct}$, so gene expression results are expressed as expression ratio relative to *PPIA* gene expression according to manufacturers' guidelines.

2.3. Western blot analysis

Subcutaneous and visceral adipocyte protein lysates were washed in ice-cold PBS followed by homogenization assay using RIPA lysis buffer (Millipore, Madrid, Spain) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Madrid, Spain) at 4°C for 30 min. Cellular debris were eliminated by centrifugation of the diluted samples at 14,000×*g* for 30 min (4°C). Protein concentration was determined using Lowry assay. RIPA protein extracts (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes by conventional procedures. Membranes were immunoblotted with $^{pSer807/811}$RB1 (Cell Signaling Technology, Isaza S.A., Barcelona, Spain), AMPK, FASN, RB1 and anti-β-actin antibodies (Santa Cruz Biotechnology, Quimigen S.L., Madrid, Spain). Antirabbit IgG and antimouse IgG coupled to horseradish peroxidase (HRP) was used as secondary antibody. HRP activity was detected by chemiluminescence, and quantification of protein expression was performed using scion image software.

2.4. RB1 activity

RB1 activity is inversely associated with the degree of RB1 phosphorylation in Ser 807/811 [21,22]. $^{pSer807/811}$RB1 was normalized with total RB1. The relative quantity of $^{pSer807/811}$RB1 and total RB1 was measured using Western blot analysis.

2.5. AMPK and ACC activity

Activation of AMPK requires phosphorylation of threonine 172 (Thr-172) within the catalytic subunit. Phosphorylation of Thr172 produces at least 100-fold activation, so that it is quantitatively much more important than the allosteric activation. As a consequence, increase ^{pSer79}ACC leads to decreased ACC activity and blunted activation of the lipogenic pathway [23].

AMPK activity was determined measuring ^{pThr172}AMPK by ELISA (KHO0651, Invitrogen). ^{pThr172}AMPK is directly associated with AMPK activity. According to the manufacturer, the analytical sensitivity of this assay is <1 U/ml of ^{pThr172}AMPK. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. The average recovery was 90%. The specificity of this assay for ^{pThr172}AMPK was confirmed by peptide competition. Intra- and interassay coefficients of variation for all these determinations were between 5% and 10%.

ACC activity was calculated measuring ^{pSer79}ACC and ACC (total) by ELISA (KHO1061 and KHO1071, respectively, Invitrogen). ^{pSer79}ACC is inversely associated with ACC activity. The analytical sensitivity of these assays is <0.5 U/ml of human ^{pSer79}ACC and human ACC, respectively. These were determined by adding two standard deviations to the mean O.D. obtained when the zero standards was assayed 30 times. The percent recovery was calculated as an average of 93% and 97%, respectively. The specificity of this assay for ^{pSer79}ACC was confirmed by peptide competition. Intra- and interassay coefficients of variation for all these determinations were between 5% and 10%.

2.6. ^{Ser473}AKT phosphorylation

To evaluate the effects of lactoferrin on insulin signaling during preadipocyte differentiation, ^{Ser473}AKT phosphorylation under insulin (100 nM) for 10 min and without insulin stimulus at the end of preadipocyte differentiation (Day 14) was investigated. Acute insulin stimulation of AKT phosphorylation has been extensively used to measure insulin action on in vitro experiments [24]. There are some evidences that lactoferrin increase AKT phosphorylation (activity). We study the effects of lactoferrin on AKT activity with and without acute insulin stimulation, measuring ^{pSer473}AKT and $_{total}$AKT to normalize the phosphorylation. ^{pSer473}AKT and $_{total}$AKT were measured using PathScan Phospho-AKT (Ser473) and Total AKT Sandwich ELISA (7160 and 7170, Cell Signaling Technology, Isaza S.A.). The PathScan Phospho-AKT (Ser473) and Total AKT Sandwich ELISA Kit are two solid-phase sandwich ELISAs that detects endogenous levels of ^{pSer473}AKT and $_{total}$AKT protein. ^{pSer473}AKT and $_{total}$AKT antibody have been coated onto the microwells. After incubation with cell lysates, ^{pSer473}AKT and $_{total}$AKT are captured by the coated antibody. Following extensive washing, AKT mouse monoclonal antibody is added to detect the captured protein in each ELISA. HRP-linked antimouse antibody is then used to recognize the bound detection antibody. HRP substrate, TMB, is added to develop color. The magnitude of optical density for this developed color is proportional to the quantity of ^{pSer473}AKT and $_{total}$AKT protein. Specificity and sensitivity were performed by the manufacturers using Western blot analysis. Intra- and interassay coefficients of variation for all these determinations were between 5% and 10%.

2.7. Glucose disappearance rate

Media glucose concentrations were measured in duplicate by the glucose oxidase method using a Beckman glucose analyzer II (Beckman Instruments, Brea, CA).

The glucose disappearance rate per cell was calculated as follows: [(baseline glucose concentration−final glucose concentration)/baseline glucose concentration]×100, divided by the no. of viable cells in each treatment.

2.8. Cell counts and LDH activity assay

Cell counts were assessed by trypan blue dye exclusion using a Neubauer hemacytometer, after 14 days differentiation of human subcutaneous preadipocytes, in triplicate.

To evaluate cell integrity, LDH activity released from damaged cells was analyzed by Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

2.9. Statistical analyses

Statistical analyses were performed using SPSS 12.0 software. Unless otherwise stated, descriptive results of continuous variables are expressed as mean and SD for Gaussian variables. Paired and unpaired *t* tests were used to evaluate the effects of lactoferrin treatments. Each lactoferrin treatment was compared individually with vehicle treatment. The experiments were performed in triplicate.

3. Results

3.1. Subcutaneous and visceral preadipocyte differentiation

As expected, adipogenic gene expression (*FASN*, *ACC*, *ADIPOQ*, *PPARγ*, *FABP4*, *STAMP2*) increased in parallel to development of lipid droplets while *IL6* and *MCP1* gene expression, and AMPK and RB activity decreased during differentiation in both subcutaneous and visceral preadipocytes (Supplemental online Figures 1 and 2).

We found several differences when comparing subcutaneous and visceral preadipocytes. The increases in adipogenic gene expression (*FASN*, *ACC*, *ADIPOQ*, *PPARγ*, *FABP4*, *STAMP2*), protein (*FASN* and *ACC*) and lipid droplets, and the decrease in *IL6* gene expression were more pronounced in subcutaneous adipocytes. Furthermore, the decreases of RB1 activity (increasing $^{pSer807/811}$RB1/total RB1), ^{pThr172}AMPK/total AMPK and ^{pSer79}ACC/total ACC were significantly more pronounced in subcutaneous adipocytes (Fig. 1). Insulin-induced ^{pSer473}AKT and the glucose disappearance rate were significantly greater in subcutaneous differentiated adipocytes in comparison with visceral adipocytes (Supplemental online Figures 1 and 2).

3.2. Human lactoferrin effects on human preadipocytes during differentiation

3.2.1. Effects on adipogenic and inflammatory gene expression

In both visceral and subcutaneous fat depots, human lactoferrin (hLf, 1 and 10 μM) increased adipogenic gene expression and protein dose-dependently in comparison with control differentiated adipocytes (Fig. 1, 2 and supplemental online Figure 3). In both visceral and subcutaneous adipocytes, hLf (10 μM) decreased significantly the expression of inflammatory genes (*IL6, IL8* and *MCP1*) in comparison with control differentiated adipocytes (Fig. 1).

hLf (1 and 10 μM) in subcutaneous and hLf (10 μM) in visceral preadipocytes increased significantly *STAMP2* gene expression in comparison with control differentiated adipocytes (Fig. 1).

3.2.2. Effects on AMPK and RB1 activity

In parallel with the increase in lipogenic genes, AMPK activity (^{pThr172}AMPK and ^{pSer79}ACC) decreased significantly after lactoferrin (1 and 10 μM) (Fig. 3). In both visceral and subcutaneous adipocytes, human lactoferrin (hLf, 1 and 10 μM) decreased RB1 activity (increasing $^{pSer807/811}$RB1/total RB1) dose-dependently in comparison with control differentiated adipocytes (Fig. 3). RB1 phosphorylation increased in both visceral and subcutaneous adipocytes. Total RB1 levels decreased significantly only in subcutaneous adipocytes.

3.2.3. Effects on insulin-induced ^{Ser473}Akt phosphorylation

In both visceral and subcutaneous fat depots, human lactoferrin (hLf, 1 and 10 μM) increased insulin-induced ^{Ser473}AKT phosphorylation dose-dependently (Fig. 4). The glucose disappearance rate was more pronounced after hLf (10 μM) in comparison with control adipocytes (Fig. 4).

3.3. Effects in nondifferentiated preadipocytes

To investigate the independent lactoferrin effects on adipogenesis, we tested hLf (10μM) in nondifferentiated subcutaneous preadipocytes, using proliferation medium for 14 days. hLf (10 μM) increased significantly lipogenic protein (FASN and ACC) and decreased significantly AMPK and RB1 activity. hLf (10μM) enhanced insulin-induced ^{pSer473}AKT and increased the glucose disappearance rate (Fig. 5).

Lactoferrin (hLf) did not show any effects on cell viability and cytotoxicity. The number of viable cells and LDH activity were similar under all treatments (Fig. 6).

4. Discussion

To the best of our knowledge, this is the first study investigating lactoferrin effects on human preadipocytes. These results suggest that lactoferrin promotes adipogenesis by enhancing insulin signaling (increasing insulin-induced ^{pSer473}AKT) and inhibiting RB1 and AMPK activities. Stimulation of AKT activity was in parallel to adipogenic gene expression and enhanced preadipocyte differentiation [25,26]. Thus, the insulin sensitizing effects of lactoferrin might explain the adipogenic effects in human preadipocytes. In fact, a significant reduction of AMPK and RB1 activities is well known during adipocyte differentiation [27-29].

The differences on adipocyte differentiation between visceral and subcutaneous preadipocyte have been extensively reported [30-32]. Subcutaneous preadipocytes are more insulin sensitive and lipogenic but less lipolitic and proinflammatory than visceral preadipocytes. Here, we corroborated these differences (Supplemental online Figures 1 and 2). Proadipogenic effects of lactoferrin were similar in both subcutaneous and visceral preadipocytes.

We had previously reported that lactoferrin treatment increased insulin action in HepG2 and 3T3-L1 cell lines. However, lactoferrin dose-dependently down-regulated 3T3-L1 differentiation, decreasing adipogenic gene expression and the development of lipid droplets, enhancing AMPK and RB1 activities [14]. Antitumoral activity of lactoferrin enhancing RB1 activity has been previously reported [33]. Lactoferrin antiadipogenic effects in 3T3-L1 cell line might be mediated by the induction of cell cycle arrest, blunting clonal expansion, the first step in the differentiation process. Previous studies under similar experimental conditions in mice myoblast cell line, C2C12, and in mice preadipocyte cell line, MC3T3-G2/PA6, showed that lactoferrin decreased adipogenesis [34,35]. These divergent effects in human preadipocytes and 3T3-L1 cell line have previously been described for other factors. For instance, Tomlinson et al. showed that exposure of human primary preadipocytes to glucocorticoids increased their sensitivity to insulin (improving insulin-mediated activation of AKT) and enhanced their subsequent response to stimuli that promote adipogenesis. This effect was observed in primary human preadipocytes but not in immortalized 3T3-L1 murine preadipocytes [36].

Anti-inflammatory effects of lactoferrin has been extensively reported in several studies [7,10,15]. In parallel with these adipogenic effects, lactoferrin displayed anti-inflammatory activity in adipocytes, reducing *IL8* and *MCP1* gene expression on visceral adipocytes and *IL6* gene expression in both visceral and subcutaneous adipocytes.

Wellen and collaborators have shown that STAMP2 (a transmembrane protein) had an important role in adipocyte physiology [37].

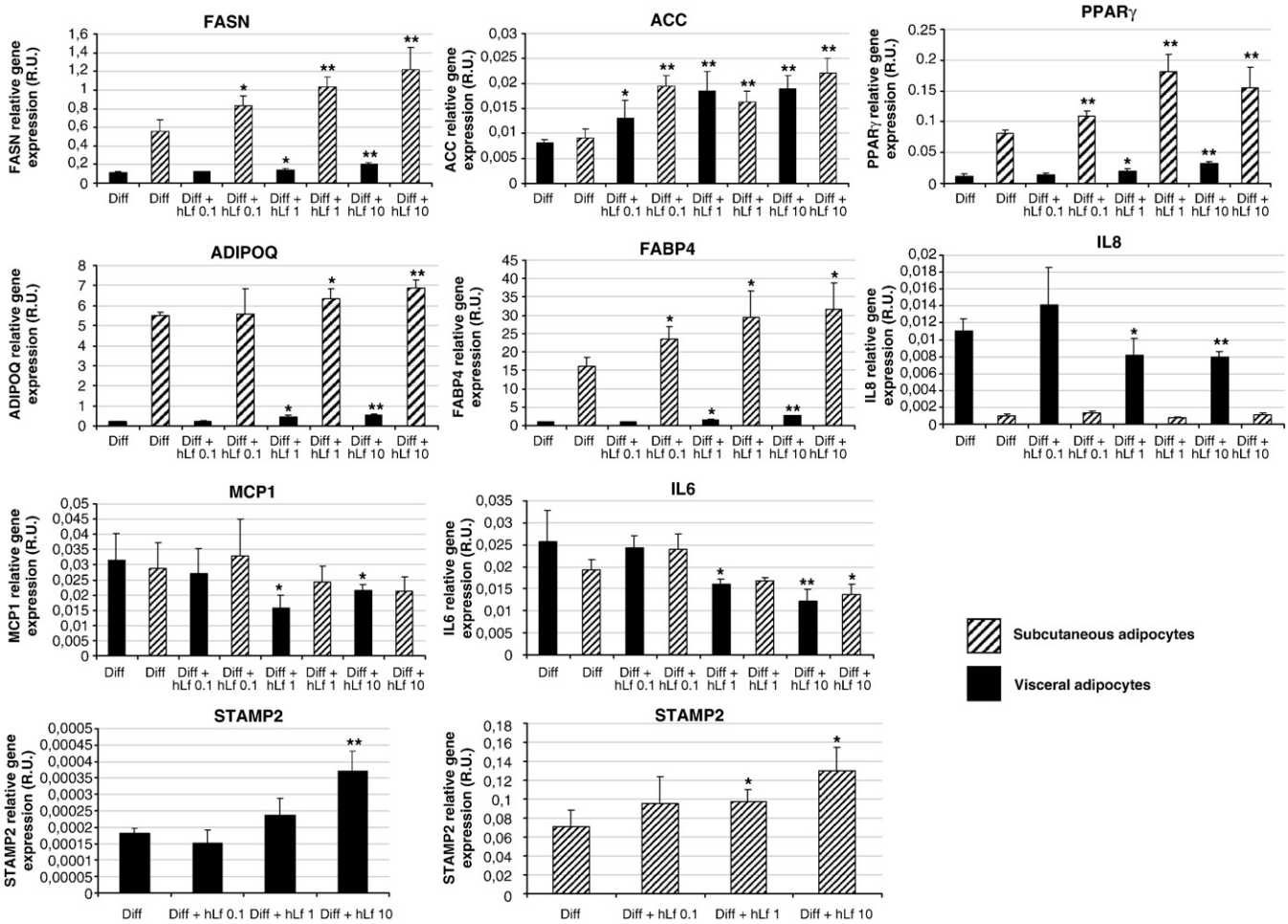

Fig. 1. Lactoferrin (hLf, 0.1, 1, 10 μM) effects on *FASN*, *ACC*, *PPARγ*, *ADIPOQ*, *FABP4*, *STAMP2*, *IL8*, *IL6* and *MCP1* gene expression on both subcutaneous and visceral preadipocytes during differentiation. *$P<.05$ and **$P<.005$ vs. control adipocytes. Paired and unpaired *t* tests were used to evaluate the effects of each lactoferrin treatment.

STAMP2 gene KO showed a deterioration of insulin sensitivity and adipogenesis, and increased nutrient-induced inflammatory responses [37]. We, here, confirmed that *STAMP2* gene expression increased during differentiation of adipocytes. Another recent study confirmed that STAMP2 has an important role mediating insulin sensitivity in adipocytes [38]. Lactoferrin treatment increased significantly *STAMP2* gene expression in comparison with differentiated control adipocytes. We might speculate that lactoferrin effects on *STAMP2* gene expression could be behind its adipogenic and insulin sensitizing effects.

In summary, lactoferrin might mediate an important role in the control of obesity-induced metabolic disturbances, impacting on adipose tissue physiology. The biological relevance of the results could explain the beneficial effects of lactoferrin observed in vivo, as the reduction of triglycerides, glucose and visceral obesity [17,18,20]. In fact, increased lactoferrin concentration has been inversely associated with insulin resistance and obesity [15].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.09.015.

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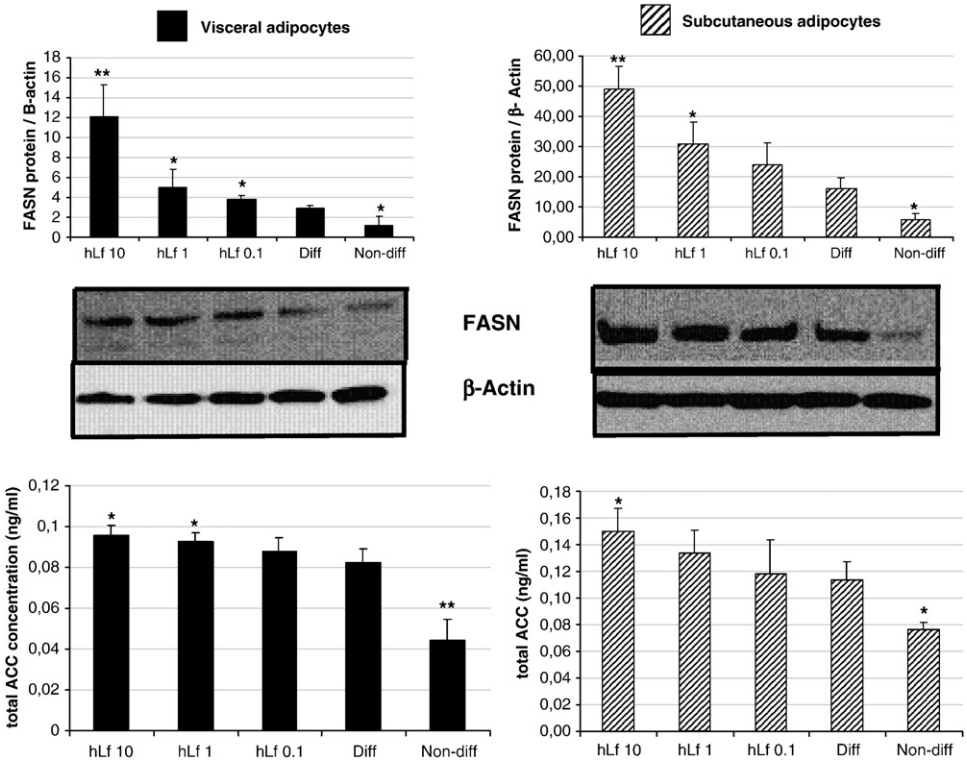

Fig. 2. Lactoferrin (hLf, 0.1, 1, 10 μM) effects on FASN and total ACC (measured by ELISA) protein on both subcutaneous and visceral preadipocytes. *P<.05 and **P<.005 vs. control adipocytes. Paired and unpaired t tests were used to evaluate the effects of each lactoferrin treatment.

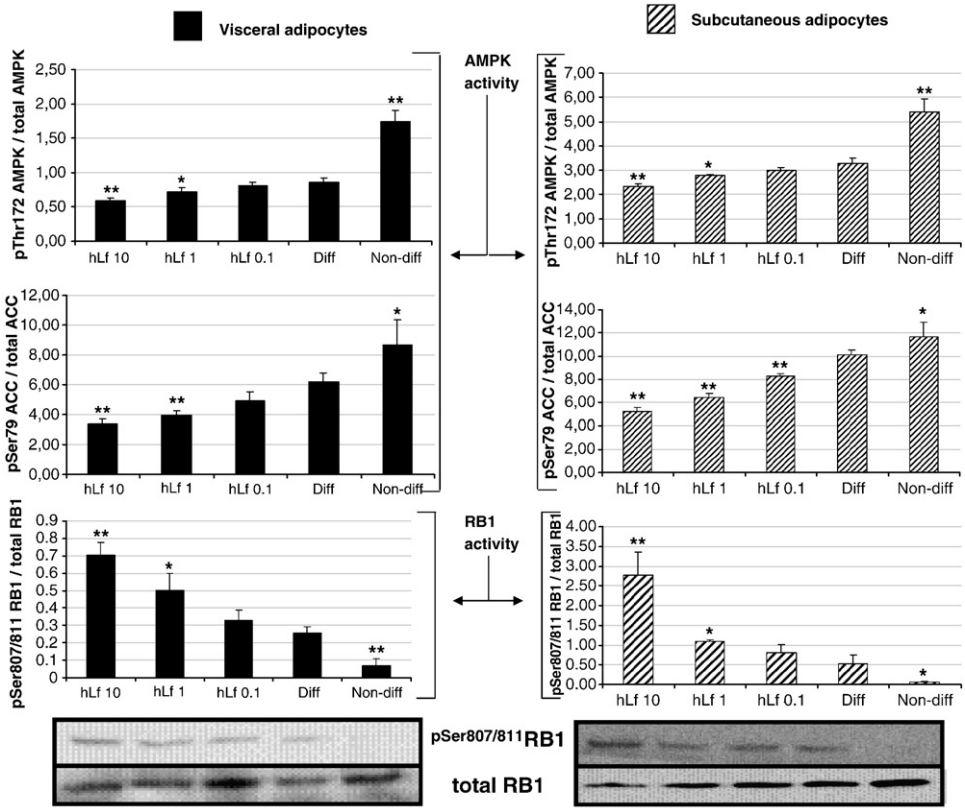

Fig. 3. Lactoferrin (hLf, 0.1, 1, 10 μM) effects on ^{p172Thr}AMPK/total AMPK, ^{p79Ser}ACC/total ACC and $^{pSer807/811}$RB1/total RB1 in subcutaneous and visceral preadipocytes. *P<.05 and **P<.005 vs. control adipocytes. Paired and unpaired t tests were used to evaluate the effects of each lactoferrin treatment.

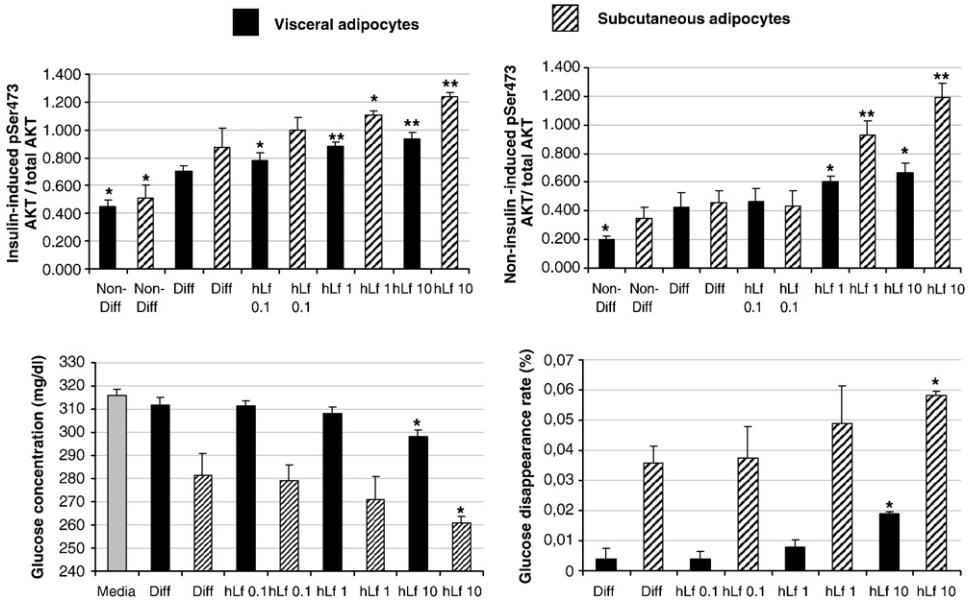

Fig. 4. Lactoferrin (hLf, 0.1, 1, 10 μM) effects on insulin and non-insulin-induced ^{pSer473}AKT/total AKT and glucose disappearance rate and absolute glucose concentration in subcutaneous and visceral preadipocytes. *P<.05 and **P<.005 vs. control adipocytes. Paired and unpaired t tests were used to evaluate the effects of each lactoferrin treatment.

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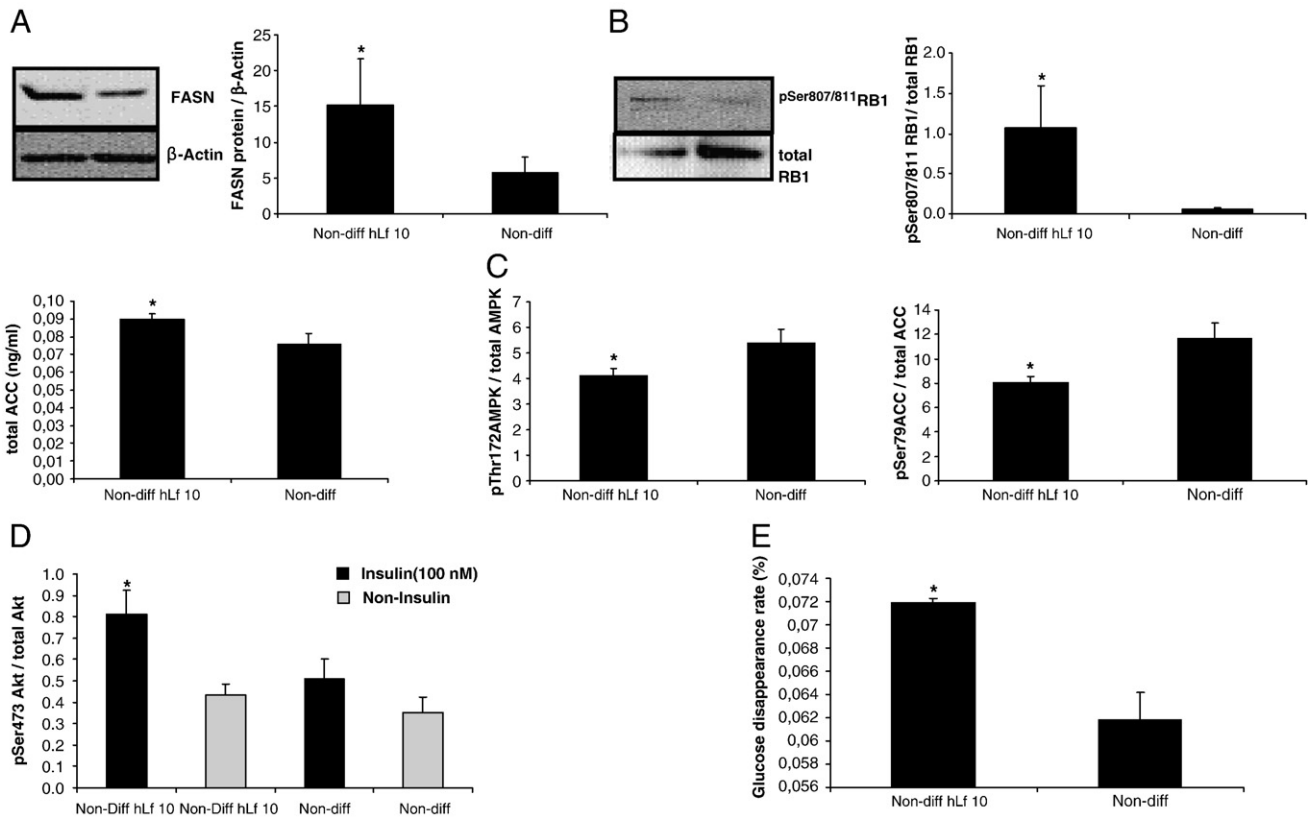

Fig. 5. Lactoferrin (hLf, 10 μM) effects on (A) FASN and total ACC level protein. (B) RB1 activity ($^{pSer807/811}$RB1/total RB1). (C) AMPK activity (^{p172Thr}AMPK/total AMPK, ^{p79Ser}ACC/totalACC). (D) Non-insulin- and insulin-induced ^{Ser473}AKT phosphorylation. (E) Glucose disappearance rate. Lactoferrin (hLf, 10 μM) effects on preadipocytes were performed with proliferation medium in parallel to differentiation process (during fourteen days). *P<.05 vs. control preadipocytes. Paired and unpaired t tests were used to evaluate the effects of each lactoferrin treatment.

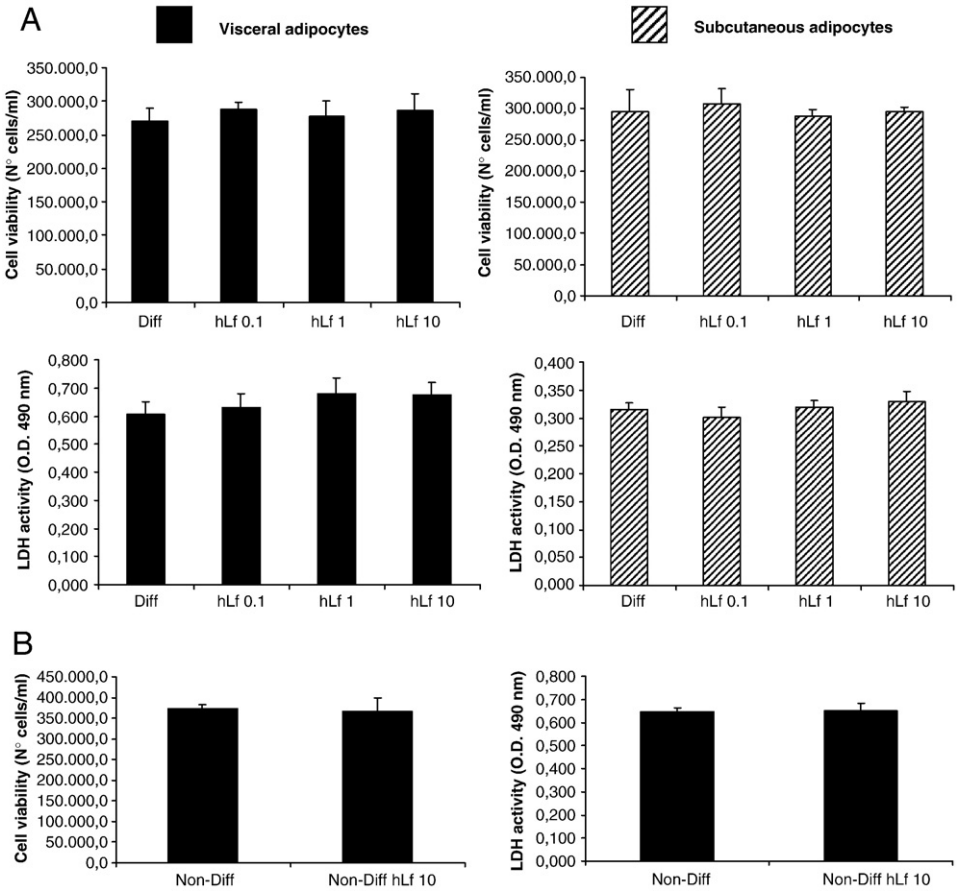

Fig. 6. Lactoferrin (hLf, 0.1, 1, 10 μM) effects on cell viability and LDH activity in both subcutaneous and visceral preadipocytes under differentiation and nondifferentiation conditions. Paired and unpaired *t* tests were used to evaluate the effects of each lactoferrin treatment.

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