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Original Paper

Effect of TNF-Alpha on Caveolin-1 Expression and Insulin Signaling During Adipocyte Differentiation and in Mature Adipocytes

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Key Words
Adipogenesis • Inflammation • Insulin receptor • Insulin-stimulated glucose uptake • DNA methylation • 3T3-L1

Abstract
Background/Aims: Tumor necrosis factor-α (TNF-α)-mediated chronic low-grade inflammation of adipose tissue is associated with obesity and insulin resistance. Caveolin-1 (Cav-1) is the central component of adipocyte caveolae and has an essential role in the regulation of insulin signaling. The effects of TNF-α on Cav-1 expression and insulin signaling during adipocyte differentiation and in mature adipocytes were studied. ***Methods:*** 3T3-L1 cells were differentiated (21 days) in the presence TNF-α (10 ng/mL) and mature adipocytes were also treated with TNF-α for 48 hours. Cav-1 and insulin receptor (IR) gene methylation were determined as well as Cav-1, IR, PKB/AKT-2 and Glut-4 expression and activation by real time RT-PCR and western blot. Baseline and insulin-induced glucose uptake was measured by the 2-[C^{14}]-deoxyglucose uptake assay. ***Results:*** TNF-α slowed down the differentiation program, hindering the expression of some insulin signaling intermediates without fully eliminating insulin-mediated glucose uptake. In mature adipocytes, TNF-α did not compromise lipid-storage capacity, but downregulated the expression of the insulin signaling intermediates, totally blocking insulin-mediated glucose uptake. Insulin sensitivity correlated with the level of activated phospho-Cav-1 in both situations, strongly suggesting the direct contribution of Cav-1 to the maintenance of this physiological response. ***Conclusion:*** Cav-1 activation by phosphorylation seems to be essential for the maintenance of an active and insulin-sensitive glucose uptake.

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Introduction

Hypertrophied and dysfunctional adipocytes from obese subjects lose their ability to respond to prolonged overnutrition and induce the secretion of several proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) or interleukin 6 (IL-6). These cytokines promote the recruitment of immune cells to adipose tissue, contributing to initiate a local and systemic inflammatory state [1]. This obesity-related chronic low-grade inflammation is closely associated with the onset and development of a cluster of abnormalities such as insulin resistance and type 2 diabetes, hyperinsulinemia, dyslipidemia or vascular abnormalities.

The cytokine TNF-α is synthesized by adipocytes and adipose tissue-infiltrated macrophages in obesity, acting as an endocrine and paracrine/autocrine mediator through interaction with type I and type II TNF-α receptors (TNFRI and II), both expressed in fat cells [2]. In this regard, it is well known that TNF-α positively contributes to maintain the proinflammatory adipocyte microenvironment, mainly by the activation of nuclear factor-κB (NFκB) signaling [3]. This mechanism causes significant changes in gene expression, suppressing adipocyte-specific genes and therefore affecting their functionality [4]. TNF-α also directly inhibits adipocyte differentiation from precursor cells [5], decreasing the expression of transcription factors essential for adipogenesis, such as nuclear peroxisome proliferator-activated receptor (PPARγ) and CCAAT/enhancer-binding protein α and δ (CEBPα & δ) among others [6]. Furthermore, TNF-α is also able to enhance Wnt/β-catenin and lipolysis-related signaling pathways [7-9], which contributes to its anti-adipogenic effect. In summary, these mechanisms triggered by TNF-α lead to an increase in circulating free fatty acid (FFAs) levels, exacerbating peripheral lipotoxicity, aggravating systemic inflammation and promoting the onset of insulin resistance and other associated disorders [10, 11].

In regard to this, TNF-α suppresses the tyrosine kinase activity of the insulin receptor (IR) in mature adipocytes, inhibiting its autophosphorylation and subsequently the activation of the downstream effector, insulin receptor substrate 1 (IRS-1). TNF-α also reduces insulin signaling by inducing the inactivating serine phosphorylation of IRS-1 [12] by the JNK-IKKβ axis [13]. Moreover, TNF-α destabilizes the interaction between IR and caveolin-1 (Cav-1) [14, 15], an integral membrane protein that enhances insulin transduction through direct binding to the beta subunit of IR [16].

Cav-1 is a key regulator of the insulin signaling pathway and is the main structural and functional component of a specific type of lipid raft called caveolae, which are flask-shaped invaginations of the plasma membrane, especially abundant in adipocytes and other highly differentiated cells [17]. Caveolae have been traditionally associated with lipid and cholesterol trafficking, endocytosis/exocytosis cycles, and compartmentalization and integration of signal transduction intermediates [18]. In addition, it has been shown that Cav-1 plays an essential role for a proper insulin response in mature adipocytes, since its depletion causes IR and glucose transporter-4 (Glut-4) degradation [19, 20], although it does not completely block Glut-4 translocation to the plasma membrane [21].

In a previous study, we showed that the induction of Cav-1 gene expression during 3T3-L1 adipogenesis can be associated with hypomethylation of its gene [22]. Since there is evidence of TNF-α regulation of gene expression in preadipocytes and mature adipocytes [21], in this study our aim was to study the influence of the TNF-α proinflammatory stimulus on Cav-1 expression and its relation to the insulin signaling pathway, during adipocyte differentiation and in mature adipocytes. For this purpose we used mouse 3T3-L1 cells differentiated to adipocytes in the presence of TNF-α and 3T3-L1 mature adipocytes treated with this cytokine for 48 hours.

Material and Methods

Cell culture and TNF-α treatment

3T3-L1 preadipocytes (ATCC, Rockville, MD) were seeded in 6-wells plates and grown to the second day postconfluence (d0) in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% calf bovine

serum (CBS) and 1% penicillin/streptomycin at 37°C, 5% CO_2 and 95% humidity. Adipocyte differentiation was induced by incubating the cells with DMEM supplemented with 10% fetal bovine serum (FBS), insulin (1 mg/mL), dexamethasone (1 mM) and 3-isobutyl-1-methylxanthine (IBMX) (0.5 mM) for 48 hours. The medium was then replaced with DMEM containing 10% FBS and insulin (1 mg/mL) for an additional 48 hours. After that point, cells were maintained in DMEM containing 10% FBS with the medium being changed every 48 hours until they were harvested for further analysis.

3T3-L1 cells were treated with recombinant human TNF-α dissolved in phosphate buffered saline solution containing 1 mg/mL bovine serum albumin (T0157, Sigma-Aldrich, St. Louis, MO, USA), at a concentration of 10 ng/mL, following two different experimental designs. In the long-term protocol, cells were chronically exposed to TNF-α during the complete adipocytic differentiation and maturation process, from day 0 to day 7 or 21. TNF-α was included in every media renewal until the cells were harvested. Secondly, in a short-term protocol, 7-day differentiated adipocytes were subjected to an acute treatment with TNF-α by incubation with this cytokine for 48 hours, with the media being replaced after the first 24 hours with fresh media containing TNF-α to ensure acute exposure to the cytokine.

Oil Red O staining

Intracellular triglyceride accumulation was measured by Oil-Red-O staining to estimate the degree of adipocytic differentiation achieved at each checkpoint (days 0, 7 and 21 for the long-term and at days 7 and 9 for the short-term TNF-α treatment). Cells were washed twice with phosphate-buffered saline (PBS) and fixed with formaldehyde 3.7% for 2 hours. After being washed three times with isopropanol 60%, cells were stained with Oil Red O (0.5% in isopropanol) diluted to 40% with water, for 30 minutes at room temperature. Excess stain was removed by washing four times with ethanol 70%. After drying, cells were photographed under a light microscope (Olympus Ck2, 40 X magnifications). The Oil-Red-O stain accumulated in lipid droplets in the cells was dissolved in isopropanol and the solution was spectrophotometrically quantified at 540 nM (Multiskan Spectrum, Thermo Electron Corporation, MA, USA).

Cell viability

Cell viability and proliferation were determined by the MTT (tetrazolium-based) colorimetric assay. For both experimental designs, long-term (d0, d7 and d21) and short-term (d7 and d9) TNF-α treatments, the cells were cultured and differentiated in 96-well plates. After washing the cells twice with PBS, the medium was replaced with a fresh serum free DMEM media containing 1 mg/mL of MTT. After 1 hour in a humidified incubator at 37 °C, 5% CO_2, the medium was removed and the formazan crystals produced were dissolved by adding DMSO (100 µL). The absorbance of each well was read at 540 nm in a spectrophotometer (Multiskan Spectrum, Thermo Electron Corporation). Empty wells were used as a background control.

Methylation analysis of Cav-1 and IR genes

The region of the Cav-1 gene under study encompasses from 619 bp 5' to 1333 bp 3' of the ATG codon and includes two CpG islands (70 CpG sites) located in the proximal promoter, exon 1 and the first intron. On the other hand, the region of the IR gene studied encompasses from 249 bp 5' to 545 bp 3' of the ATG codon, including 40 CpG dinucleotides located in the first exon and the first intron. Both Cav-1 and IR gene sequences have been described in detail in a previous work of our group [22]. Genomic DNA was extracted and purified from 3T3-L1 cells using the QIAamp DNA kit (Qiagen, Hilden, Germany) at checkpoints established for both experimental designs, days 0, 7 and 21 for the long-term and days 7 and 9 for the short-term TNF-α treatment. DNA concentration and quality were measured by Picogreen (Invitrogen). MassArray Epityper technique (Sequenom Inc., San Diego, CA, USA) was performed in the Central Research Unit of the School of Medicine (UCIM) of the University of Valencia (Spain) in order to determine the methylation percentage of each CpG dinucleotide under study, although 9 CpG sites were excluded in the Cav-1 gene for technical reasons. All the measurements were performed in triplicate.

Real Time RT-PCR

In the case of the long-term TNF-α treatment throughout 3T3-L1 adipocyte differentiation, total RNA was extracted from preadipocytes (d0) and adipocytes (d7, d21) using the DNA/RNA/Protein Mini Kit (Qiagen). Total RNA from 3T3-L1 mature adipocytes subjected to the short-term TNF-α treatment (d7, d9) was isolated using TRIzol® reagent (Invitrogen). RNA concentration and quality were determined using a

Nanodrop ND-1000 spectophotometer (NanoDrop Technologies, Wilmington, DE, USA). Each RNA sample was subsequently treated with DNase I (DNA-Free Kit, Ambion, Carlsbad, CA, USA) to remove possible contaminating genomic DNA. Two micrograms of purified total RNA were used for the synthesis of first-strand cDNA using M-MLV reverse transcriptase (Invitrogen) and random primers (Invitrogen), according to the manufacturer's instructions. Predesigned TaqMan® probes (Applied Biosystems, Foster City, CA, USA) were used to perform real-time PCR for caveolin-1 (Cav-1, Mm_00483057_m1), insulin receptor (IR, Mm_01211875_m1), protein kinase B (AKT-2, Mm02026778_g1) and glucose transporter type-4 (Glut-4, Mm00436615_m1). The cDNA was amplified in an ABI Prism 7300 HT Sequence Detection System (Applied Biosystems), using the TaqMan Universal PCR Master Mix (Applied Biosystems) according to standard conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Cyclophilin A (*Ppia*, Mm02342430_g1) was used as invariant internal control for RT-PCR and subsequent normalization. Relative quantification of gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Data from long-term TNF-α treatment were referenced to day 0 whereas data from shot-term TNF-α treatment were referenced to day 7 as the calibrator sample.

Western Blot analysis

In the case of the long-term TNF-α treatment throughout 3T3-L1 adipocyte differentiation, proteins were extracted from 3T3-L1 preadipocytes (d0) and adipocytes (d7, d21) using the DNA/RNA/Protein Mini Kit (Qiagen). Proteins from 3T3-L1 mature adipocytes subjected to the short-term TNF-α treatment (d7, d9) were extracted by cell lysis with a specific protein extraction buffer (glycerol 87%, Tris-HCl 2M, NaCl 5M, Triton 10%, NaF 1M and sodium orthovanadate 0,2M) supplemented with protease inhibitor cocktail, phosphatase inhibitor cocktail and phenylmethanesulfonyl fluoride (Pierce, Rockford, IL, USA) after washing cells with ice-cold PBS. Proteins were purified from the lysate by centrifugation at 13,000 rpm, 4 ºC for 5 minutes. After quantification by the bicinchoninic acid (BCA) assay (Pierce), isolated proteins were subjected to Western Blot analysis performed with primary antibodies specific for Cav-1 (sc-894, Santa Cruz Biotechnology, Dallas, TX, USA), IR (sc-711, Santa Cruz Biotechnology), AKT-2 (# 9272, Cell Signaling, Beverly, MA, USA) and Glut-4 (G4048, Sigma-Aldrich). Total proteins, ranging from 10 to 50 μg depending on the protein assayed, were fractionated by SDS-PAGE and electrotransferred onto a Hybond-C Extra nitrocellulose membrane (Amersham GE-Health, Uppsala, Sweden). After blocking the membrane with 5% milk in 0.05% Tris buffered saline with Tween 20 (TBST) for 2 hours, it was incubated overnight with the suitable primary antibody dilution at 4 ºC. Next, the membrane was washed three times with TBST before being incubated with an appropriate dilution of the horseradish peroxidase (HRP)-conjugated secondary antibody. Finally, after three more washes, specific immunoreactive bands were detected by a chemiluminescent ECL assay kit (Amersham-Pharmacia). Specific secondary HRP-labeled rabbit (170-6515, Bio-Rad, Hercules, CA, USA) or mouse (NXA931, GE Healthcare, Little Chalfont, UK) antibodies were employed depending on the primary antibody origin. β-Actin (A1978, Sigma-Aldrich) was used as an invariant internal control for sample normalization.

Relative protein activation by phosphorylation, before and after stimulating cells with 50 nm insulin for 10 min, was measured using the same methodology. Specific primary anti-phospho protein antibodies for Cav-1 (Tyr14, 611339, BD Transduction Laboratories, Franklin Lakes, NJ, USA), IR (Tyr1146, # 3021, Cell Signalling) and AKT-2 (Ser473, # 9271, Cell Signaling) were used.

2-[C^{14}]-Deoxyglucose uptake

Baseline and insulin-induced glucose uptake was measured by the 2-[C^{14}]-deoxyglucose uptake assay. At first, cells were incubated in serum and glucose-free DMEM for 2 hours in an incubator at 37 ºC, 5% CO_2, 95% humidity. Subsequently, cells were incubated in the presence or absence of insulin (50 nM) for 10 min, before 2-[C^{14}]-deoxyglucose uptake assay was initiated by the addition of serum and glucose-free DMEM containing 2-deoxyglucose (50 μM) and 2-[C^{14}]-deoxyglucose (0.075 μCi /mL, ARC0111, American Radiolabeled, Saint Louis, MO, USA). Cells were incubated for another 10 min and the reaction was terminated by washing three times with pre-cold PBS containing 0.05 M glucose. Next, cells were incubated with lysis buffer (0.1 M NaOH, 0.1 % SDS) at 37 °C for two hours and the cell lysate (100 μL) was transferred to a tube containing 2 mL of scintillation liquid for radioactivity counting using a scintillation counter (Wallac 1214 Rackbeta Conter, Perkin Elmer Life Sciences, Waltham, MA, USA). 2-Deoxyglucose uptake was reported as [C^{14}] radioactivity, normalized to protein content from the remaining cell lysate as determined by BCA

analysis (Pierce). Measurements were performed in triplicate under conditions where hexose uptake was linear.

Secreted adipokines in the supernatant

ELISA kits from Millipore (Billerica, MA, USA) were employed to determine leptin (Cat. # EZML-82K), interleukin 6 (IL-6) (Cat. # EZMIL6) and adiponectin (Cat. # EZMAKP-60K) secreted to the culture media.

Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M). Depending on the number of samples analyzed, statistical significance of differences among the groups was checked by using the ANOVA for a single factor and Dunnett test or applying the Wilcoxon signed rank test. All analyses were performed using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Modulation of adipokine secretion pattern by TNF-alpha

TNF-α treatment had no effect on 3T3-L1 adiponectin secretion whereas it severally inhibited leptin and IL-6 secretion during the initial differentiation process (d7). After 21 days, leptin and IL-6 secretion by the fully mature 3T3-L1 cells was greatly attenuated and the presence of TNF-α did not potentiate this effect (Fig. 1A, C, E).

After 48-h exposure of mature adipocytes to TNF-α, leptin secretion was dramatically reduced while, in contrast, the production of IL-6 was greatly increased. In this case, adiponectin secretion was significantly reduced, although not as much as leptin (Fig. 1B, D, F).

Effect of TNF-α on 3T3-L1 intracellular lipid accumulation and viability

Differentiation of 3T3-L1 preadipocytes to adipocytes was analyzed by measuring intracellular triglycerides accumulation by Oil-red O staining. Long-term exposure to the proinflammatory cytokine TNF-α during the differentiation process induced a clear reduction of stored lipids both at day 7 and at day 21 (Fig. 2A, C), whereas short-term (48-h) TNF-α treatment of 7-day adipocytes slightly increased the amount of triglycerides at the end of the experiment (d9) (Fig. 2B, D). Cell viability was not affected by TNF-α with either treatment (Fig. 2E).

TNF-α induces caveolin-1 gene hypermethylation during adipocyte differentiation

The same experimental designs were performed to evaluate DNA methylation changes in a CpG island located along the promoter, exon 1 and intron 1 of the Cav-1 gene. The sequence analyzed encompasses from -619 bp to +1333 bp from the initiation codon ATG and contains 70 CpG dinucleotides. As shown in Fig. 3, continuous exposure to TNF-α (d21) significantly increased the methylation of Cav-1 gene in 16 out of 70 CpG sites. On the other hand, short-term (48-h) exposure to TNF-α only altered the methylation percentages of three CpG sites (Fig. 4).

Methylation status of a selected region (-249 bp to +545 bp from the initiation codon ATG) of the IR gene, encompassing a sequence which includes a part of its first exon and first intron, was also analyzed after the 21-day treatment with TNF-α. No significant changes were observed and for this reason IR methylation was not analyzed in the short-term (48-h) TNF-α treatment (data not shown).

Effects of TNF-α on caveolin-1 and insulin signaling pathway intermediates expression levels

Cav-1, IR, AKT-2 and Glut-4 mRNA significantly increased throughout the adipogenic differentiation and cell maturation process (Fig. 5A, 7A). In general, the presence of TNF-α during this process did not affect the expression of these genes, except for Cav-1, which was

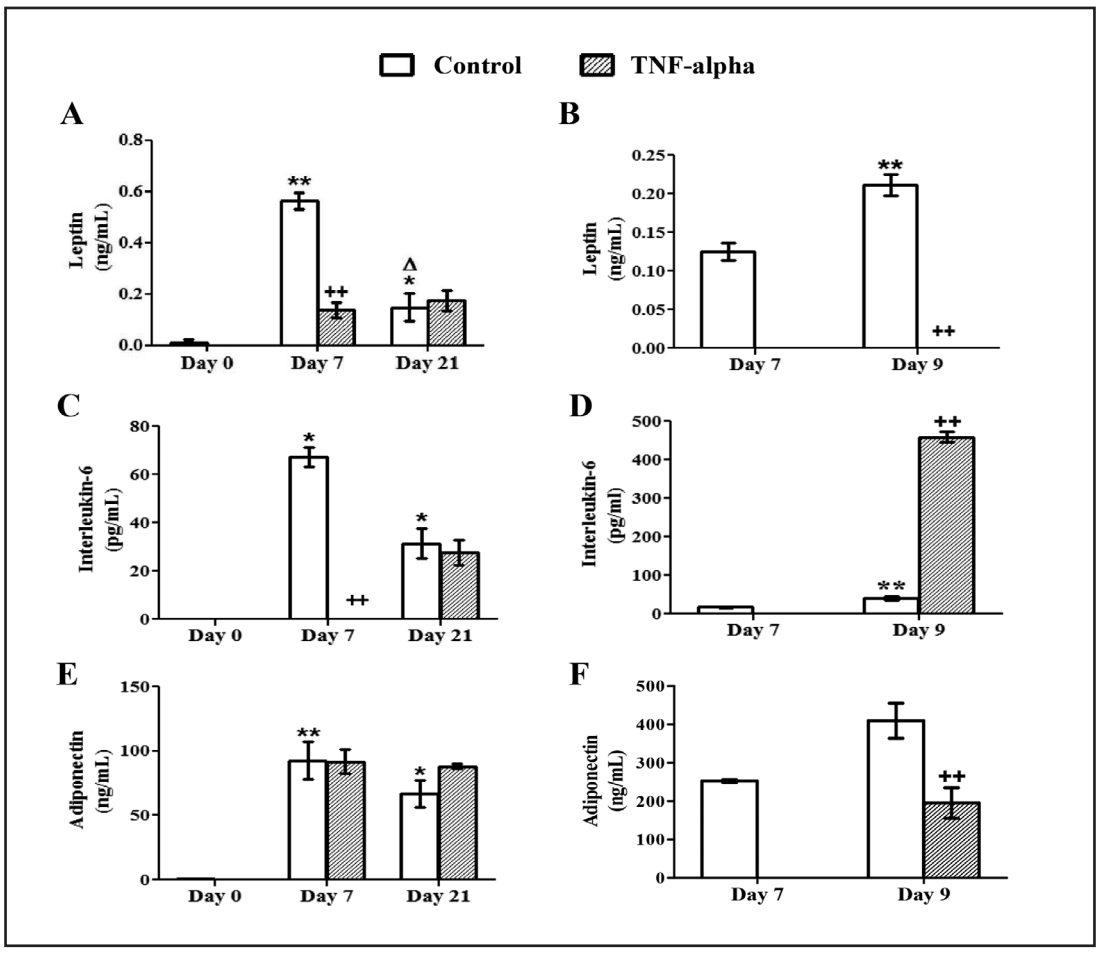

Fig. 1. Effect of TNF-α on secreted leptin, interleukin 6 (IL-6) and adiponectin levels during 3T3-L1 cell adipogenesis and after an acute treatment on 3T3-L1 mature adipocytes. Culture medium samples of (A, C, E) 3T3-L1 preadipocytes (d0) and control/TNF-α treated (10 ng/mL) adipocytes (d7, d21) and of (B, D, F) 3T3-L1 control (d7, d9)/TNF-α treated (10 ng/mL, 48 hours) adipocytes (d9) were obtained for adipokine ELISA assays. Data are means ± SEM of the concentration of each adipokine secreted to the cell medium. Groups were compared by using the ANOVA for a single factor and Dunnett test. (A, C, E) Data from control groups are compared to day 0 *. $p < 0.05$; **. $p < 0.01$ or to day 7 Δ. $p < 0.05$. TNF-α treated cells are compared to the control group from the same differentiation day ++. $p < 0.01$. (B, D, F) Data from control cells at day 9 were compared to control cells at day 7 **. $p < 0.01$. Data from TNF-α treated cells were compared to their same day control group ++. $p < 0.01$. The number of independent samples analyzed is ≥10 for each adipokine and condition evaluated.

induced by the cytokine after 7 days. The levels of all four proteins also increased significantly at day 7. However, only AKT-2 continued this tendency up to day 21, while Cav-1, IR and Glut-4 protein levels were reduced. This was especially evident in the case of IR and Glut-4, whose expression was brought down to the levels of non-differentiated cells (day 0). The presence of TNF-α reduced the expression of all these proteins, with the notable exception of IR and Glut-4, whose levels increased at day 21 (Fig. 5B, 7C).

The short-term (48-h) treatment of 7-day differentiated adipocytes with TNF-α markedly decreased the mRNA levels of all the insulin signaling intermediates under study (Fig. 6A, 7B). A reduction in the protein levels was also observed, although the difference did not reach statistical significance for Glut-4 (Fig. 6B, 7D).

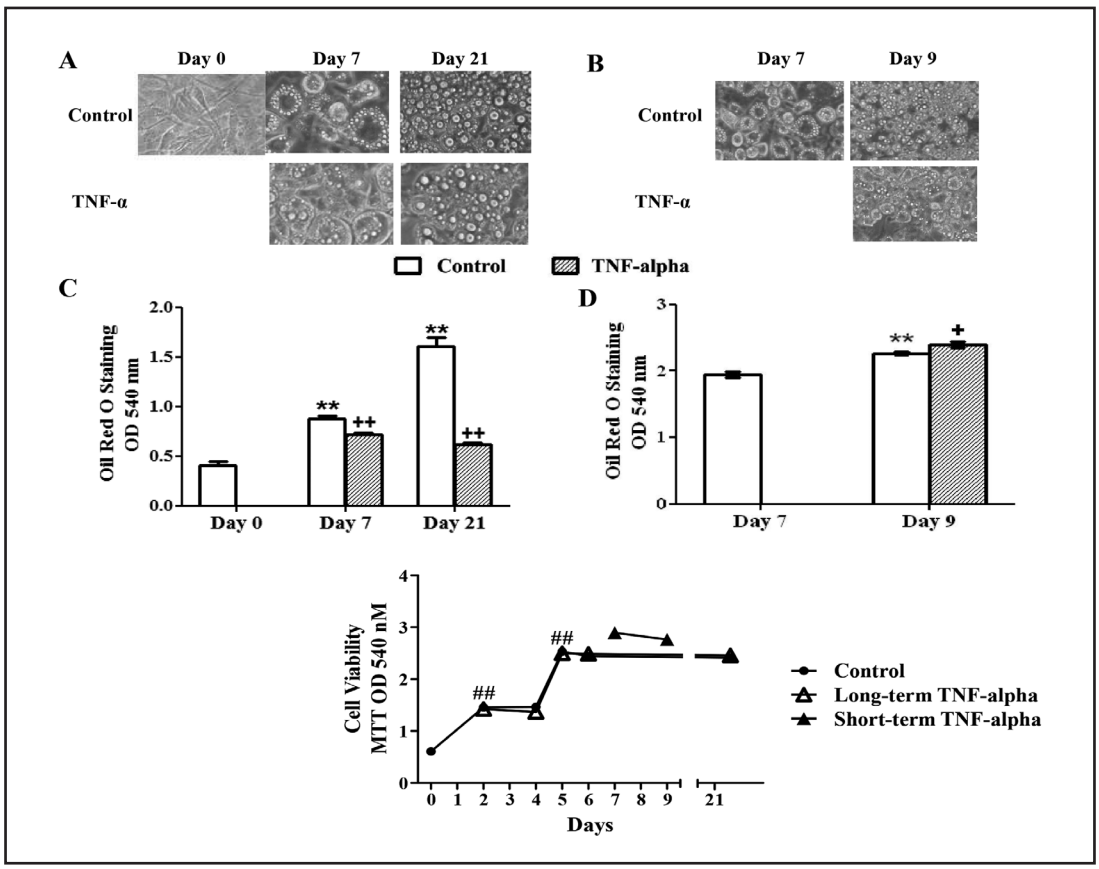

Fig. 2. Effect of TNF-α on cell differentiation, triglyceride storage and viability during 3T3-L1 cell adipogenesis and after an acute treatment on 3T3-L1 mature adipocytes. (A) 3T3-L1 preadipocytes (d0) and control/TNF-α treated (10 ng/mL) adipocytes (d7, d21) and (B) 3T3-L1 control (d7, d9)/TNF-α treated (10 ng/mL, 48 hours) adipocytes (d9) were photographed using a light microscope (40X magnification). (C) Intracelullar tryglicerides quantification of the preadipocytes (d0) and control/TNF-α treated (10 ng/mL) adipocytes (d7, d21) and (D) of the 3T3-L1 control (d7, d9)/TNF-α treated (10 ng/mL, 48 hours) adipocytes (d9) using Oil Red O Staining. Data are means ± SEM of dye OD at 540 nm. (E) Cell viability measurement at days 0, 2, 4, 5, 6 and 21 for the long-term TNF-α treatment and at days 7 and 9 for the acute TNF-α treatment using MTT assay. Data are means ± SEM of dye OD at 540 nm. Groups were compared by using the ANOVA for a single factor and Dunnett test. (C) Data from control groups are compared to day 0 **. $p < 0.01$ and TNF-α treated groups are compared to the control groups from the same differentiation day ++. $p < 0.01$. (D) Data from control group are compared to day 7 **. $p < 0.01$ and from TNF-α treated group are compared to the control group of the same differentiation day +. $p < 0.05$. (E) Data are compared to the previous checking point ##. $p < 0.01$. The number of independent samples analyzed is ≥10 for each determination and condition evaluated.

Effect of TNF-α on caveolin-1 and insulin signaling pathway intermediates activation and insulin sensitivity

In agreement with previous results [22], the baseline level of Cav-1 phosphorylation was significantly increased in mature adipocytes (day 21) and the response to insulin was small and not significant, while, on the other hand, the IR and AKT-2 insulin response was enhanced in the mature cells (day 21). The addition of TNF-α during the differentiation process significantly reduced Cav-1 baseline phosphorylation, but maintained its capacity to respond to insulin. Interestingly, TNF-α also significantly reduced the insulin response of IR, but not that of AKT-2 in mature cells (Fig. 5C). Results obtained with the short-term (48-h) TNF-α treatment of differentiated adipocytes (d7) were similar, with the insulin

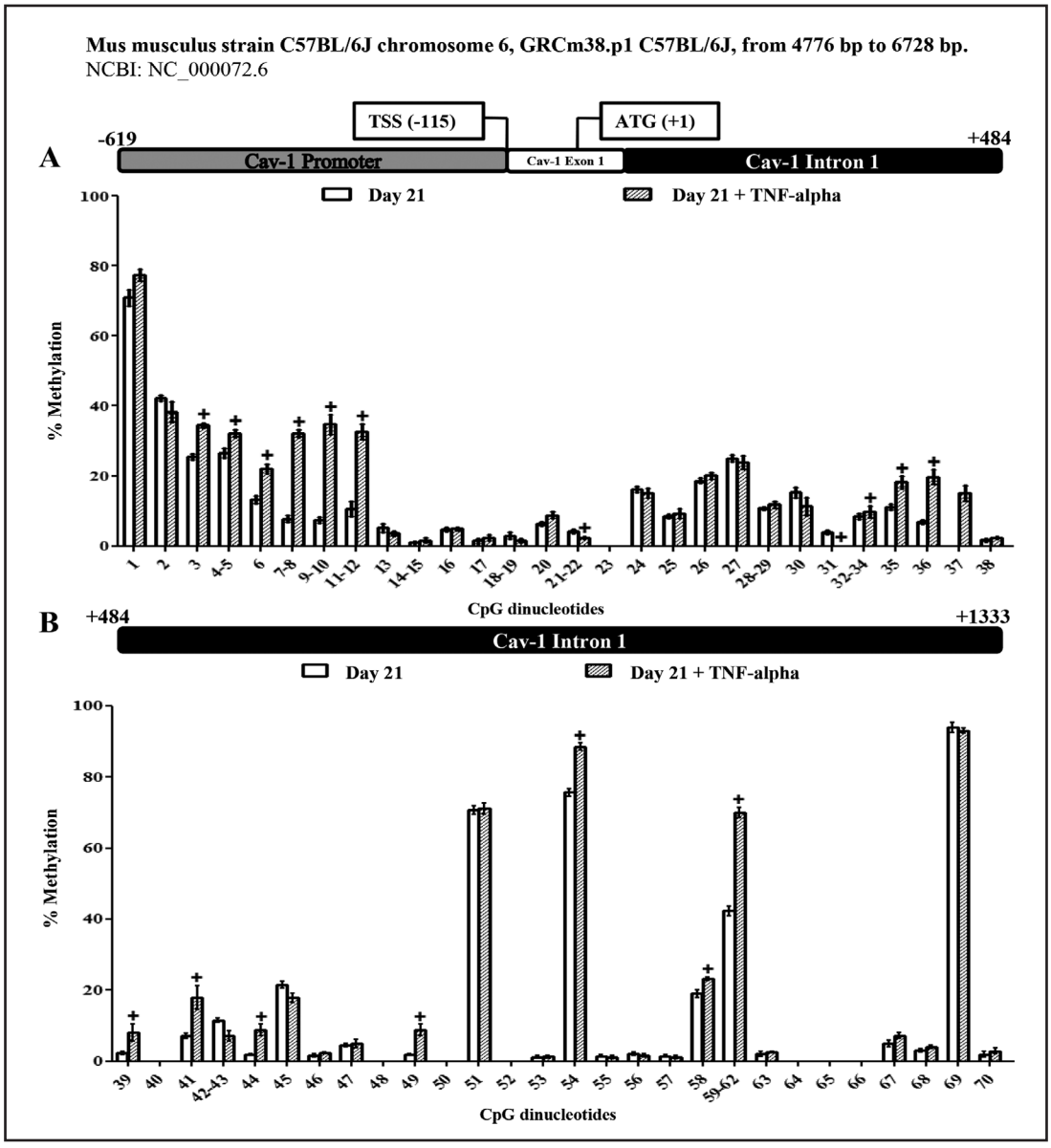

Fig. 3. Methylation levels of CpG dinucleotides located in the caveolin-1 promoter, exon 1 and intron 1 in 3T3-L1 mature adipocytes treated or not with TNF-α (10 ng/mL) during cell differentiation. The methylation levels of 70 CpG sites in caveolin-1 promoter, exon 1 and intron 1 were compared in control/TNF-α treated 3T3-L1 mature adipocytes at day 21. MassARRAY system was used for the quantitative methylation analysis. (A) CpG 1 to 38 (B) CpG 39 to 70 of the sequence under study. Data are means ± SEM of the methylation percentage of each CpG dinucleotide specified in the figure. Groups were compared by using the Mann-Whitney U test. Significant differences between control and TNF-α treated cells +. $p < 0.05$. Gene structure is schematized over the graphs indicating the Transcription Start Site (TSS) and the initiation codon (ATG) position. The number of independent samples analyzed is between 5-8, measured in triplicate for each CpG dinucleotide under study.

response capacity of Cav-1 and AKT-2 being maintained and that of IR not even being reduced (Fig. 6C).

The uptake of 2-deoxyglucose was determined before and after insulin stimulation in the two experimental designs in order to analyze Glut-4 response to insulin. We have previously reported that baseline 2-deoxyglucose uptake increased during adipocyte

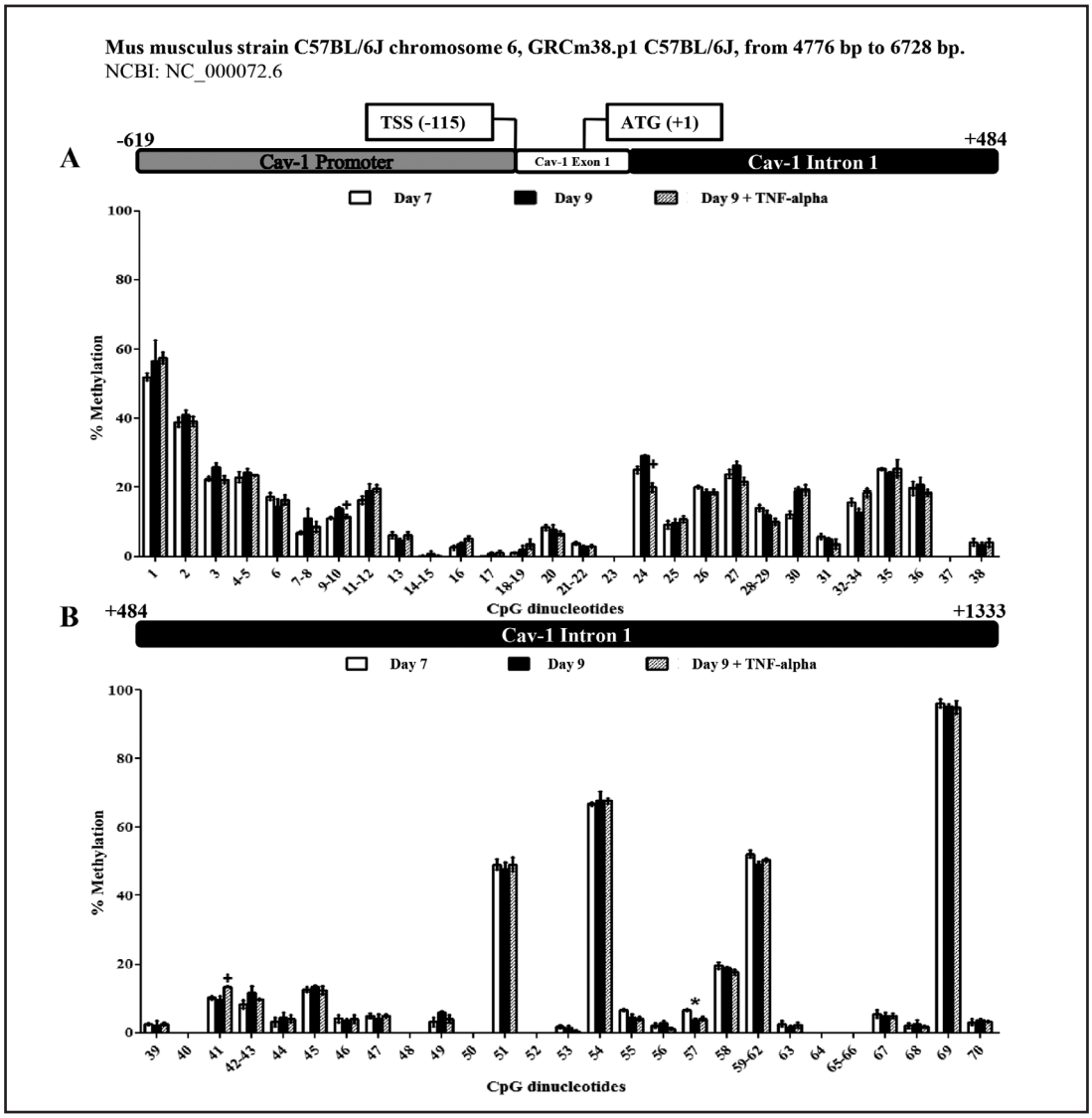

Fig. 4. Methylation levels of CpG dinucleotides in the caveolin-1 promoter, exon 1 and intron 1 in 3T3-L1 mature adipocytes treated or not with TNF-α (10 ng/mL) during 48 hours. The methylation levels of 70 CpG sites in caveolin-1 promoter, exon 1 and intron 1 were compared in control (d7)/TNF-α treated (48 hours, d9) 3T3-L1 mature adipocytes. MassARRAY system was used for the quantitative methylation analysis. (A) CpG 1 to 38 (B) CpG 39 to 70 of the sequence under study. Data are means ± SEM of the methylation percentage of each CpG dinucleotide specified in the figure. Groups were compared by using the Mann-Whitney U test. Significant differences between control day 7 and day 9 cells *. $p < 0.05$. Significant differences between control and TNF-α treated cells +. $p < 0.05$. Gene structure is schematized over the graphs indicating the Transcription Start Site (TSS) and the initiation codon (ATG) position. The number of independent samples analyzed is between 5-8, measured in triplicate for each CpG dinucleotide under study.

differentiation (d7), but decreased in long-term fully mature adipocytes (d21), and that insulin was always able to significantly induce hexose uptake, either in preadipocytes (d0) or in the differentiated cells (d7, d21) (20). In the present study, however, we observed that TNF-α treatment during the differentiation process clearly inhibited glucose uptake by the cells, both in the baseline and in the insulin-stimulated states (Fig. 7E). On the other hand, short-term TNF-α (48-h) treatment of differentiated adipocytes (d7) did not reduce baseline glucose transport activity, but was able to completely block its response to insulin (Fig. 7F).

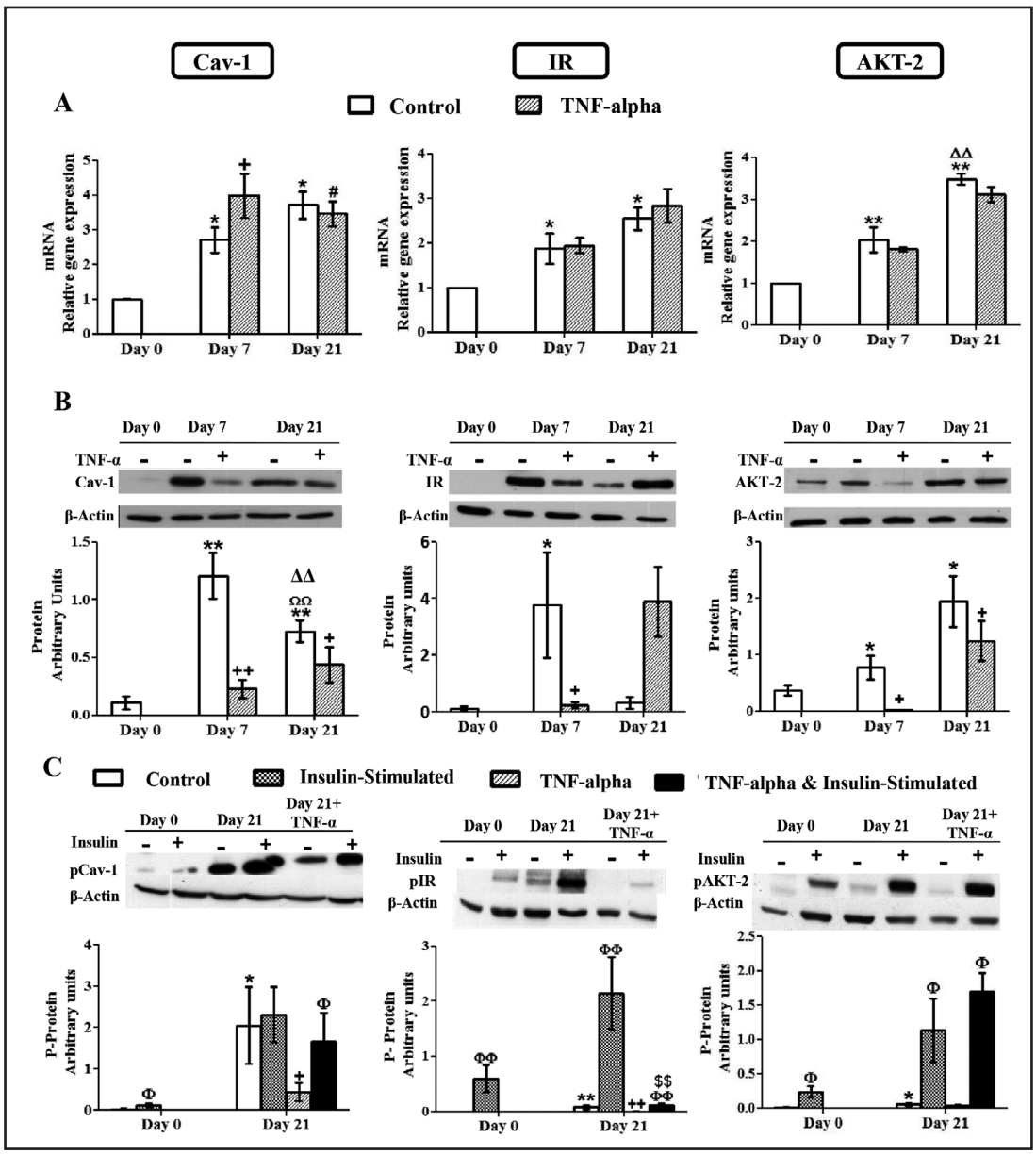

Fig. 5. Caveolin-1 and insulin signaling intermediates expression and activation during 3T3-L1 cell adipogenesis in the presence or in the absence of TNF-α (10 ng/mL). (A) mRNA levels. Data are means ± SEM of the ratio between each gene and cyclophilin expression at differentiation days 0, 7 and 21. Groups were compared by using the ANOVA for a single factor and Dunnett test. (B) Protein levels. Data are means ± SEM of the ratio between each protein and β-Actin expression at differentiation days 0, 7 and 21. Groups were compared by using the Wilcoxon signed rank test. (C) Phosphorylation levels. Results represent data from cells before and after stimulation with insulin (50 nM, 10 minutes). Data are means ± SEM of the ratio between each phopho-protein and β-Actin expression at the differentiation days 0 and 21. Groups were compared by using the Wilcoxon signed rank test. Data from control cells were compared to day 0 *. $p < 0.05$; **. $p < 0.01$ or to day 7 ΔΔ. $p < 0.01$. Data from TNF-α treated groups were compared to their same day control group +. $p < 0.05$; ++. $p < 0.01$. Data from TNF-α treated group at day 21 were compared to TNF-α treated group at day 7 #. $p < 0.05$. Data from insulin-stimulated groups were compared to their unstimulated control group Φ. $p < 0.05$, ΦΦ. $p < 0.01$. Data from insulin-stimulated TNF-α treated group were compared to their same day insulin-stimulated control group $$. $p < 0.01$. The number of independent samples analyzed for (A) and (B) is ≥10 for each determination and condition evaluated, whereas the number of independent samples analyzed for (C) is between 5-10 for each protein and condition evaluated.

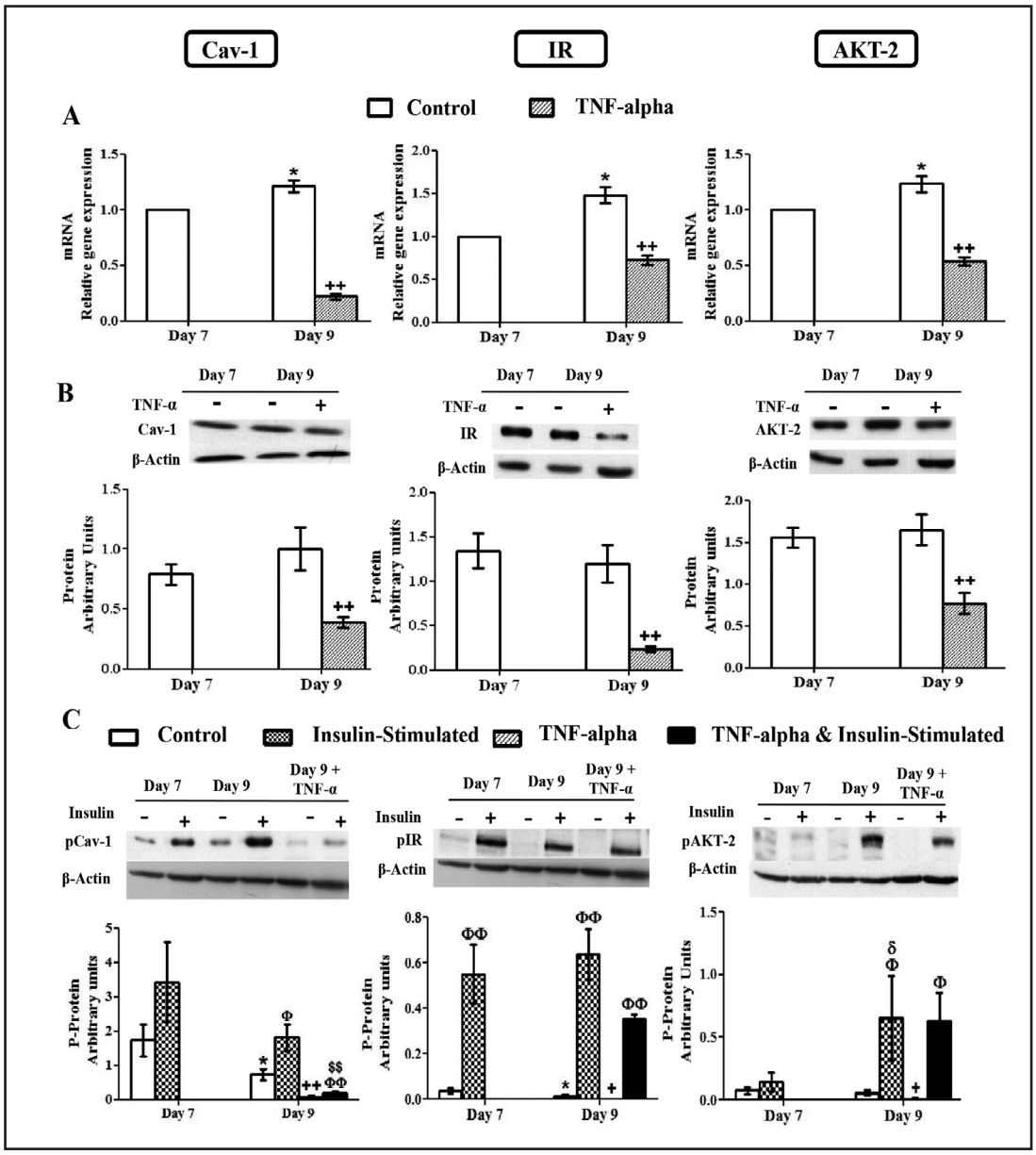

Fig. 6. Caveolin-1 and insulin signaling intermediates expression and activation in non-treated and TNFα-treated (10 ng/mL, 48 hours) 3T3-L1 mature adipocytes. (A) mRNA levels. Data are means ± SEM of the ratio between each gene and cyclophilin expression at differentiation days 7 and 9. (B) Protein levels. Data are means ± SEM of the ratio between each protein and β-Actin expression at differentiation days 7 and 9. Groups from (A) and (B) were compared by using the ANOVA for a single factor and Dunnett test. (C) Phosphorylation levels. Results represent data from cells before and after stimulation with insulin (50 nM, 10 minutes). Data are means ± SEM of the ratio between each phopho-protein and β-Actin expression at the differentiation days 7 and 9. Groups were compared by using the Wilcoxon signed rank test. Data from control cells at day 9 were compared to control cells at day 7 *. $p < 0.05$. Data from TNF-α treated groups were compared to their same day control group +. $p < 0.05$; ++. $p < 0.01$. Data from insulin-stimulated groups were compared to their unstimulated control group Φ. $p < 0.05$, ΦΦ. $p < 0.01$. Data from insulin-stimulated control group at day 9 was compared to insulin-stimulated control group at day 7 δ. $p < 0.05$. Data from insulin-stimulated TNF-α treated group were compared to their same day insulin-stimulated control group $$. $p < 0.01$. The number of independent samples analyzed for (A) and (B) is ≥10 for each determination and condition evaluated, whereas the number of independent samples analyzed for (C) is between 5-10 for each protein and condition evaluated.

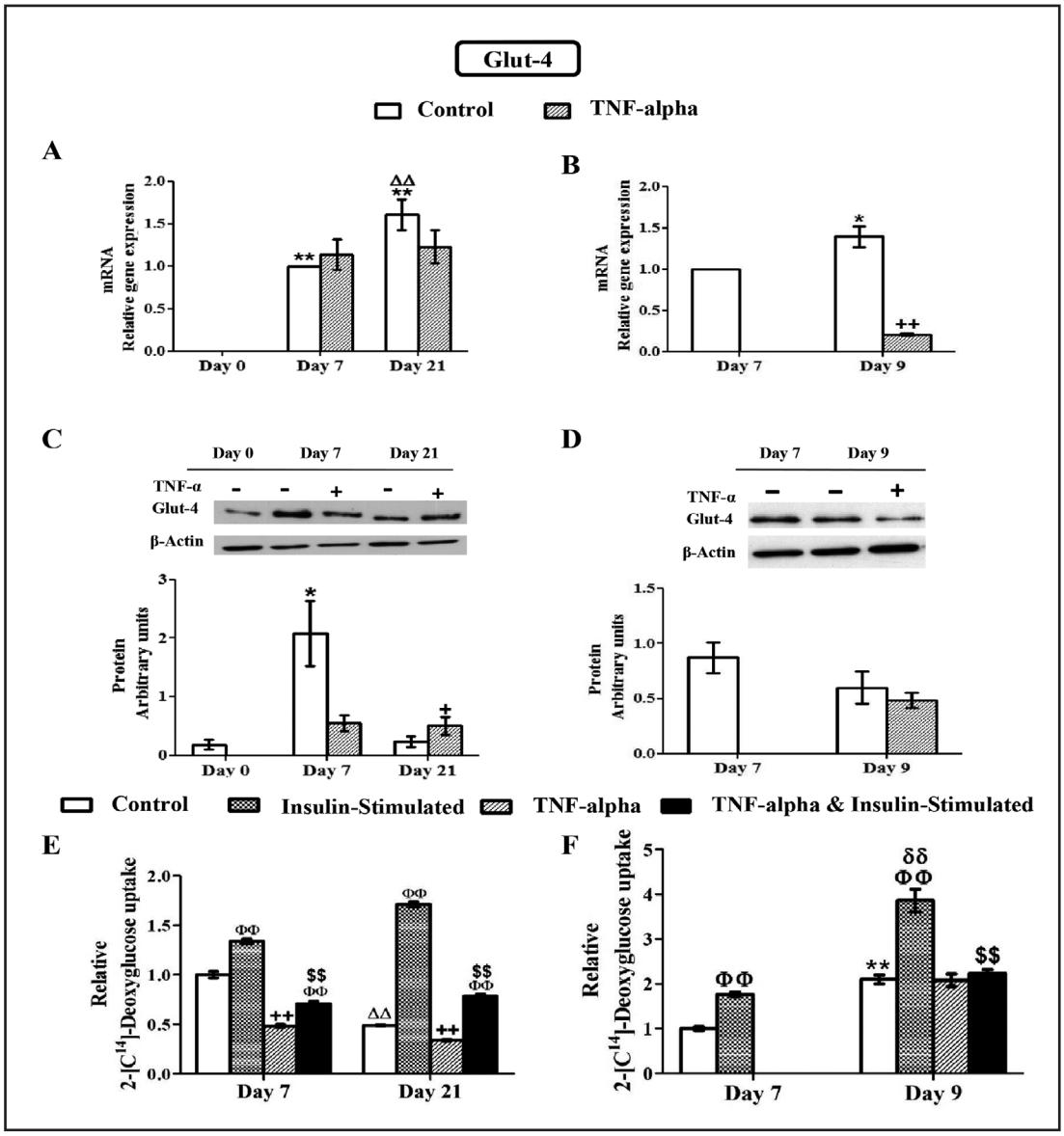

Fig. 7. Effect of TNF-α on glucose transporter type-4 (Glut-4) expression and deoxyglucose uptake during 3T3-L1 cell adipogenesis (A, C, E) and after an acute treatment on 3T3-L1 mature adipocytes (B, D, F). Results from A and C correspond to preadipocytes (d0) and control/TNF-α treated (10 ng/mL) adipocytes (d7, d21) whereas results from E correspond to control/TNF-α treated (10 ng/mL) adipocytes (d7, d21). Results from B, D and F correspond to control (d7, d9) /TNF-α treated (10 ng/mL, 48 hours) adipocytes (d9). (A, B) mRNA level. Data are means ± SEM of the ratio between Glut-4 and cyclophilin expression. Groups were compared by using the ANOVA for a single factor and Dunnet test. (C, D) Protein level. Data are means ± SEM of the ratio between Glut-4 and β-Actin expression. Groups were compared by using the Wilcoxon signed rank test. (E, F) Deoxyglucose uptake by 3T3-L1 control and TNF-α treated cells before and after insulin stimulation (50 nM, 10 min). Data are relative quantification of means ± SEM of 2-[C^{14}]- deoxyglucose (in µmol) incorporated by cells after 10 minutes, adjusted by total protein in grams. All groups were refered to control d7 and were compared by using the ANOVA for a single factor and Dunnett test. (A, C, E) Data from control groups are referred to control day 0 *. $p < 0.05$; **. $p < 0.01$ or to control day 7 ΔΔ. $p < 0.01$. Data from TNF-α treated groups were compared to their same day control group +. $p < 0.05$; ++. $p < 0.01$. (E) Data from insulin-stimulated groups were compared to their unstimulated group, ΦΦ. $p < 0.01$. Data from insulin-stimulated TNF-α treated groups were compared to the same day insulin-stimulated control groups $$. $p < 0.01$. (B, D, F) Data from control groups at day 9 were compared to control cells at day 7 *. $p < 0.05$;

**. p < 0.01. Data from TNF-α treated groups were compared to their same day control group ++. p < 0.01. (F) Data from insulin-stimulated groups were compared to their unstimulated group, ΦΦ. p < 0,01. Data from insulin-stimulated TNF-α treated groups were compared to the same day insulin-stimulated control groups $$. p < 0.01. Data from insulin-stimulated control group at day 9 was compared to insulin-stimulated control group at day 7 δδ. p < 0.01. The number of independent samples analyzed for (A), (B), (E) and (F) is ≥10 for each determination and condition evaluated whereas the number of independent samples analyzed for (C) and (D) is between 5 and 10 for each protein and condition evaluated.

Discussion

Role of methylation in the regulation of Cav-1 gene expression

In a previous study, we described that the Cav-1 gene induction that takes place during 3T3-L1 adipocyte differentiation is accompanied by a significant demethylation of a CpG island extending from the proximal promoter to the first exon and a substantial portion of the first intron (20). In this study, we analyzed the same sequence and the methylation percentages of 16 out of the 70 CpGs present were significantly higher after 21 days of differentiation in the presence of TNF-α (Fig. 3), although Cav-1 gene expression was not affected (Fig. 5A). Furthermore, treatment of differentiated adipocytes (7 days) with TNF-α for 48 hours, which actually induced an important decrease in Cav-1 expression (Fig. 6A), only produced significant methylation percentage changes in three CpG sites (Fig. 4). Taken together, these results indicate that in the presence of TNF-α, DNA methylation of the sequence under study does not seem to be of relevance for the modulation of Cav-1 expression. In agreement with this, expression changes of another crucial gene for insulin signaling, such as IR during 3T3-L1 adipogenesis in the presence of TNF-α, did not correlate with the absence of significant methylation pattern changes (Data not shown). Therefore, although epigenetic DNA methylation may contribute to some extent to the regulation of 3T3-L1 adipocyte differentiation process, other mechanisms become more important under certain conditions.

TNF-α effect during adipocyte differentiation

The presence of TNF-α during the adipogenic differentiation of 3T3-L1 cells seems to limit the degree of maturation of these cells which, after 7 days, are no longer able to continue accumulating triglycerides (Fig. 2C) and indeed, some preadipocytes never initiated the differentiation program. This effect is also observed in the secretion pattern of two characteristic adipokines that positively correlate with adiposity, obesity, insulin resistance and the development of type 2 diabetes, such as leptin and IL-6 [23-26], while on the other hand adiponectin does not seem to be affected (Fig. 1A, C, E). TNF-α and leptin seem to maintain a double-sense regulatory loop, since leptin has been reported to act as a protective factor against LPS-induced TNF-α release [27]. Interestingly, long-term mature adipocytes (d21) exhibited lower levels of leptin, IL-6 and adiponectin release than mature fat cells from day 7. This decrease could be related to the hypertrophy that the cells undergo as a consequence of prolonged maturation, which has been proposed to cause important physiological alterations [28, 29]. The decreased expression pattern observed for other proteins characteristic of mature adipocytes, such as Cav-1, IR and Glut-4 (Fig. 5 and 7), correlates well with this effect. Our results indicate that, despite the fact that non-mature preadipocytes appear to express very low levels of the two types of TNF-α receptors (TNFRI and TNFRII) [30], TNF-α signaling in these cells seems to slow down the differentiation process.

The inhibitory role of TNF-α in adipogenesis is well known, since this cytokine inhibits the expression of the key adipogenic mediators C/EBPα and PPARγ [31, 32]. Recently, Wei et al. [33] have shown that when preadipocytes are cocultured with adipocytes, the latter block 3T3-L1 preadipocyte differentiation through overexpression of PREF-1, a factor that maintains the decline of C/EBPα and PPARγ mRNA. Interestingly, these events were promoted under conditions of inflammation or insulin resistance and they were specially

induced when both conditions were present at the same time. In addition, the slower response of preadipocytes to the proadipogenic stimulus might be also related to the TNF-α-mediated inhibition of growth-arrest genes [4] and the attenuation of STAT1 and STAT5, both considered to be inducers of the development and maintenance of the adipocyte phenotype [34].

Enhanced Cav-1 expression is a hallmark of adipocyte differentiation [35], which is accompanied by IR, AKT-2 and Glut-4 gene induction (Fig. 5A and 7A), as we have reported previously [22]. Surprisingly, the presence of TNF-α did not significantly reduce the mRNA levels of any of these genes, despite slowing down and limiting the 3T3-L1 maturation process. However, protein levels seem more consistent with a less differentiated state, since they are lower after 7 days in the presence of TNF-α and continued increasing in late mature adipocytes (21 days) (Fig. 5B). This result is compatible with the idea that TNF-α is delaying the adipocytic differentiation program but not completely blocking it, since maximum expression levels are reached earlier in non-treated cells. This effect seems to be mediated mainly by reducing mRNA translation, perhaps shortening the half-life of mRNAs, or by accelerating protein degradation.

We have reported previously that Cav-1 is phosphorylated and activated during adipogenesis [22], which explains why, after 21 days of differentiation, although IR and AKT-2 insulin responses are markedly increased, insulin has little effect on Cav-1 activation (Fig. 5C). However, when differentiation takes place in the presence of TNF-α total and phosphorylated Cav-1 are reduced, but remarkably its insulin sensitivity is retained (Fig. 5 B and C). In contrast, TNF-α actually reduces insulin response, shrinking induced IR phosphorylation and significantly diminishing insulin stimulated glucose uptake (Fig. 5C and 7E), despite total Glut-4 protein levels remaining higher in treated cells than in control ones (Fig. 7C). In this regard, it is important to note that TNF-α has been previously reported to cause a total depletion of the Glut-4 protein in the adipocyte membrane [36, 37], retaining it in an inactive form in intracellular membrane compartments. The differences observed in control cell glucose uptake during adipocyte differentiation were discussed in a previous study by our group in relation to the facilitative insulin-independent glucose transporter-1 (Glut-1) [22]. Nonetheless, insulin-stimulated AKT-2 phosphorylation was not affected by TNF-α. In regard to this, AKT-2 activation through PI3K (phosphatidylinositol-3 kinase) has also been described as part of TNF-α signaling, in order to activate its main effector, NF-κB [38, 39]. All these results are also consistent with the delay that TNF-α seems to be causing on the 3T3-L1 cells differentiation process.

TNF-α effect on mature adipocytes

The addition of TNF-α to 7 day differentiated adipocytes during 48 hours does not seem to interfere with the rate of lipid accumulation by these cells (Fig. 2D), despite there being evidence of a dose-dependent lipolytic effect of TNF-α in fully differentiated adipocytes [9, 40]. However, this acute TNF-α treatment produces in the differentiated adipocytes quite important and opposite responses in cytokine secretion, totally blocking leptin induction and greatly inducing IL-6 production, while adiponectin was significantly reduced (Fig. 1B, D, F). These results are in line with previous studies in mouse adipocytes [41] or cultured rat adipocytes [42], which found a significant decrease or a complete drop, respectively, in leptin secretion after treatment with high concentrations of TNF-α. It has also been suggested that TNF-α might act as a paracrine mediator in adipocytes stimulating the secretion of IL-6, which would be responsible of the systemic effects mediated by inflammation [24]. In addition, circulating adiponectin is generally reduced in obesity [43], and levels of this protein have been found to be inversely correlated with IL-6 [44, 45].

The two days incubation of differentiated adipocytes with TNF-α induced a significant reduction of Cav-1, IR, AKT-2 and Glut-4 mRNA expression, which in this case correlated well with the sharp drop observed in protein levels, except for Glut-4 (Fig. 6A, B and 7B, D). These results agree with the TNF-α induced lower expression of some insulin signaling intermediates reported by other groups [36, 37]. Nevertheless, this TNF-α treatment also

reduced phosphorylated Cav-1 maintaining its insulin sensitivity (Fig. 6C). TNF-α is a main mediator of the inflammatory process associated with obesity that, if sustained in time, can lead to insulin resistance and indeed the results obtained point to a certain impairment of the insulin signal transmission. Therefore, besides reducing the expression of all the insulin intermediates measured, TNF-α also reduces the phosphorylation of Cav-1 linked to adipocyte differentiation, and diminishes its capacity to respond to insulin. Indeed, after the two days of TNF-α treatment, sensitivity of glucose uptake to insulin is lost (Fig. 7F), although TNF-α in this case does not reduce so severely IR response (Fig. 6C).

It has been previously reported that TNF-α is able to destabilize the interaction between IR and Cav-1 [14], resulting in the elimination of the majority of IR proteins from caveolae in 3T3-L1 adipocytes [46]. These data could explain the lower levels of phosphorylated IR in TNF-α treated cells in both experimental conditions used in this study (Fig. 5C and 6C). In addition, it is also known that TNF-α contributes to disruption of the insulin signaling pathway by causing IR dephosphorylation [12] and NF-κB-mediated induction of the protein tyrosine phosphatase PTP1B expression [5]. Moreover, PTP1B has been shown to catalyze efficiently phospho-Cav-1 tyrosine dephosphorylation [47]. This could partly explain the drop in phospho-Cav-1 levels caused by TNF-α even after insulin stimulation and the following fall in the capacity of insulin to stimulate glucose uptake (Fig. 5C, 6C and 7E, F).

The capacity of IR to phosphorylate Cav-1 has been demonstrated [48-50], and we have shown that Cav-1 is also phosphorylated during adipogenic differentiation in an insulin independent way [22]. Therefore, it seems that the phosphorylation of Cav-1 is not necessary for its interaction with IR and is more related to Glut-4 and the facilitation of glucose uptake by this transporter.

In summary, we have shown that TNF-α is able to slow down the pace of 3T3-L1 adipocyte differentiation. There seems to be a certain adaptation to the proinflammatory environment that does not preclude adipocyte differentiation, but which interferes with its physiological responses. The final outcome is that cells, although still able to elicit a certain response, have partially lost glucose uptake sensitivity to insulin. The direct role of Cav-1 in facilitating the functional integration of Glut-4 in the plasma membrane after insulin stimulation is strengthened, since the presence of phosphorylated Cav-1 is associated with insulin-stimulated glucose uptake, even when IR response is diminished.

On the other hand, when differentiated adipocytes are exposed to TNF-α there is a sharp response, manifested by marked changes in adipokine secretion and pronounced falls in the expression of the insulin signaling intermediates measured. The insulin sensitivity of glucose uptake in these cells is completely lost. The implication of Cav-1 in this lack of response is evident, since insulin-induced Cav-1 phosphorylation is less prominent although phosphorylation of IR is still maintained.

Taken together, our results strongly suggest that Cav-1 activation by phosphorylation is essential for the maintenance of an active and insulin-sensitive glucose uptake. How direct this action of Cav-1 on glucose transporter Glut-4 might be in the context of membrane caveolae, is something that will need further work to be determined. Nonetheless, it is important to point out that in our model, 3T3-L1 cells are isolated from the interaction with the other cell types present in the adipose tissue, such as infiltrating immune cells, which can have a profound influence on the chronic inflammation associated with obesity and which might modulate these responses.

Disclosure Statement

The authors declare no conflict of interests.

Abbreviations

Tumor Necrosis Factor-α (TNF-α); Caveolin-1 (Cav-1); Insulin receptor (IR); Glucose transporter-4 (Glut-4); Protein kinase B (AKT-2); Interleukin 6 (IL-6).

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