Interleukin-6 is a positive regulator of tumor necrosis factor α-induced adipose-related protein in 3T3-L1 adipocytes

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Abstract Tumor necrosis factor (TNF) α-induced adipose-related protein (TIARP) is a novel TNFα-stimulated protein in adipocytes. Besides TNFα, interleukin (IL)-6 has recently been shown to be another adipocytokine implicated in insulin resistance. Therefore, the impact of IL-6 on TIARP gene expression in 3T3-L1 adipocytes was determined by quantitative real-time reverse transcription-polymerase chain reaction. Interestingly, TIARP mRNA expression was stimulated up to 3.8-fold by IL-6 in a dose-dependent fashion with significant stimulation detectable at effector concentrations as low as 3 ng/ml and maximal effects seen at 100 ng/ml IL-6. Induction of TIARP mRNA by IL-6 was time-dependent with significant upregulation occurring as early as 2 h after effector addition and maximal effects observed at 4 h. In parallel, TIARP protein synthesis was upregulated with maximal effects seen after 8 h of IL-6 treatment. Furthermore, the Janus kinase 2 inhibitor AG490 decreased TIARP mRNA expression. The increase of TIARP mRNA could be reversed by withdrawal of IL-6 for 24 h. Furthermore, TIARP mRNA induction by IL-6 was also seen in brown adipocytes but not in muscle and liver cells. Taken together, these results show that TIARP is acutely regulated in adipose tissue not only by TNFα but also by IL-6 which has been shown to be another important cytokine implicated in the pathogenesis of insulin resistance.

Key words: Adipocyte; Insulin resistance; Interleukin-6; Obesity; Tumor necrosis factor α; Tumor necrosis factor α-induced adipose-related protein

1. Introduction

Obesity-associated insulin resistance is an important risk factor in the development of atherosclerosis, hypertension, and type 2 diabetes [1]. The connection between insulin resistance and increased body weight is unclear. However, recent studies suggest that adipocytes secrete various biologically active proteins which influence insulin sensitivity thereby altering glucose homeostasis. Among those so-called adipocytokines, tumor necrosis factor (TNF) α [2], interleukin (IL)-6 [3], and resistin [4] have been suggested to induce insulin resistance whereas adiponectin [5] appears as an endogenous insulin sensitizer. Elucidating downstream signaling molecules of these adipocytokines has become a focus of current research since novel targets for the treatment of insulin resistance and associated metabolic disorders might arise from these studies. Recently, in an elegant experimental setting, a novel TNFα-responsive gene was cloned from fat cells by differential display technique [6]. It was named TNFα-induced adipose-related protein (TIARP), and the authors demonstrated convincingly that it is induced by TNFα in a dose- and time-dependent fashion and during adipogenic conversion [6]. Furthermore, TIARP is a transmembrane protein preferentially present at the plasma membrane and its general structure is reminiscent of various channels or transporters [6]. Although the biological function of TIARP is presently unknown, these data suggest that this protein might participate in adipocyte maturation and metabolism and may mediate TNFα effects on fat cells. However, a potential regulation of TIARP gene expression by other important adipocytokines with profound effects on insulin sensitivity has not been elucidated so far.

In the current study, we, therefore, examined the effect of IL-6 on TIARP synthesis in vitro. We demonstrate for the first time that IL-6 potently and reversibly induces TIARP expression in fat cells.

2. Materials and methods

2.1. Materials

AG490, dexamethasone, IL-6, isobutylmethylxanthine, LY294002, PD98059, and SB203580 were obtained from Sigma Chemical Co. (St. Louis, MO, USA), insulin from Roche Molecular Biochemicals (Mannheim, Germany). Cell culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY, USA), oligonucleotides from MWG-Biotech (Ebersberg, Germany).

2.2. Cell culture and differentiation

3T3-L1 cells (American Type Culture Collection, Rockville, MD, USA) were grown and differentiated into adipocytes as previously described [7]. In brief, preadipocytes were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM

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Abbreviations: IL-6, interleukin-6; IRS, insulin receptor substrate; Jak, Janus kinase; MAP, mitogen-activated protein; PI, phosphatidylinositol; TIARP, tumor necrosis factor α-induced adipose-related protein; TNF, tumor necrosis factor

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glucose, 10% fetal bovine serum, and antibiotics (culture medium). Confluent cells were induced for 3 days in culture medium further supplemented with 1 μM insulin, 0.5 mM isobutylmethylxanthine, and 0.1 μM dexamethasone before they were maintained for another 3 days in culture medium with 1 μM insulin and for additional 4–8 days in culture medium. After this period, more than 90% of the cells showed fat droplet accumulation. Brown adipocytes and C2C12 myocytes were cultured and differentiated as described recently [8,9].

2.3. Analysis of TIARP gene expression

TIARP mRNA was quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) in a fluorescent temperature cycler (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany) as previously described [10]. In brief, total RNA was isolated with Trizol reagent (Life Technologies, Inc., Grand Island, NY, USA), reverse transcribed in a 20 μl reaction using standard reagents (Life Technologies, Inc., Grand Island, NY, USA), and 10% of each RT reaction was loaded into capillary tubes. Amplification was performed in a 20 μl PCR containing 3 mM $MgCl_2$, 0.5 μM each primer and 1× LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals, Mannheim, Germany) using the following PCR parameters: An initial denaturation at 94°C for 30 s, followed by 40 PCR cycles, each consisting of 95°C for 1 s, 61°C for 7 s, and 72°C for 11 s. The following primers were used: TIARP (accession number AJ319746) TAGGGTGTAGGCGAGCAGCAGT (sense) and TCAGTGACACGCGGGAAGATT (antisense); 36B4 (accession number NM007475) AAGCGCGTCCTGGCATTGTCT (sense) and CCGCAGGGGCAGCAGTGGT (antisense). After each cycle, SYBR Green I fluorescence emission readings were monitored and TIARP, as well as 36B4 mRNA levels, were quantified by the second derivative maximum method of the LightCycler Software (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, the crossing points of individual samples were determined by an algorithm identifying the first turning point of the fluorescence curve. TIARP expression was calculated relative to 36B4 which has been frequently used as an internal control due to its resistance to hormonal regulation [11].

Amplification of specific transcripts was confirmed by melting curve profiles (cooling the sample to 68°C and heating slowly to 95°C with continuous measurement of fluorescence) at the end of each PCR and by subjecting the amplification products to agarose gel electrophoresis.

2.4. Western blotting

Detection of TIARP synthesis by Western blotting was performed essentially as described previously [12]. Briefly, after the stimulation period cells were harvested in lysis buffer (50 mM HEPES, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM ethylenediamine tetraacetic acid (EDTA), 10% glycerol, 1% Igepal CA-630, 2 mM vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, pH 7.4). Lysates were clarified and equal amounts of protein were solubilized in Laemmli sample buffer. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes, blocked for 1 h, and immunoblotted with TIARP antibody [6]. Specifically bound primary antibodies were detected with peroxidase-coupled secondary antibody and enhanced chemiluminescence.

2.5. Statistical analysis

Results are expressed as means ± S.E.M. Differences between various cell treatments were analyzed by unpaired Student's *t*-tests. *P* values <0.01 are considered highly significant, <0.05 significant.

3. Results

3.1. IL-6 stimulates TIARP mRNA and protein expression in 3T3-L1 adipocytes

Since it has recently been shown that TIARP mRNA expression is upregulated by TNFα, we tested whether the adipocytokine IL-6 might influence TIARP synthesis in 3T3-L1 adipocytes in vitro. In fact, IL-6 stimulated TIARP gene expression in a dose-dependent fashion. Thus, a significant 1.9-fold induction of TIARP mRNA synthesis was detectable at

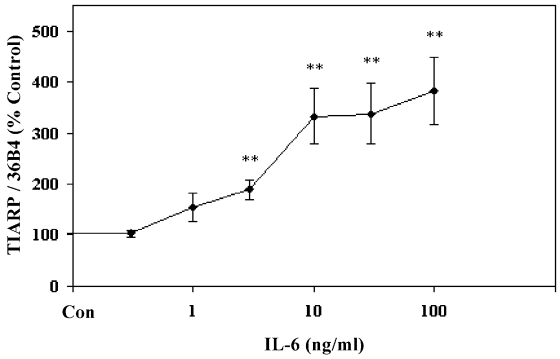

Fig. 1. Dose-dependent stimulation of TIARP gene expression by IL-6. 3T3-L1 adipocytes were serum-starved for 6 h before the indicated concentrations of IL-6 were added for 16 h. Total RNA was isolated and TIARP mRNA expression normalized to 36B4 was determined by quantitative real-time RT-PCR as described in Section 2. Data are given relative to untreated control (Con) cells (= 100%). Results are the means ± S.E.M. of three independent experiments. $^{**}P<0.01$ comparing IL-6-treated with non-treated cells.

IL-6 concentrations as low as 3 ng/ml ($P<0.01$) (Fig. 1). A maximal 3.8-fold increase was found at 100 ng/ml of the adipocytokine ($P<0.01$) (Fig. 1). Furthermore, 30 ng/ml IL-6 upregulated TIARP mRNA time dependently with significant 2.7-fold stimulation detectable as early as 2 h after effector addition, a maximal 3.2-fold induction after 4 h of hormone treatment, and stimulation persisting for up to 24 h ($P<0.01$) (Fig. 2A). Moreover, TIARP protein expression was upregulated time dependently with maximal stimulation seen after 8 h of IL-6 incubation (Fig. 2B).

3.2. IL-6 induces TIARP mRNA in brown adipocytes but not in C2C12 myocytes

Next, we determined whether IL-6 upregulated TIARP mRNA in other insulin-sensitive tissues. Similar to 3T3-L1 cells, IL-6 induced TIARP mRNA expression in differentiated brown adipocytes time dependently with significant 3.3-fold stimulation seen as early as 1 h after effector addition and a maximal almost 10-fold increase detectable after 24 h ($P<0.05$) (Fig. 2C). In contrast, no induction of TIARP mRNA expression was detected when C2C12 myocytes were treated with IL-6 (Fig. 2D). Moreover, mRNA expression of the human TIARP homolog was not altered by IL-6 in HepG2 liver cells (data not shown).

3.3. Stimulation of TIARP gene expression by IL-6 is reversible

We determined whether the stimulatory effect of IL-6 on TIARP gene expression was reversible. Fully differentiated 3T3-L1 adipocytes were treated with IL-6 (30 ng/ml) for 16 h, and the medium was then replaced by DMEM containing 25 mM glucose with (Fig. 3A) or without (Fig. 3B) 10% fetal bovine serum (FBS) for an additional 24 h. Addition of IL-6 again increased TIARP mRNA expression more than 3-fold as compared to untreated control cells (Fig. 3A and B, columns 1 and 2). However, removal of IL-6 from the medium for 24 h decreased TIARP mRNA expression to control levels (Fig. 3A and B, columns 3 and 4). Interestingly, addition of serum-containing medium for an additional 24 h stimulated TIARP gene expression more than 2-fold (Fig. 3A, columns 1

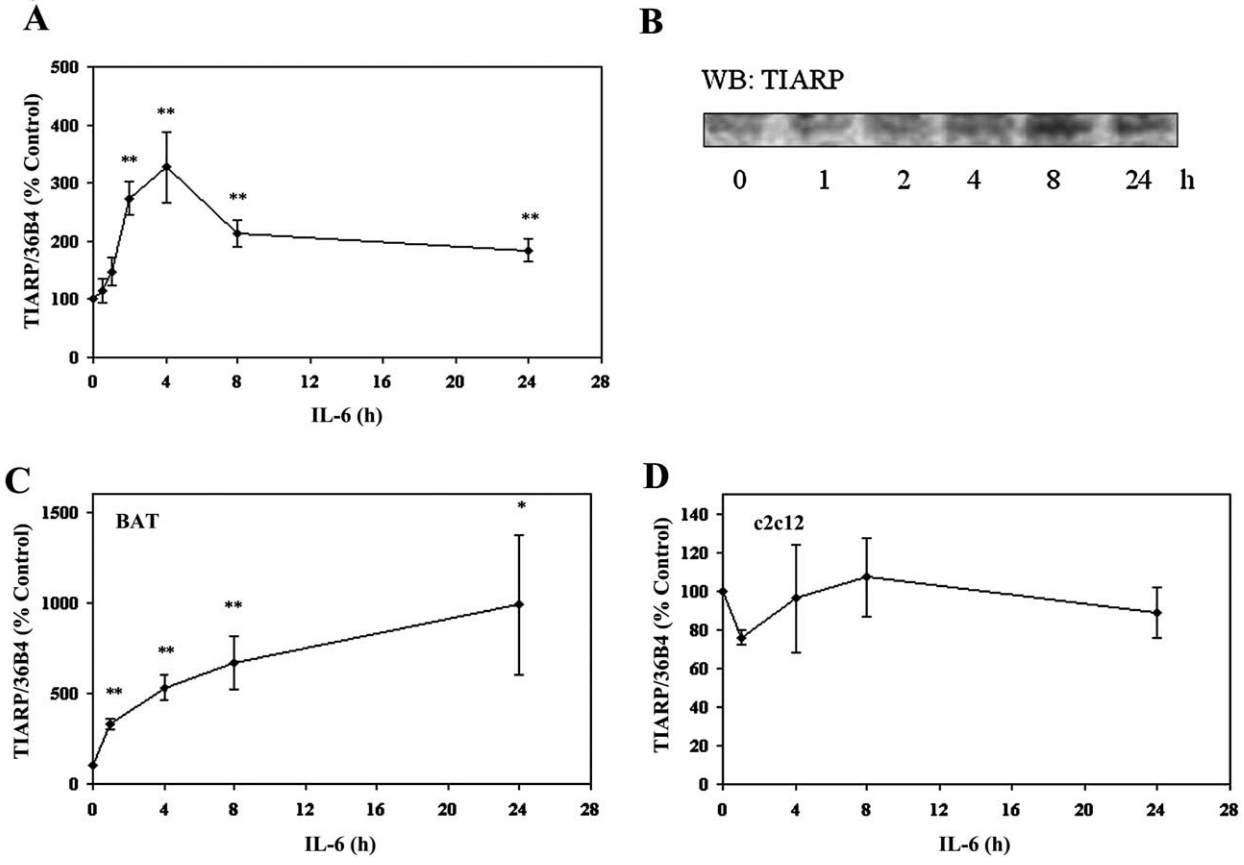

Fig. 2. Time-dependent induction of TIARP synthesis by IL-6. Fully differentiated (A, B) 3T3-L1 adipocytes, (C) brown adipocytes, and (D) C2C12 myocytes were serum-deprived overnight before 30 ng/ml IL-6 was added for different periods of time. A, C, D: After total RNA extraction, TIARP synthesis normalized to 36B4 mRNA was determined by quantitative real-time RT-PCR and expressed relative to untreated controls (=100%) as described in Section 2. Results are the means ± S.E.M. of at least three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ comparing IL-6-treated with untreated control cells. B: Total protein was isolated and immunoblotted as described in Section 2. A representative blot of three independent experiments is shown.

and 3), an effect which was not detectable in the absence of FBS (Fig. 3B, columns 1 and 3).

3.4. The Janus kinase (Jak) 2 inhibitor AG490 downregulates TIARP mRNA expression

We tested whether signaling proteins such as Jak2, p44/42 mitogen-activated protein (MAP) kinase, p38 MAP kinase, and phosphatidylinositol (PI) 3-kinase, which have been implicated in IL-6 signaling, may play a role in upregulation of TIARP gene expression. For this purpose, 3T3-L1 adipocytes were pretreated with specific pharmacological inhibitors for 1 h before IL-6 (30 ng/ml) was added for 16 h. The Jak2 inhibitor AG490 alone (10 μM) significantly downregulated basal TIARP mRNA expression to 29% of the levels seen in untreated control cells ($P < 0.01$) (Fig. 4). In contrast, inhibition of p44/42 MAP kinase, p38 MAP kinase, and PI 3-kinase with PD98059 (50 μM), SB203580 (20 μM), and LY294002 (10 μM), respectively, did not significantly influence basal TIARP mRNA synthesis (Fig. 4). Again, TIARP mRNA was upregulated to 436% of control levels after 16 h of IL-6 treatment ($P < 0.01$) (Fig. 4). Interestingly, inhibition of Jak2 by AG490 significantly reversed this stimulation by almost 70–137% of the expression level seen in untreated adipocytes ($P < 0.01$) (Fig. 4). In contrast, inhibition of p44/42 MAP kinase, p38 MAP kinase, and PI 3-kinase did not significantly influence induction of TIARP gene expression by IL-6 (Fig. 4).

4. Discussion

Recently, TIARP was described by Moldes et al. as a novel protein strongly induced by TNFα and adipose conversion which might mediate some effects of TNFα on insulin sensitivity and adipose metabolism [6]. In the current study, we demonstrate for the first time that IL-6 is another potent stimulator of TIARP expression in insulin-sensitive white and brown adipocytes. This induction appears to be tissue-specific since no stimulatory effect is observed in muscle and liver cells.

IL-6 is a proinflammatory cytokine, and it has been shown that plasma concentrations of this protein correlate with the development of type 2 diabetes mellitus [3,13]. IL-6 is also regarded an adipocytokine since about 25% of systemic IL-6 originates from subcutaneous fat cells in vivo and omental as compared to subcutaneous adipocytes that secrete even 2- to 3-fold more IL-6 in vitro [14,15]. IL-6 affects glucose metabolism profoundly. Thus, administration of recombinant IL-6 in rodent models induces hepatic gluconeogenesis which, in turn, leads to hyperglycemia and compensatory hyperinsulinemia [16]. Moreover, increased glucose levels have been described in humans after IL-6 treatment [13]. Recently, direct effects of IL-6 on insulin signaling molecules such as insulin receptor substrate (IRS) proteins have been elucidated. These proteins have been shown to be essential for insulin action

[17,18]. Thus, IL-6, similar to TNFα, decreases IRS-1 and Glut4 mRNA and protein expression and IRS-1 tyrosine phosphorylation in 3T3-L1 cells [19,20]. Moreover, Senn et al. reported impaired insulin signaling and insulin-induced glycogen synthesis in hepatocytes after IL-6 pretreatment [21]. However, signaling pathways and proteins mediating these effects are far from clear. In the current study, we show upregulation of TIARP by IL-6. Given our findings, further experiments specifically stimulating or suppressing TIARP synthesis are now required to establish whether this protein might be involved in regulating IL-6- and TNFα-dependent insulin sensitivity and adipocyte metabolism.

Recently, major steps in IL-6 signaling have been elucidated. Thus, it has been shown that IL-6 induces gp130 homodimerization at the plasma membrane [22]. Upon binding of IL-6, gp130-associated kinases such as Jak1, Jak2, and Tyk2 become activated and phosphorylate the cytoplasmic tail of gp130 [23]. In the current study, we show that pharmacological inhibition of Jak2 by AG490 inhibits both, basal and IL-6-induced TIARP mRNA expression but we also demonstrate that TIARP synthesis is still stimulated by IL-6 in the presence of AG490. Thus, it is possible that this compound may have an independent repressive effect on the TIARP promoter and that IL-6 is not able to completely reverse this effect. Signal transducer and activator of transcription (Stat) 1 and 3, as well as SH2 domain-containing tyrosine phosphatase 2, bind to the tyrosine-phosphorylated gp130 and stimulate downstream signaling proteins such as p44/42 MAP kinase, p38 MAP kinase, and PI 3-kinase [23]. Since pharmacological inhibition of p44/42 MAP kinase, p38 MAP kinase, and PI 3-kinase does not influence IL-6-induced TIARP expression they are probably not involved in TIARP mRNA regulation by IL-6. Furthermore, a 'dedifferentiation' or other toxic effect of IL-6 is unlikely since induction of TIARP synthesis is reversible upon removal of the hormone. Moreover, preliminary studies do not reveal a strong effect of rosiglitazone on the induction of TIARP mRNA by IL-6 (data not shown).

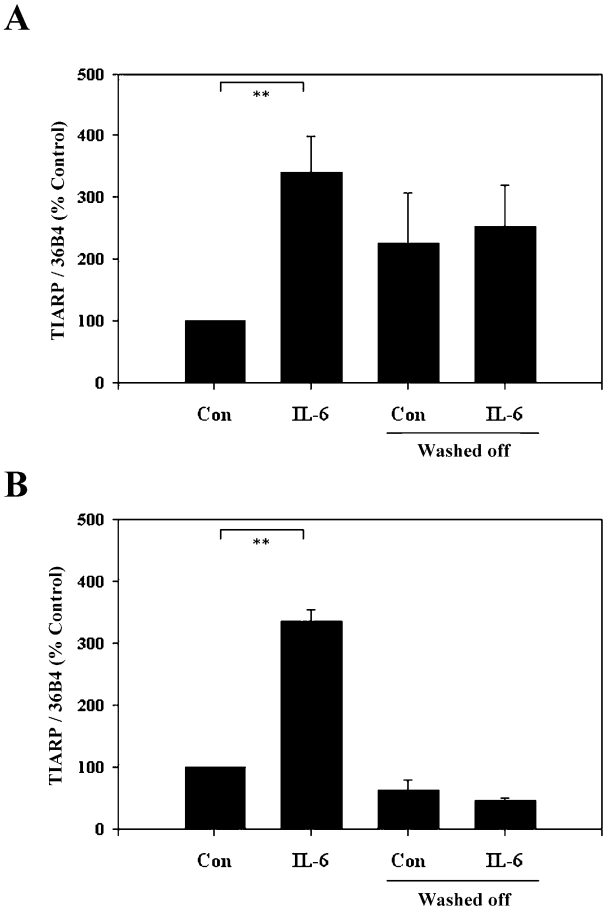

Fig. 3. Stimulation of TIARP expression by IL-6 is reversible. 3T3-L1 adipocytes were serum-starved for 6 h before IL-6 (30 ng/ml) was added for 16 h (columns 1, 2). After this period cells were washed and maintained in A: culture medium or B: serum-free DMEM for an additional 24 h (columns 3, 4). Total RNA was extracted and quantitative real-time RT-PCR was performed as described in Section 2. TIARP gene expression normalized to 36B4 mRNA levels is expressed relative to untreated control (Con) cells (= 100%). Results are the means ± S.E.M. of at least three independent experiments. ** denotes $P < 0.01$ comparing non-treated with IL-6-treated adipocytes.

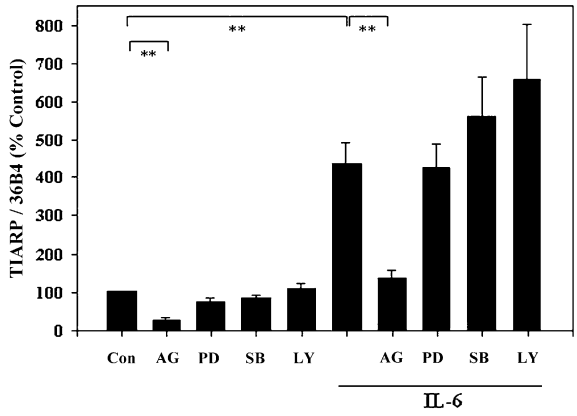

Fig. 4. TIARP mRNA expression is downregulated by the Jak2 inhibitor AG490. After serum starvation for 5 h, 3T3-L1 adipocytes were maintained in the presence or absence of AG490 (AG, 10 μM), PD98059 (PD, 50 μM), SB203580 (SB, 20 μM), or LY294002 (LY, 10 μM) for 1 h before IL-6 (30 ng/ml) was added for 16 h. After extraction of total RNA, quantitative real-time RT-PCR was performed as described in Section 2. TIARP gene expression normalized to 36B4 mRNA is given relative to untreated control (Con) cells (= 100%). Results are the means ± S.E.M. of three independent experiments. ** denotes $P < 0.01$ comparing untreated with AG490-pretreated or IL-6-treated cells, as well as comparing IL-6-treated with AG490-pretreated adipocytes.

Taken together, we demonstrate for the first time a significant induction of TIARP mRNA and protein expression by IL-6 in 3T3-L1 cells. Furthermore, we present evidence that the positive effect of IL-6 is reversible. These data indicate that TIARP expression is acutely and selectively regulated in adipose tissue by a cytokine implicated in insulin resistance. Further studies are needed to more clearly define its function in insulin sensitivity and adipocyte metabolism.

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