GH 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) has recently been cloned as a TNF α -stimulated protein expressed in adipocytes. Its expression is differentiation-dependent and potentially involved in mediating TNF α -induced insulin resistance. To further characterize regulation of TIARP gene expression, 3T3-L1 adipocytes were treated with key hormones modulating insulin sensitivity and influencing adipocyte metabolism, and TIARP gene expression was determined by quantitative real-time RT-PCR. Interestingly, TIARP mRNA expression was stimulated almost 9-fold after 500 ng/ml GH were added for 16 h whereas addition of 10 μ M isoproterenol, 100 nM insulin and 100 nM dexamethasone for 16 h significantly decreased TIARP gene expression to between 35 and 50% of control levels. In contrast, angiotensin 2 (10 μ M) and triiodothyronine

(1 μ M) did not have any effect. The stimulatory effect of GH was time- and dose-dependent with stimulation occurring as early as 1 h after effector addition and at concentrations as low as 5 ng/ml GH. Moreover, pharmacological inhibition of Janus kinase 2 and p42/44 mitogen-activated protein kinase reversed the stimulatory effect of GH, suggesting that both signaling molecules are involved in activation of TIARP gene expression by GH. Furthermore, an increase of TIARP mRNA could be completely reversed to control levels by withdrawal of GH for 24 h. Taken together, these results show that TIARP is not only responsive to TNF α but also to important other hormones influencing glucose homeostasis and adipocyte metabolism. Thus, this factor may play an integrative role in the pathogenesis of insulin resistance and its link to obesity.

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Introduction

Insulin resistance is an important risk factor in the development of atherosclerosis, hypertension and type 2 diabetes. Insulin resistance is frequently associated with obesity. Moreover, various factors linking increased adiposity and reduced sensitivity of target tissues to insulin have been described recently (Kahn *et al.* 2000). Thus, it has been shown that adipocytes secrete biologically active molecules such as tumor necrosis factor (TNF) α (Hotamisligil 1999), resistin (Steppan *et al.* 2001) and adiponectin (Yamauchi *et al.* 2001), which profoundly influence insulin sensitivity. However, downstream targets of these adipocytokines potentially mediating their effect on glucose homeostasis and adipocyte metabolism are not completely clear. Recently, a novel TNF α -responsive gene was cloned from fat cells by differential display technique and named TNF α -induced adipose-related protein (TIARP) (Moldes *et al.* 2001). It could be demonstrated convincingly that TIARP is induced dose- and time-dependently by TNF α and during adipogenic con-

version of 3T3-L1 cells (Moldes *et al.* 2001). Furthermore, it was shown that TIARP is a transmembrane protein preferentially present at the plasma membrane (Moldes *et al.* 2001). Moreover, sequence analysis demonstrated that the general structure of TIARP is reminiscent of various channels or transporters (Moldes *et al.* 2001). Although the biological function of TIARP is presently unknown, these data suggest that TIARP might participate in adipocyte maturation and metabolism and mediate some TNF α effects on fat cells.

Besides TNF α , various other hormones such as insulin, isoproterenol, dexamethasone, angiotensin (AT) 2, growth hormone (GH) and triiodothyronine (T3) have been shown to cause insulin resistance *in vivo* and *in vitro* and affect adipocyte metabolism profoundly (Fasshauer *et al.* 2002a,b). In the current study, we, therefore, examined the effect of these hormones on TIARP gene expression in 3T3-L1 adipocytes *in vitro*. We demonstrate for the first time that GH potently and reversibly induces TIARP gene expression whereas isoproterenol, insulin and dexamethasone suppress TIARP mRNA. Furthermore, we

Figure 1 Differentiation-dependent TIARP gene expression. Confluent 3T3-L1 cells (day 0) were differentiated as described in Materials and Methods. At the days indicated total RNA was subjected to quantitative real-time RT-PCR and TIARP mRNA levels were determined relative to cells on day 0 (=100%). Results are the means $\pm$ S.E. of four independent experiments. ** $P < 0.01$ comparing confluent cells (day 0) with differentiated adipocytes (day 6, 9). Inset, agarose gel electrophoresis of the PCR product at cycle 25. d, day.

present evidence that the stimulatory effect of GH is mediated via Janus kinase (JAK) 2- and p44/42 mitogen-activated protein (MAP) kinase-dependent pathways. These results indicate that TIARP is highly regulated by a number of insulin resistance-inducing hormones besides TNF α . Thus, our data may indicate a potential role of this novel adipose-expressed molecule in the control of adipocyte metabolism and glucose homeostasis.

Materials and Methods

Materials

TNF α , PD98059, SB203580, LY294002, AG490, isoproterenol, GH, dexamethasone, T3 and AT2 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Insulin was from Roche Molecular Biochemicals (Mannheim, Germany). Oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany), cell culture reagents from Life Technologies, Inc. (Grand Island, NY, USA).

Cell culture and differentiation

3T3-L1 adipocytes (American Type Culture Collection, Rockville, MD, USA) were grown and differentiated under conditions previously described (Fasshauer *et al.*

2001a). Briefly, cells were grown to confluence in DMEM containing 25 mM glucose, 10% fetal bovine serum and antibiotics (culture medium) and confluent preadipocytes were induced for 3 days in culture medium further supplemented with 1 μ M insulin, 0.5 mM isobutylmethylxanthine, and 1 μ M dexamethasone before cells were maintained for another 3 days in culture medium with 1 μ M insulin alone. After an additional 4–8 days in culture medium, more than 90% of cells showed adipocyte morphology.

Analysis of TIARP gene expression

TIARP mRNA expression was analyzed by real-time RT-PCR in a fluorescent temperature cycler (LightCycler; Roche Molecular Biochemicals) as previously described (Fasshauer *et al.* 2001b). Briefly, total RNA was isolated with TRIzol reagent (Life Technologies) and reverse transcribed in a 20 μ l reaction using standard reagents (Life Technologies). Two microliters of each RT reaction were loaded into capillary tubes and amplified in a 20 μ l PCR containing 3 mM MgCl₂, 0.5 μ M each primer and 1 $\times$ LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals). Samples were incubated in the LightCycler for an initial denaturation at 94 $^{\circ}$ C for 30 s, followed by 40 PCR cycles, each consisting of 95 $^{\circ}$ C for 1 s, 61 $^{\circ}$ C for 7 s, and 72 $^{\circ}$ C for 11 s.

Figure 2 TIARP mRNA expression is induced by GH. Fully differentiated 3T3-L1 adipocytes were serum-deprived for 6 h before TNF α (TNF, 10 ng/ml), GH (500 ng/ml), isoproterenol (Iso, 10 μ M), insulin (Ins, 100 nM), dexamethasone (Dexa, 100 nM), AT2 (10 μ M) and T3 (1 μ M) were added for 16 h. Total RNA was subjected to quantitative real-time RT-PCR and TIARP mRNA levels normalized to 36B4 were determined as described in Materials and Methods. TIARP gene expression is expressed relative to untreated control (Co) cells (=100%). Results are the means $\pm$ S.E. of at least four independent experiments. **Significant ($P < 0.01$) upregulation, XXsignificant ($P < 0.01$) downregulation comparing hormone-treated with non-treated cells.

The following primers were used: TIARP (accession no. AJ319746) TAGGGTGTAGGCGAGCAGCAGT (sense) and TCAGTGACACGCGGGAAGATT (anti-sense); 36B4 (accession no. NM007475) AAGCGCGTC CTGGCATTGTCT (sense) and CCGCAGGGGCAG CAGTGGT (antisense). SYBR Green I fluorescence emission readings were monitored after each cycle and TIARP and 36B4 mRNA levels were quantified by the second derivative maximum method of the LightCycler Software (Roche Molecular Biochemicals). This method determines the crossing points of individual samples by an algorithm identifying the first turning point of the fluorescence curve. TIARP expression is given relative to 36B4, which has been frequently used as an internal control due to its resistance to hormonal regulation (Lin *et al.* 2002).

Melting curve profiles (cooling the sample to 68 $^{\circ}$ C and heating slowly to 95 $^{\circ}$ C with continuous measurement of fluorescence) were produced at the end of each PCR to confirm amplification of specific transcripts. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Western blotting

Detection of phosphorylated p44/42 MAP kinase by Western blotting was performed essentially as described

previously (Klein *et al.* 1999). Briefly, after the stimulation period cells were harvested in lysis buffer (50 mM Hepes, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM Na₄P₂O₇, 10 mM NaF, 2 mM EDTA, 10% glycerol, 1% of the nonionic detergent Igepal CA-630, 2 mM vanadate, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 2 mM phenyl-methylsulfonyl fluoride, pH 7.4). Lysates were clarified and equal amounts of protein were solubilized directly in Laemmli sample buffer. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, blocked for 1 h, and immunoblotted with a phospho-specific p44/42 MAP kinase antibody. Specifically bound primary antibodies were detected with peroxidase-coupled secondary antibody and enhanced chemiluminescence.

Statistical analysis

Results are expressed as means $\pm$ S.E. Comparisons between groups were carried out by unpaired Student's *t*-test and in the case of multiple time points and treatments by one-way ANOVA. *P* values < 0.05 are considered significant, < 0.01 highly significant.

Results

TIARP is induced during adipogenesis

TIARP gene expression was analyzed during the course of differentiation. As shown in Fig. 1, TIARP mRNA

Figure 3 Dose- and time-dependent stimulation of TIARP gene expression by GH and TNF α . Fully differentiated 3T3-L1 adipocytes were serum-deprived before the indicated concentrations of (A) GH and (B) TNF α were added for 16 h or (C) 50 ng/ml GH and (D) 20 ng/ml TNF α were added for different periods of time. After total RNA extraction, TIARP expression normalized to 36B4 mRNA was determined by quantitative real-time RT-PCR as described in Materials and Methods. Data are expressed relative to untreated control cells (= 100%). Results are the means $\pm$ S.E. of at least three independent experiments. * $P < 0.05$ and ** $P < 0.01$ comparing non-treated with GH- or TNF α -treated cells.

synthesis was strongly induced during differentiation with a rapid exponential increase in mRNA levels within the last 6 days of a 9 day differentiation course ($P < 0.01$).

GH is a potent stimulator of TIARP gene expression

As TIARP has recently been shown to be upregulated by TNF α , we tested whether different hormones which also induce insulin resistance might influence TIARP gene expression in 3T3-L1 adipocytes *in vitro*. Consistent with previous findings, TIARP gene expression was stimulated more than 5-fold by treatment of adipocytes with 10 ng/ml TNF α ($P < 0.01$) (Fig. 2). Interestingly, TIARP mRNA synthesis was stimulated almost 9-fold after 500 ng/ml GH were added for 16 h ($P < 0.01$) (Fig. 2). In contrast, addition of 10 μ M isoproterenol, 100 nM insulin and 100 nM dexamethasone for 16 h significantly decreased TIARP gene expression to between 35 and 50%

of control levels ($P < 0.01$) (Fig. 2). AT2 (10 μ M) and T3 (1 μ M) did not significantly influence expression of TIARP (Fig. 2).

Stimulation of TIARP gene expression by GH and TNF α is dose- and time-dependent

GH stimulated TIARP gene expression in a dose-dependent fashion. Thus, a significant 2.3-fold increase of TIARP mRNA was detectable at GH concentrations as low as 5 ng/ml ($P < 0.01$) (Fig. 3A). A maximal more than 8-fold increase was found at 500 ng/ml GH ($P < 0.01$) (Fig. 3A). TNF α increased TIARP gene expression dose-dependently with a significant 2-fold stimulation detectable already at 1 ng/ml effector ($P < 0.01$) (Fig. 3B). Furthermore, GH induced TIARP synthesis time-dependently with significant activation detectable as early as 2 h after effector addition and a maximal effect after 8 h

Figure 4 Stimulatory effect of GH is mediated via JAK2 and p44/42 MAP kinase. After 5 h serum-starvation, 3T3-L1 cells were cultured in the presence or absence of AG490 (AG, 10 μ M), PD98059 (PD, 50 μ M), SB203580 (SB, 20 μ M) or LY294002 (LY, 10 μ M) for 1 h before GH (500 ng/ml) was added for 1 h. After extraction of total RNA, quantitative real-time RT-PCR was performed as described in Materials and Methods. TIARP gene expression normalized to 36B4 mRNA is expressed relative to untreated control (Con) cells (=100%). Results are the means $\pm$ s.e. of three independent experiments. ** $P < 0.01$ comparing GH-treated with untreated or AG490- and PD98059-pretreated adipocytes.

of hormone treatment ($P < 0.05$) (Fig. 3C). Similarly, maximal more than 10-fold induction of TIARP mRNA was detectable after 8 h of TNF α -treatment ($P < 0.01$) (Fig. 3D).

Stimulation of TIARP gene expression by GH is mediated via JAK2 and p44/42 MAP kinase

Various effects of GH have been shown to be exerted via activation of JAK2, p44/42 MAP kinase, p38 MAP kinase or phosphatidylinositol 3-kinase (PI 3-kinase) (Frank 2001). To test whether these signaling molecules might play a role in stimulating TIARP mRNA expression, 3T3-L1 adipocytes were pretreated with specific pharmacological inhibitors for 1 h before GH (500 ng/ml) was added for another 1 h. None of the pharmacological inhibitors alone significantly affected basal TIARP gene expression (Fig. 4). Treatment of 3T3-L1 adipocytes with GH for 1 h increased TIARP mRNA expression almost 3-fold. Interestingly, inhibition of JAK2 and p44/42 MAP kinase with AG490 (10 μ M) and PD98059 (50 μ M) respectively, significantly and almost completely blocked this stimulatory effect of GH ($P < 0.01$) (Fig. 4). In contrast, treatment with the p38 MAP kinase inhibitor SB203580 (20 μ M) and the PI 3-kinase inhibitor LY294002 (10 μ M) did not impair the stimulatory effect of GH on TIARP gene expression (Fig. 4).

GH stimulates p44/42 MAP kinase at least partially via protein kinase C (PKC)

Since p44/42 MAP kinase appears to be involved in GH-induced upregulation of TIARP its regulation by GH was determined. As shown in Fig. 5A, GH (50 ng/ml) stimulated p44/42 MAP kinase as assessed with a phospho-specific antibody and with maximal effects seen at 15 min after effector addition. Furthermore, it was determined whether JAK2 and PKC might mediate stimulation of p44/42 MAP kinase by GH. Interestingly, pretreatment of 3T3-L1 adipocytes with the JAK2 inhibitor AG490 (10 μ M) did not significantly influence GH-induced MAP kinase phosphorylation (Fig. 5B). In contrast, addition of the PKC inhibitor GF109203X (5 μ M) significantly decreased p44/42 MAP kinase activation by 53% as compared with GH alone ($P < 0.01$) (Fig. 5B). As expected, PD98059 completely blocked MAP kinase activation ($P < 0.01$) (Fig. 5B).

The stimulatory effect of GH on TIARP gene expression is reversible

Finally, we determined whether the stimulatory effect of GH on TIARP gene expression was reversible. Fully differentiated 3T3-L1 adipocytes were treated with 500 ng/ml (Fig. 6A) or 50 ng/ml GH (Fig. 6B) for 16 h and the medium was then replaced by DMEM containing 25 mM glucose and 10% fetal bovine serum for an additional 24 h. Addition of GH again increased TIARP mRNA expression by about 8-fold (Fig. 6A, columns 1 and 2) and 3-fold (Fig. 6B, columns 1 and 2) respectively, as compared with untreated control cells. However, removal of GH from the medium for 24 h decreased TIARP mRNA expression to control levels (Fig. 6, columns 3 and 4). Interestingly, addition of serum-containing medium for an additional 24 h appeared to stimulate TIARP gene expression about 2-fold (Fig. 6, columns 1 and 3).

Discussion

Recently, Moldes *et al.* (2001) described a novel interesting protein, TIARP, which is strongly induced by TNF α and adipose conversion and might mediate some effects of TNF α on insulin sensitivity and adipose metabolism. In the current study, we show for the first time that GH is another potent stimulator of TIARP gene expression in insulin-sensitive 3T3-L1 adipocytes. GH is produced primarily in the anterior pituitary gland as a 22 kDa polypeptide (Frank 2001). Interestingly, GH, like TNF α , potently antagonizes insulin action on insulin-sensitive tissues such as muscle, fat and liver *in vivo* and *in vitro*. Thus, it has been shown that patients with GH excess due to pituitary tumors are insulin-resistant (Rizza *et al.* 1982, Hansen *et al.* 1986). The role of GH in common insulin

Figure 5 GH activates p44/42 MAP kinase via PKC. After serum-starvation for 16 h, (A) GH (50 ng/ml) or insulin (100 nM) was added for the indicated periods of time or (B) the cells were cultured in the presence or absence of AG490 (AG, 10 μ M), GF109203X (GFX, 5 μ M), and PD98059 (PD, 50 μ M) for 1 h before addition of GH (50 ng/ml) for 15 min. After extraction of total protein, Western blotting with a phospho-specific p44/42 MAP kinase antibody was performed as described in Materials and Methods. In (A) a representative blot of two independent experiments is shown. In (B) the amount of phosphorylated p44/42 MAP kinase relative to GH-treated cells (=100%) is shown. Results are the means $\pm$ S.E. of three independent experiments. ** $P < 0.01$ comparing GH-treated with non-treated, GF109203X- and PD98059-pretreated cells. Furthermore, a representative blot is shown.

resistance and obesity is less clear. On the one hand, it has been suggested that nocturnal GH secretion in diabetic patients may contribute to nocturnal hyperglycemia

(Campbell *et al.* 1985). On the other hand, in obese insulin-resistant individuals basal and stimulated GH secretion have been shown to be low (Bjorntorp &

Figure 6 Stimulation of TIARP expression by GH is reversible. 3T3-L1 adipocytes were serum-starved for 6 h before (A) 500 ng/ml GH or (B) 50 ng/ml GH were added for 16 h (columns 1, 2). After this period cells were washed and maintained in culture medium for an additional 24 h (columns 3, 4). After extraction of total RNA, quantitative real-time RT-PCR was performed as described in Materials and Methods. TIARP gene expression normalized to 36B4 mRNA levels is expressed relative to untreated control (Con) cells (=100%). Results are the means $\pm$ S.E. of (A) two and (B) four independent experiments. ** $P < 0.01$ comparing GH-treated with non-treated and GH-washed-off cells.

Rosmond 1999, Franco *et al.* 2001). *In vivo* studies have consistently shown that insulin resistance in rats caused by chronic GH treatment is accompanied by a decrease in insulin-stimulated insulin receptor (IR) activity and IR substrate (IRS) protein phosphorylation (Smith *et al.* 1997, Thirone *et al.* 1997). However, signaling pathways and proteins mediating this effect are far from clear. Although little is known about TIARP function, its strong upregulation by both GH and TNF α indicates that it might

participate in controlling insulin sensitivity and adipocyte metabolism.

Binding of GH to its receptor monomer results in receptor dimerization and activation of the cytosolic tyrosine kinase JAK2 (Frank 2001). Consistent with JAK2 being the major intracellular mediator of GH action, inhibition of this tyrosine kinase by AG490 abolishes GH-induced stimulation of TIARP gene expression in the current study. Various proteins have been implicated in the signaling downstream of JAK2. Thus, it has been demonstrated that signal transducer and activator of transcription 5b is tyrosine phosphorylated in the cytoplasm upon GH treatment, dimerizes via phosphotyrosine-SH2 interactions and translocates to the nucleus affecting target gene activation (Frank 2001). Furthermore, it was shown that GH potently induces PI 3-kinase activity and that inhibition of PI 3-kinase by wortmannin completely blocked the anti-lipolytic effect of GH in 3T3-L1 adipocytes (Yamauchi *et al.* 1998). Moreover, both p38 and p44/42 MAP kinases are stimulated by GH and have been implicated in GH-stimulated mitogenesis (Zhu & Lobie 2000). In the current study, we show that inhibition of p44/42 MAP kinase by PD98059 almost entirely abrogates GH-mediated stimulation of TIARP mRNA expression. However, we also demonstrate that p44/42 MAP kinase is not stimulated downstream of JAK2 since pharmacological inhibition of JAK2 does not influence its activation. In contrast, inhibition of PKC by GF109203X blunts p44/42 MAP kinase stimulation in accord with previous reports (MacKenzie *et al.* 1997). These findings point to the fact that both JAK2- and PKC-p44/42 MAP kinase-dependent pathways are necessary for activation of TIARP by GH. In contrast, inhibition of p38 MAP kinase by SB203580 or PI 3-kinase by LY294002 does not significantly alter induction of TIARP mRNA by GH indicating that these proteins do not play major roles in the regulation of TIARP gene expression. Furthermore, the effect of GH on TIARP gene expression is reversible as removal of the hormone for 24 h decreases TIARP mRNA to control levels.

Besides GH and TNF α , various other hormones have been shown to induce insulin resistance and affect adipocyte metabolism profoundly. Thus, growing evidence suggests that increased activity of the sympathetic nervous system contributes to insulin resistance (Reaven *et al.* 1996). We have recently delineated molecular mechanisms potentially involved in crosstalk between adrenergic and insulin signaling (Klein *et al.* 1999, 2000). Furthermore, our group recently demonstrated that patients with pheochromocytoma show insulin resistance due to increased serum levels of catecholamines, which can be reduced by surgical removal of the tumors in most cases (Bluher *et al.* 2000). Furthermore, hyperinsulinemia caused by peripheral insulin resistance contributes to the development of type 2 diabetes (Gerich 1998). Moreover, glucocorticoids have long been known to cause insulin

resistance *in vivo* and our group recently suggested that downregulation of insulin-sensitizing adiponectin in adipocytes might be one mechanism by which they mediate their effect (Andrews & Walker 1999, Fasshauer *et al.* 2002b). In the present study, we show for the first time that isoproterenol, insulin and dexamethasone potently downregulate TIARP gene expression in 3T3-L1 adipocytes. Therefore, our data indicate that TIARP might also mediate some cellular effects of β -adrenoceptor agonists, insulin and glucocorticoids. Furthermore, the inhibitory effect of isoproterenol on TIARP gene expression is mediated via increased intracellular levels of cAMP (data not shown) pointing to the fact that a β -adrenergic receptor- G_s -protein-adenylyl cyclase pathway downregulates TIARP mRNA synthesis whereas JAK2- and p44/42 MAP kinase-dependent pathways activate TIARP expression.

T3 and AT2 also affect glucose tolerance at least partly via decreased activity of insulin signaling molecules such as IRS proteins essential for insulin action (Fickova *et al.* 1997, Folli *et al.* 1997, Fasshauer *et al.* 2000, 2001c). However, both hormones do not appear to influence TIARP gene expression in our experimental system *in vitro*.

Taken together, we demonstrate for the first time significant upregulation of TIARP gene expression by GH in 3T3-L1 adipocytes whereas isoproterenol, insulin and dexamethasone potently downregulate TIARP mRNA. Furthermore, we present evidence that the positive effect of GH is mediated via JAK2- and p44/42 MAP kinase-dependent pathways whereas isoproterenol inhibits TIARP gene expression via a classical G_s -protein-coupled pathway. These data indicate that TIARP expression is a selectively regulated mechanism that might constitute an important element in the pathogenesis of insulin resistance and obesity. Further studies are needed to more clearly define TIARP function in insulin sensitivity and adipocyte metabolism.

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