FoxO1 Controls Insulin-dependent Adipose Triglyceride Lipase (ATGL) Expression and Lipolysis in Adipocytes

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FoxO1 represents a central regulator of metabolism in several cell types. Although FoxO1 is abundant in adipocytes, its biological functions in these cells remain largely unknown. We show here that the promoter region of the rate-limiting lipolytic enzyme, adipose triglyceride lipase (ATGL), has two FoxO1-binding sites, and co-transfection with wild type and unphosphorylated FoxO1 mutant activates the expression of luciferase driven by the ATGL promoter. In 3T3-L1 adipocytes, insulin controls nucleo-cytoplasmic shuttling of FoxO1 and regulates its interaction with endogenous ATGL promoters. Knockdown of FoxO1 in 3T3-L1 adipocytes decreases the expression of ATGL and attenuates basal and isoproterenol-stimulated lipolysis. Infection of mouse embryonic fibroblasts with FoxO1-encoding lentivirus increases ATGL expression and renders it sensitive to regulation by insulin. Thus, FoxO1 may play an important role in the regulation of lipolysis in adipocytes by controlling the expression of ATGL.

One of the key physiological functions of insulin in the mammalian organism is to inhibit lipolysis and to promote accumulation and storage of triglycerides in fat tissue. Control of lipolysis plays an important role in energy partitioning and balance and maintains the size of fat depots in the body. In patients with insulin resistance and type 2 diabetes, insulin cannot suppress high levels of free fatty acids (FFA) in the bloodstream (1) so that the availability of FFA exceeds the energy requirements of the body. As a result, FFA are accumulated in the form of lipids in non-adipose peripheral tissues, such as liver and skeletal muscle, aggravating insulin resistance and causing multiple hazardous metabolic effects (2, 3). In addition, excess of FFA may lead to overproduction of VLDL in the liver and predispose the organism to cardiovascular disease.

The complete hydrolysis of triglycerides to glycerol and FFA is performed jointly by tri-, di-, and monoacylglyceride lipases (4, 5). Recently discovered adipose triglyceride lipase ATGL (6–8) is responsible for the bulk of triacylglycerol hydrolysis activity in cells and has low affinity to di- and monoacylglycerides (4, 5). Importantly, ATGL is now considered the rate-limiting lipolytic enzyme in mammals (9), flies (10), and yeast (11). The major diacylglyceride lipase in adipocytes is hormone-sensitive lipase, or HSL (5). ATGL- and HSL-mediated hydrolysis activity is controlled by catecholamines primarily via the cAMP-mediated-phosphorylation of perilipin (12, 13), the major lipid droplet-forming protein in adipocytes (14). Monoacylglyceride lipase in adipocytes is believed to be hormone-independent (4).

The effect of insulin on lipolysis is attributed to the stimulation of activity and/or the expression of cyclic nucleotide phosphodiesterases 3B and 4 (15–17), which decrease intracellular levels of cAMP and reverse the stimulatory effect of cAMP-dependent protein kinase on lipolysis (5). In parallel, insulin may suppress lipolysis in a cAMP-independent fashion by stimulating protein phosphatase 1 and by promoting re-esterification of fatty acids (reviewed in Ref. 5).

It has also been shown that insulin decreases the expression of ATGL at the level of transcription (18–20). The molecular mechanism of this effect has not yet been elucidated. Although it has been recently shown that transcription of ATGL is regulated by PPARγ (20, 21), it is yet not clear whether and to what extent PPARγ is responsible for the regulation of ATGL by insulin. Here, we decided to explore the role of the insulin-responsive transcription factor, FoxO1, in the regulation of ATGL expression and lipolysis.

FoxO1 represents a central regulator of metabolism in several cell types and tissues (22, 23). Its activity is negatively regulated by insulin and growth factors via Akt-mediated phosphorylation and nuclear exclusion (24). Although FoxO1 is abundant in adipocytes, its biological functions in these cells remain largely unknown. We are showing here that insulin controls the expression of the rate-limiting lipolytic enzyme, ATGL, via FoxO1.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antibodies against ATGL and aP2 were kind gifts of Dr. A. Greenberg (Tufts University) and Dr. S. Farmer (Boston University School of Medicine). A rabbit polyclonal antibody against cellugyrin was described previously (25). Polyclonal anti-FoxO1, anti-i4E-BP, anti-PPARγ, and CEBPα antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-V5 tag antibody and monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody were obtained from Sigma. Monoclonal antibody against RNA polymerase II was from Millipore Corp. (Bedford, MA).

Cell Culture—3T3-L1 preadipocytes were cultured, differentiated, and maintained as described previously (25). Mouse embryonic fibroblasts (MEFs) and 293T cells were cultured in...
DMEM supplemented with 10% fetal bovine serum in 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. MEFs were infected with pLenti-m1-V5-FoxO1 according to the Invitrogen protocol. Selection of cells was performed with blasticidin (10 μg/ml) for 7–10 days, and pooled clones were used for further experiments.

Cell Fractionation—Nuclear and cytoplasmic fractions were isolated from 3T3-L1 adipocytes using NE-PER nuclear and cytoplasmic extraction reagents (Pierce).

Quantitative PCR—Total RNA was extracted from differentiated 3T3-L1 cells using the TRIzol reagent (Invitrogen). Reverse transcription of 500 ng of total RNA was performed using random decamers (RETROscript kit; Ambion, Austin, TX), and ATGL mRNA was determined by quantitative PCR with the help of the MX4000 multiplex qPCR system (Stratagene, La Jolla, CA). Reactions were performed in triplicate in the total volume of 25 μl containing 2.5 μl of 1:10 diluted cDNA, 1× SYBR green master mix (Brilliant II SYBR Green qPCR master mix; Stratagene), and gene-specific primers (for ATGL, 5’-AAGACACATCCGTTCCAA-3’ and 5’-GGTTCAGTAGCCATTCTCC-3’; for 36B4, 5’-TCACTCAGGCTGTGGTACCA-3’ and 5’-GGGACGAGGCAACAGTT-3’). ATGL expression was normalized by 36B4 expression for the ΔΔCt method. Deoxyribonuclease-treated samples and no-template controls were analyzed in parallel experiments to confirm specificity.

Chromatin Immunoprecipitation (ChIP)—ChIP studies were carried out in 3T3-L1 adipocytes using the EZ-chIP kit (Millipore) according to the manufacturer’s instructions. Briefly, proteins were cross-linked to DNA with 18.5% formaldehyde, lysed, and sonicated seven times for 15 s. FoxO1 proteins were then immunoprecipitated from precleared lysates. Protein-DNA complexes were eluted and treated with proteinase K. Purified DNA was subjected to PCR using the following primers: 5’-ATCTTTAAAGGCAATTAAGCTG-3’ and 5’-TCATCCAGGCTGTGGTACCA-3’ and 5’-GGGACGAGGCAACAGTT-3’. ATGL expression was normalized by 36B4 expression for the ΔΔCt method. Deoxyribonuclease-treated samples and no-template controls were analyzed in parallel experiments to confirm specificity.

Results—Differentiated 3T3-L1 adipocytes were incubated in DMEM with 2% fatty acid-free bovine serum albumin for 2 h at 37 °C in the presence or in the absence of 10 μM isoproterenol. Glycerol content in the media was measured colorimetrically at 540 nm using the triglyceride (glycerol phosphate oxidase) reagent set (Pointe Scientific, Canton, MI) against a set of glycerol standards. Cells were then washed with cold PBS and lysed in 1% Triton X-100 buffer, and the protein concentration was determined and used to normalize glycerol release. All the experiments were carried out in triplicates.

Gel Electrophoresis and Western Blotting—Proteins were separated in SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore) in 25 mM Tris, 192 mM glycine. After transfer, the membrane was blocked with 10% nonfat milk in PBS with 0.5% Tween 20 for 2 h. The blots were probed overnight with specific primary antibodies at 4 °C and 1 h with horseradish peroxidase-conjugated secondary antibodies (Pierce) at room temperature and detected with an enhanced chemiluminescence substrate kit (PerkinElmer Life Sciences) using a Kodak image station 440CF (Eastman Kodak Co.).

Statistics—Student’s paired two-tailed t test was used to evaluate the statistical significance of the results.

RESULTS

Incubation of 3T3-L1 cells with insulin for 16 h decreases the rate of basal and isoproterenol-stimulated lipolysis in these cells (supplemental Fig. 1A). On the contrary, removal of serum from the incubation media for the same period of time increases lipolysis (supplemental Fig. 1B). The effect of isoproterenol on lipolysis in adipocytes that have been serum-starved for 16 h is undetectable (not shown). In accord with these results, we have found that insulin administration decreases the expression of the rate-limiting lipolytic enzyme, ATGL, at the level of both mRNA (supplemental Fig. 1C) and protein (supplemental Fig. 1D), whereas serum starvation has the opposite effect (supplemental Fig. 1, E and F) (see also Refs. 18–20).

As treatment of 3T3-L1 adipocytes with the PI3 kinase inhibitor wortmannin completely reverses insulin action on ATGL expression and lipolysis (supplemental Fig. 1, A and D), we have...
suggested that ATGL could be a target of FoxO1, which represents the most abundant FoxO isoform in adipocytes (27) and is regulated by insulin via the PI3 kinase-Akt pathway (28). Analysis of the ~3-kb 5′-promoter region of mouse ATGL using Genomatix software demonstrates the presence of at least two FoxO1-binding sites (supplemental Fig. 2). Furthermore, the expression of FoxO1 in differentiating 3T3-L1 adipocytes reaches the maximum on day 3 of differentiation (Fig. 1A) (see also Ref. 27) simultaneously with the induction of ATGL (Fig. 1A). To determine whether or not FoxO1 can regulate the expression of ATGL mRNA, we co-transfected 293T cells with the luciferase reporter construct containing the ATGL promoter (20) together with FoxO1 cDNA. As is shown in Fig. 1B, wild-type FoxO1 significantly increases the expression of luciferase (ca. 6-fold), and constitutively active unphosphorylated FoxO1 mutant (AAA) demonstrates even higher activity (ca. 10-fold). This effect has been further confirmed in 3T3-L1 adipocytes and mouse embryonic fibroblasts (not shown).

Insulin regulates functional activity of FoxO1 in several cell types by controlling its nucleo-cytoplasmic distribution (28). It is yet unknown, however, whether or not this mechanism is active in adipose cells. Using immunofluorescence staining

FIGURE 1. ATGL is a transcriptional target of FoxO1. A, 3T3-L1 adipocytes were differentiated for the indicated time periods, and FoxO1 and ATGL protein levels were detected in whole cell lysates (50 μg). CEBPα was used as positive control for differentiation and cellugyrin as loading control. B, 293T cells were transiently transfected with 2979/–21 luciferase (LUC) ATGL promoter construct together with empty vector (EV), FoxO1, or FoxO1-AAA constructs. For normalization of transfection efficiency, cells were co-transfected with eGFP. After 48 h, cells were harvested in the reporter lysis buffer. Luciferase activity in cell lysates was assayed as described under "Experimental Procedures" and normalized by GFP fluorescence. Data are presented for triplicate samples as mean ± S.D. (error bars). **, p < 0.001. C, differentiated 3T3-L1 cells were left in complete medium (Control), incubated in DMEM without serum (Starve), or treated with 100 nM insulin (Ins) for 16 h. Fixed cells were stained with FoxO1 antibody and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (red). 4′,6-Diamidino-2-phenylindole (DAPI) (blue) was incorporated in the mounting solution. D, 3T3-L1 adipocytes were treated as indicated for panel C, and nuclear and cytosolic fractions were isolated and analyzed by Western blotting (50 μg per lane). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and RNA polymerase II (Pol II) were used as controls for cytosolic and nuclear fractions, respectively. E, ChIP assays were performed in 3T3-L1 adipocytes incubated either in complete medium (Serum +) or in DMEM without serum (Serum –) for 16 h. Following cross-linking and sonication, genomic fragments were immunoprecipitated with antibody against FoxO1 or rabbit IgG, and then 221 bp between nucleotides −1004 and −1225 of the ATGL promoter was amplified by PCR, separated in a 3% agarose gel, and visualized by ethidium bromide staining. F, 3T3-L1 cells were incubated in DMEM (Starve) or DMEM with 100 nM Ins for 16 h. The region between nucleotides −1041 and −1158 of the ATGL promoter in the immunoprecipitated genomic fragments was amplified by SYBR green reaction as described under "Experimental Procedures" and analyzed by quantitative PCR. The experiment was performed in triplicate, and data were expressed as mean ± S.D. (error bars) normalized by input. *, p < 0.05.
mediated knockdown of FoxO1 in differentiated cells does not show to affect adipocyte differentiation (27). However, siRNA-use this effect as a positive control for siRNA action.

Because 4E-BP is a known target of FoxO1 (29, 30), we note that knockdown of FoxO1 also decreases the expression of 4E-BP (Fig. 2C). Thus, although PPARγ is known to regulate the expression of ATGL (20, 21), the effect of FoxO1 knockdown apparently takes place in a PPARγ-independent fashion.

As expected, FoxO1-mediated decrease in the expression of ATGL attenuates the rate of lipolysis in adipocytes (Fig. 2D). Note that this effect does not involve other lipolytic components, such as HSL, perilipin, and aP2, levels of which do not markedly change upon knockdown of FoxO1 (Fig. 2C). We conclude, therefore, that FoxO1 controls lipolysis in adipocytes by regulating the expression of the rate-limiting enzyme, ATGL.

To determine how essential the FoxO1-mediated pathway is for the insulin regulation of ATGL expression, we used mouse embryonic fibroblasts where the expression of FoxO1 is low and, in fact, virtually undetectable (Fig. 3A). Unlike the situation in adipocytes, neither insulin nor serum withdrawal regulates ATGL expression in these cells (Fig. 3A). Stable overexpression of FoxO1 with the help of the lentiviral vector increased ATGL expression in MEFs (Fig. 3B). Moreover, in FoxO1-expressing MEFs, we observed a clear inhibitory effect of insulin on ATGL expression, whereas withdrawal of serum increased the expression of ATGL (Fig. 3B). Thus, FoxO1 is largely responsible for the effect of insulin on ATGL expression.

DISCUSSION

Experiments in vivo (9) and in vitro (19, 31) have demonstrated that the rate of lipolysis in adipocytes and other cells depends on the levels of ATGL expression. As insulin inhibits the expression of ATGL at the level of transcription (19, 20), this phenomenon may help to explain the inhibitory action of insulin on lipolysis as well as its opposing effect on lipid storage.

![FIGURE 2. Knockdown of FoxO1 with siRNA decreases lipolysis and ATGL expression in adipocytes.](image)

![FIGURE 3. FoxO1 confers insulin sensitivity to regulation of ATGL expression in mouse embryonic fibroblasts.](image)

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**FIGURE 2.** Knockdown of FoxO1 with siRNA decreases lipolysis and ATGL expression in adipocytes. A, differentiated 3T3-L1 adipocytes (day 7) were transfected with either 30 nM scrambled (sc) or two different siRNAs against mouse FoxO1 (si#1 and si#2), and total cell lysates were harvested after 48 h. Levels of ATGL protein upon knockdown of FoxO1 were analyzed by Western blotting. B, quantitative analysis of relative band intensities of FoxO1 and ATGL in three independent experiments as described for panel A. Data were presented as mean ± S.D. (error bars). C, analysis of adipocyte differentiation markers and proteins associated with lipolysis upon knockdown of FoxO1. D, glycerol release in the media was determined in cells transfected with either scrambled (sc) or specific (si#2) siRNA for 2 h. Data were expressed as mean ± S.D. (error bars) relative to non-stimulated scrambled cells. *p < 0.05.

**FIGURE 3.** FoxO1 confers insulin sensitivity to regulation of ATGL expression in mouse embryonic fibroblasts. A, Western blot analysis of FoxO1 and ATGL expression in total cell lysates prepared from 3T3-L1 adipocytes (left panels) and MEFs (right panels) incubated either in complete medium (Control) or in DMEM in the absence (Starve) or presence of 100 nM Ins for 16 h. B, MEFs stably overexpressing FoxO1 (MEF-FoxO1) were treated as described for panel A, and total cell lysates were analyzed by Western blotting. Wild-type MEF was shown as control.
However, the mechanism of insulin action on transcription of ATGL has not been understood.

We show here that the expression of ATGL is directly regulated by FoxO1 and that insulin inhibits the expression of ATGL in adipocytes by restraining the nuclear localization of FoxO1. As up-regulation of ATGL results in the depletion of lipid stores (31), we believe that our results are consistent with the earlier report by Nakae et al. (27), who have demonstrated that FoxO1 inhibits fat accumulation in differentiating adipocytes.

The regulatory connection between FoxO1 and lipolysis is conserved in evolution. Thus, in Caenorhabditis elegans, the forkhead transcription factor DAF-16 increases the expression of triglyceride lipase K04A8.5 (32) and, in Drosophila, dFOXO directly up-regulates lipase 4 (33). Intriguingly, activation of K04A8.5 expression in fat storage tissues significantly contributes to the increase in the life span of C. elegans produced by reduced signaling of the insulin receptor/daf-2 (32). Given that genetic ablation of the insulin receptor selectively in fat tissue reduces sufficient amounts of fatty acids for combustion.

Fat-derived fatty acids are not only metabolized in skeletal muscle but also are used for biosynthesis of triglycerides and VLDL in the liver. In this tissue, FoxO1 increases VLDL production (hence, delivery of triglycerides to skeletal muscle for oxidation) by up-regulating the expression of microsomal triglyceride transfer protein (35). Thus, FoxO1 increases lipolysis in fat tissue, stimulates VLDL production in the liver, and boosts lipid oxidation in skeletal muscle; in other words, FoxO1 may be considered as an important regulator of lipid homeostasis and partitioning of triglycerides between different tissues of the mammalian organism.

Finally, it appears that the effects of insulin on gluconeogenesis in the liver and lipolysis in adipocytes are regulated via the same signaling pathway: insulin receptor → insulin receptor substrate → PI3 kinase → Akt → FoxO1. This notion may bring together the “glucocentric” and the “lipocentric” theories of the development of diabetes (36) as the same defect in the insulin signaling pathway may cause abnormalities in both lipid and glucose homeostasis and simultaneously lead to hyperglycemia and hyperlipidemia.

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