Insulin Inhibits Lipolysis in Adipocytes via the Evolutionarily Conserved mTORC1-Egr1-ATGL-Mediated Pathway

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One of the basic functions of insulin in the body is to inhibit lipolysis in adipocytes. Recently, we have found that insulin inhibits lipolysis and promotes triglyceride storage by decreasing transcription of adipose triglyceride lipase via the mTORC1-mediated pathway (P. Chakrabarti et al., Diabetes 59:775–781, 2010), although the mechanism of this effect remained unknown. Here, we used a genetic screen in Saccharomyces cerevisiae in order to identify a transcription factor that mediates the effect of Tor1 on the expression of the ATGL ortholog in yeast. This factor, Msn4p, has homologues in mammalian cells that form a family of early response transcription factors. One member of the family, Egr1, is induced by insulin and nutrients and directly inhibits activity of the ATGL promoter in vitro and expression of ATGL in cultured adipocytes. Feeding animals a high-fat diet increases the activity of mTORC1 and the expression of Egr1 while decreasing ATGL levels in epididymal fat. We suggest that the evolutionarily conserved mTORC1-Egr1-ATGL regulatory pathway represents an important component of the antilipolytic effect of insulin in the mammalian organism.

Current epidemics of metabolic diseases, such as type 2 diabetes, cardiac dysfunction, hypertension, hepatic steatosis, etc., are largely caused by widespread obesity. Although obesity can affect human health via several different mechanisms, the best-established connection between obesity and metabolic disease is elevated and/or dysregulated levels of circulating free fatty acids (FFA). In addition to their direct pathological effects, superfluous FFA accumulate in the form of lipids, and their metabolic products in nonadipose peripheral tissues, such as liver, skeletal muscle, heart, and pancreas and cause detrimental effects on human health via mechanisms that are currently under intense investigation (1–5).

The levels of circulating FFA depend primarily on the rates of lipolysis in the adipose tissue. One of the key physiological functions of insulin as the major anabolic hormone in the body is to restrain lipolysis and to promote fat storage in adipose tissue in the postprandial state. The failure of insulin to suppress lipolysis in adipocytes has been long considered as a very serious metabolic defect and one of the most important if not the most important causative factor of insulin resistance and diabetes mellitus (6, 7).

Complete hydrolysis of triglycerides to glycerol and fatty acids is performed jointly by tri-, di-, and monoacylglyceride lipases (8–11). The recently discovered enzyme, adipose triglyceride lipase (ATGL; also known as desnutrin, PNPLA2, TTS2.2, and iPLA2) (12–14), is responsible for the bulk of triacylglycerol hydrolase activity in various cells and represents the rate-limiting lipolytic enzyme. In every experimental model tested thus far, elevated ATGL expression increases, while attenuated ATGL expression decreases, both basal and cAMP-stimulated lipolysis (12–22). At the same time, ATGL has low affinity to di- and monoacylglycerides (8, 9). The major diacylglyceride lipase in adipocytes is hormone-sensitive lipase (HSL). Monoacylglyceride products of HSL are hydrolyzed by monoacylglyceride lipase (8, 9).

According to current views, lipolysis is regulated primarily at the posttranslational level with the cyclic AMP (cAMP)-mediated signaling pathway playing the key role in this process. Briefly, phosphorylation of perilipin and HSL by protein kinase A leads to the recruitment of HSL to the lipid droplet and activation of the enzyme. At the same time, a protein cofactor of ATGL, CGI-58, dissociates from phosphorylated perilipin and activates ATGL (10). Jointly, both processes rapidly and significantly stimulate lipolysis. Within this model, the inhibitory effect of insulin on lipolysis is attributed primarily to the inhibition of cAMP-mediated signaling to HSL via Akt-dependent (9, 23) and -independent (24) mechanisms.

However, in order to have a lasting effect on lipolysis, insulin has to suppress its rate-limiting enzyme, ATGL. Indeed, regulation of lipolysis in vivo by physiological stimuli, such as insulin, physical exercise, feeding, and fasting (13, 19, 25–29), are accompanied and likely to be mediated by changes in ATGL expression. Thus, not only posttranslational regulation of the enzymatic activity but also tight control of ATGL expression is necessary for the lipolytic control and FFA homeostasis. However, unlike posttranslational regulation that has been studied in much detail (8–11), very little is known about regulation of ATGL expression.

In order to fill this gap, we initiated a search for the pathways that regulate expression of ATGL by nutrients and insulin. We have found two novel pathways: the mTORC1-mediated pathway that inhibits lipolysis by decreasing transcription of ATGL (22) and the Sirt1/FoxO1-mediated pathway that activates lipolysis by increasing transcription of ATGL (30, 31). Since FoxO1 directly binds to and stim-
ulates the activity of the ATGL promoter (30), the mechanism of its action seems evident. However, the mechanism of mTORC1 action on transcription of ATGL remained obscure. Importantly, a similar regulatory link between dTORC1 and the ATGL homologue, Brummer, exists in Drosophila (32, 33). Conservation of this regulatory pathway in the evolution suggests that it plays an essential role in animal physiology and makes it an attractive target for investigation. Here, we report that mTORC1 suppresses lipolysis in cultured adipocytes via the immediate-early response transcription factor Egr1 that directly inhibits ATGL gene expression.

**MATERIALS AND METHODS**

**Antibodies.** Polyclonal antibodies against ATGL and perilipin were from Cell Signaling (Beverly, MA). Polyclonal antibody against Egr1 (sc110) was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-β-actin antibody was from Sigma (St. Louis, MO). A rabbit polyclonal antibody against celluigalin was described previously (34). Polyclonal antibody against perilipin and all phospho-specific antibodies were from Cell Signaling (Beverly, MA).

**Yeast strains and media.** TheSaccharomyces cerevisiae wild-type strain BY4742 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and single gene deletion mutants (Invertigo yeast gene deletion library) were cultured in YP medium (1% yeast extract and 2% peptone containing 2% glucose as a carbon source) at 30°C with rotational shaking at 200 rpm.

**Cell culture.** 3T3-L1 preadipocytes were cultured, differentiated, and maintained as described previously (34). HEK 293T cells and mouse embryonic fibroblasts (MEFs) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 2 mM l-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml.

**Animals and procedures.** Age-matched, 8-week-old C57BL/6J male mice were purchased from Jackson Laboratory, maintained at three mice per cage at a controlled temperature (22°C) and a 12-h light-dark cycle (light on from 7 a.m. to 5 p.m.), and allowed free access to food and water. At 10 weeks of age, the mice were fed under either high-fat (HF; 45% of calories from fat) or low-fat (LF; 10% of calories from fat) conditions. At 10 weeks of age, the mice were fed under either high-fat (HF; 45% of calories from fat) or low-fat (LF; 10% of calories from fat) conditions. At 10 weeks of age, the mice were fed under either high-fat (HF; 45% of calories from fat) or low-fat (LF; 10% of calories from fat) conditions.

**RESULTS AND DISCUSSION**

Previously, we found that the activation of mTORC1 suppresses ATGL gene expression and lipolysis, whereas the inhibition of mTORC1 has the opposite effect (22). In order to dissect the

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mechanism of mTORC1 action, we expressed several truncated mutants of the ATGL promoter linked to the luciferase cDNA (25) in wild-type and TSC2−/− MEFs that have hyperactive mTORC1 (37). In agreement with our previous observations (22), the expression of luciferase driven by the full-size ATGL promoter is suppressed in TSC2−/−/− MEFs (Fig. 1). Interestingly, the negative effect of mTORC1 on transcription of the reporter gene is maintained even in the case of the shortest ATGL promoter that includes only the proximal 48 bp (Fig. 1). This suggests that neither of the transcription factors that are known to control ATGL expression, namely, peroxisome proliferator-activated receptor γ (PPARγ) (25, 38–40), FoxO1 (30), and interferon regulatory factor 4 (41), play a major role in mediating the effect of mTORC1 because their binding sites are located between 1 and 3 kb upstream of the transcription initiation site (data not shown).

In order to search for a mechanism of mTORC1 action, we turned to yeast. ATGL has a functional ortholog in S. cerevisiae, a triglyceride lipase called Tgl4p (21). In order to determine whether or not the expression of Tgl4 is regulated via the Tor1-mediated pathway, we grew S. cerevisiae in the absence and in the presence of the specific Tor1/mTORC1 inhibitor, rapamycin, for 30 and 90 min and determined the expression levels of mRNA for Tgl4p and two related lipases, Tgl3p and Tgl5p, by qPCR. As shown in Fig. 2A, rapamycin strongly and specifically increased expression of Tgl4p mRNA. Thus, the transcriptional control of ATGL expression by mTORC1 should be essential for regulation of metabolism, or it would not be conserved in evolution from yeast (Fig. 2A) through Drosophila (32, 33) to mammals (22).

To determine which transcription factors specifically control the expression of Tgl4, we screened the S. cerevisiae deletion library. It turned out that the deletion of Msn4 not only elevated the basal expression level of Tgl4 (Fig. 2B) but also dramatically inhibited the effect of rapamycin on Tgl4 expression (Fig. 2C). Still, rapamycin had a small positive effect on Tgl4 expression even in the absence of Msn4 (Fig. 2C), suggesting that not all effects of TOR1 on Tgl4 are mediated by Msn4.

Mammalian homologues of Msn4p form a family of early growth response factors with the best-studied members called...
Egr1 (Krox24) and Egr2 (Krox20) (42). Although specific targets of the Egr transcription factors remain largely unknown, this family has recently been implicated in adipogenesis (43–45), insulin resistance (46), and cholesterol biosynthesis (47). Furthermore, two polymorphisms in Egr1 have been associated with impaired lipid metabolism in humans (48). In order to determine whether or not mammalian Egr1 and Egr2 can regulate expression of ATGL, we analyzed their activity in vitro toward ATGL promoter deletion constructs shown in Fig. 1. We found that Egr1 is a potent inhibitor of ATGL transcription in vitro, whereas Egr2 is significantly less effective (Fig. 3A and B). Note that Egr1 exerts a negative effect on the “minimal” 48-bp ATGL promoter (Fig. 3A) that has a consensus Egr1 binding site (Fig. 3C) (see also references 35 and 49). Indeed, the replacement of five nucleotides in this site for T’s strongly decreases the inhibitory effect of Egr1 on the activity of both full-size and minimal ATGL promoters but does not change the effect of Egr2 (Fig. 3D).

Since the same proximal region of the ATGL promoter is responsible for the transcriptional inhibition by mTORC1 (Fig. 1) and Egr1 (Fig. 3A), we hypothesize that mTORC1 and Egr1 are engaged in the same regulatory pathway. In support of this idea, we found that the expression of Egr1 is very low in wild-type MEFs but is significant in TSC2−/− MEFs (Fig. 3E), which may explain the results shown in Fig. 1. Also, rapamycin inhibits the expression of Egr1 in TSC2−/− MEFs (Fig. 3E).

In order to test the newly discovered regulatory link between mTORC1, Egr1, and ATGL in physiologically relevant cells and conditions, we analyzed the effect of insulin in cultured adi-
mTORC1 Inhibits ATGL Transcription via Egr1

FIG 4 Insulin-dependent Egr1 expression controls ATGL expression in 3T3-L1 adipocytes. (A) Differentiated 3T3-L1 adipocytes were treated with 100 nM insulin for the indicated periods of time. (Top panel) The levels of ATGL and Egr1 mRNA were determined in triplicate by quantitative PCR and normalized based on 36B4 mRNA. (Bottom panel) Total cell lysates were analyzed by Western blotting for Egr1 and ATGL. Actin served as a loading control. (B) 3T3-L1 adipocytes were treated with 100 nM insulin in the presence or absence of actinomycin D (AcD; 0.2 μg/ml) for 16 h. Total cell lysates were analyzed by Western blotting for ATGL. Perilipin served as a control for AcD action, and celluargin and actin served as loading controls. (C) 3T3-L1 adipocytes were infected with adenovirus expressing Egr1 (AdEgr1) and GFP (AdGFP) and cultured for 48 and 72 h. Total cell lysates were analyzed by Western blotting for Egr1 and ATGL. Actin served as a loading control. (D) 3T3-L1 adipocytes infected with adenovirus expressing Egr1 (AdEgr1) and GFP (AdGFP) and cultured for 48 h. Cells were then incubated in phenol red-free DMEM with 2% fatty acid-free BSA without (white bars) or with (black bars) 10 μM isoproterenol (Iso) for 2 h. Glycerol was measured in medium aliquots in triplicate and normalized by protein concentration in whole-cell lysates. Data are expressed as means ± the SD. (E) ChIP assays were performed in 3T3-L1 adipocytes treated with 100 nM insulin for 4 h. Genomic fragments were immunoprecipitated with antibody against Egr1 or rabbit IgG, amplified by PCR, separated in a 3% agarose gel, and visualized by ethidium bromide staining.

Insulin-stimulated induction of Egr1 in adipocytes is mediated, at least partially, by mTORC1 as rapamycin and PP242 inhibit this effect (Fig. 5A). In parallel, both inhibitors block the negative effect of insulin on ATGL expression (Fig. 5A) and lipolysis (Fig. 5B) in insulin-treated adipocytes.

In order to confirm the connection between mTORC1 and Egr1, we determined the effect of nutrients on the expression of Egr1 mRNA in the absence and in the presence of insulin. We found that withdrawal of nutrients blocked insulin-stimulated induction of Egr1 mRNA (Fig. 5C). On the contrary, treatment of cultured 3T3-L1 adipocytes with leucine alone activated the mTORC1 pathway and stimulated Egr1 expression (Fig. 5D).

In the next experiment, we analyzed protein expression in mice that received a high-fat diet for 14 weeks. As reported earlier (52), the activity of mTORC1 in the fat tissues of these animals is increased (Fig. 6A). According to our model, activation of mTORC1 should decrease expression of ATGL, and ATGL levels are indeed decreased in mice fed high-fat diet (Fig. 6A). This observation, although supportive of the previous findings (41, 53), may seem to contradict a well-known fact that plasma FFA are commonly increased in obesity (10). This, however, may reflect the overall increase in adipose tissue, whereas lipolysis normalized per kg of fat mass is actually decreased in obesity (reviewed in reference 54), a finding consistent with results shown in Fig. 6A. Finally, we have determined, by qPCR, that a high-fat diet increases the expression of Egr1 (Fig. 6B).

Thus, our experiments identify the evolutionarily conserved mTORC1-Egr1-ATGL regulatory axis (Fig. 6C) as a novel mecha-
anism of the antilipolytic effect of insulin. We want to emphasize here that our results do not negate the previously established mechanism of short-term insulin action on lipolysis by inhibition of cAMP-mediated signaling to HSL and perilipin (9, 23, 24). Rather, these findings demonstrate a new level of such regulation that is essential for better understanding and restraining the metabolic disease. For example, the downregulation of ATGL via this pathway may represent an essential compensatory mechanism that may be needed for maintaining physiological concentrations of circulating FFA in obesity.

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