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Insulin Efficiently Stores Triglycerides in Adipocytes by Inhibiting Lipolysis and Repressing PGC-1α Induction.

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White adipose tissue (WAT) is important as an energy reservoir in mammals, but the precise mechanism by which energy storage in WAT is controlled remains unclear. It is well known that representative anabolic hormone insulin efficiently stores triglyceride in adipocytes. We showed that insulin inhibited β-agonist-induced lipolysis at least in part by inhibiting phosphorylation of perilipin and hormone-sensitive lipase (HSL) in 3T3-L1 adipocytes. Furthermore, insulin inhibited β-agonist-induced increase of PGC-1α expression, which is important for mitochondrial biogenesis and energy expenditure. These results suggest the possibility that insulin efficiently stores triglyceride in adipocytes by decreasing lipolysis and repressing energy expenditure.

Obesity characterized by the storage of excess triglyceride in adipose tissue is a complex and chronic disorder that has become a global epidemic (1). Especially, visceral obesity, with fat accumulation in abdomen among viscera, is often associated with insulin resistance, dyslipidemia, and hypertension and with an increased risk of accelerated atherosclerosis, which is called the metabolic syndrome (10). Adipose tissue is originally an important organ as an energy reservoir in mammals and takes in glucose and free fatty acid (FFA) in the post prandial period and stores them as triglyceride by lipogenesis. In the fasting, adipose tissue in turn releases FFA and glycerol by lipolysis of stored triglycerides into the circulating blood.

Lipolysis in adipocytes is regulated by a number of hormones, such as epinephrine, norepinephrine, and glucagon (6). Catecholamine-induced lipolysis is well characterized, initiated by stimulation of β-adrenergic receptors, which are coupled to activation of adenylyl cyclase by the heterotrimeric G_s protein, which in turn converts ATP to cAMP. cAMP-dependent protein kinase A (PKA) then phosphorylates two main targets, hormone-sensitive lipase (HSL), the primary lipase responsible for hydrolysis of triglycerides, as well as perilipin A, the coating protein of lipid droplets (6). In response to β-adrenergic activation, phosphorylated perilipin A undergoes a conformational change, which is essential for proper translocation of HSL from the cytosol to the surface of lipid droplets and subsequent attachment to triglycerides, leading to initiation of triglyceride hydrolysis. The mobilization of FFA and glycerol from adipose tissue as a result of lipolysis supplies other tissues with metabolites and energy substrates.

In energy metabolism, mitochondria are important organs. Mitochondria oxidize glucose metabolites and FFA to produce ATP. Thus, energy consuming organs including brain, heart, and skeletal muscle possess well-developed mitochondrial systems. In contrast, development of mitochondria is not appropriate for energy storing adipose tissue. Phosphorylated CREB

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at Ser^{133} by PKA promotes the expression of PGC-1α which is a key regulator of mitochondrial biogenesis, leading to the increase of energy expenditure (5,11,13,14).

Therefore, examination of energy storage in adipose tissue is important to clarify the mechanism of systemic glucose metabolism and the development of obesity. Here we demonstrate the mechanism in which the representative anabolic hormone, insulin efficiently stores energy as triglyceride in adipocytes.

MATERIALS AND METHODS

Cell culture

3T3-L1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 μg/ml streptomycin, and 50 units/ml penicillin. Adipogenesis was induced by treatment with insulin, dexamethasone, and isobutylmethylxanthine as described previously (2), and the cells were subjected to experiments 7 to 13 days after differentiation.

Measurement of lipolysis

3T3-L1 adipocytes cultured in six-well plates were incubated with or without insulin (2×10^{-8}M) for 24 h. Then, cells were stimulated with 1 μM isoprotenerol or 1 mM 8-bromoadenosine 3', 5'-cyclic monophosphate (8 Br-cAMP) in DMEM containing 1% bovine serum albumin (BSA) in the absence of serum for 20 min and for 40 min, respectively. FFAs in the culture medium were measured with an acyl-CoA oxidase–based colorimetric kit (Wako). Lipolysis was estimated by the increase of FFA in the culture medium (16).

Western blot analysis

3T3-L1 adipocytes were solubilized with the lysis buffer [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride]. Total cell lysates were subjected to immunoblot analysis with antibodies to perilipin (Affinity BioReagents), phospho-(Ser/Thr) PKA substrate (Cell Signaling), cAMP responsive element binding protein (CREB) (Upstate), phospho-HSL (Cell-Signaling), phospho-CREB (Ser133) (Upstate), β-actin (Sigma) and HSL (Chemicon). Immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence (ECL; Amersham, Little Chalfont, UK).

Isolation of RNA and quantitative RT-PCR

Total RNA was extracted from 3T3-L1 adipocytes with the use of an RNeasy kit (Qiagen). Complementary DNA synthesized from total RNA was analyzed in a Sequence Detector (model 7900, PE Applied Biosystems) with specific primers and SYBR Green PCR Master reagents (Perkin Elmer Life Sciences). The relative abundance of mRNAs was calculated with 36B4 mRNA as the invariant control. The primers (sense and antisense, respectively) were as follows: mouse PGC-1α, 5'-GGAGCTGGATGGCTTGGGACAT-3' and 5'-TTCGCAGGCTCATTGTTGTACTGGT-3'; mouse 36B4, 5'-GAGGAATCAGATGA GGATATGGGA-3' and 5'-AAGCAGGCTGACTTGGTTGC-3'.

RESULTS

Insulin inhibits β-agonist-induced lipolysis in 3T3-L1 adipocytes

Inhibition of lipolysisis is expected to result in the increased triglyceride content in adipocytes. Thus, we at first examined whether insulin inhibits catecholamine-induced lipolysis in 3T3-L1 adipoctytes. Treatment of 3T3-L1 adipocytes with 1 μM isoprotenerol

for 20 min remarkably induced FFA release into culture medium (Figure 1a, compare lanes 1 and 2), implying that isoprotenerol activated lipolysis. In this condition, pretreatment of the cells with insulin for 24 h dramatically inhibited isoprotenerol-induced FFA release (Figure 1a, compare lanes 2 and 4). Insulin is reported to activate phosphodiesterase 3B (PDE3B) through Akt (8). The activation of PDE3B by insulin was thought to result in the decrease of the cellular cAMP level, leading to the decrease of PKA activation and the following inhibition of lipolysis. Thus, to further investigate the effects of insulin to the downstream signals of PKA, we next examined whether insulin also inhibit the cAMP analogue-induced lipolysis in 3T3-L1 adipocytes. A cell-permeable and PDE-resistant cAMP analogue, 8-bromoadenosine 3', 5'-cyclic monophosphate (8-Br-cAMP) (12) which directly activates PKA induced remarkable lipolysis (Figure 1a, compare lanes 5 and 6). Insulin also inhibited 8-Br-cAMP-induced lipolysis almost completely (Figure 1a, compare lanes 6 and 8). These results suggest that insulin can inhibit 8-Br-cAMP-induced lipolysis mainly by repressing PKA or the downstream signals of PKA, although we can not exclude the involvement of insulin-induced PDE3B activation and the subsequent decrease of intracellular cAMP level. In addition, we can not exclude the possibiliy that insulin inhibited lipolysis through other pathways than PKA signaling, either.

Insulin inhibits β-agonist-induced phosphorylation of CREB, perilipin and HSL

We next examined whether insulin treatment actually affects the PKA-induced phosphorylation of perilipin which is important for lipolysis in 3T3-L1 adipocytes. Phosphorylated perilipin was demonstrated as a slower-migrating band compared with non-phosphorylated form in immunoblot analysis using anti-perilipin antibody (3). 1 μM isoprotenerol for 20 min induced phosphorylation of perilipin (Fig.1b, 1st panel, compare lanes 1 and 2). However, treatment with insulin for 24 h decreased isoprotenerol-induced phosphorylation form of perilipin (Fig.1b, 1st panel, compare lanes 2 and 4). In addition, A 62-kD protein band which migrated coincident with immunoreactive perilipin was actually detected by using anti-phospho-PKA substrate antibody in the treatment with isoprotenerol (Fig.1b, 2nd panel, compare lanes 1 and 2). In addition, this phosphorylation of perilipin was inhibited by insulin treatment for 24h (Fig. 1b, 2nd panel, compare lanes 2 and 4). Furthermore, treatment with insulin for 24 h showed the tendency to decrease isoprotenerol-induced phosphorylation form of HSL (Fig.1b, 4th panel, compare lanes 2 and 4, and Fig. 1c, $p = 0.08$). These results indicate that insulin inhibition of β-agonist induced lipolysis results at least in part from the decreased phosphorylation level of perilipin and HSL.

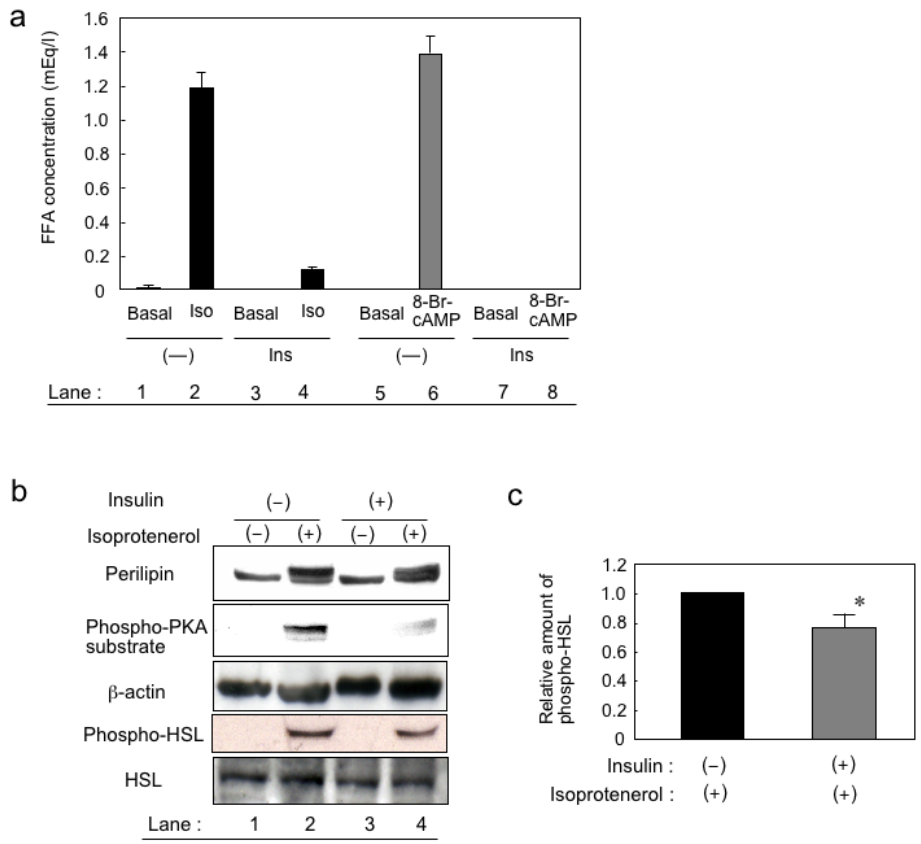

Figure 1. Inhibitory effects of insulin to β-agonist-induced lipolysis and to β-agonist-induced phosphorylation of perilipin and HSL in 3T3-L1 adipocytes.
(a) 3T3-L1 adipocytes incubated with or without 2×10^{-8} M insulin for 24 h were stimulated with 1μM isoprotenerol (Iso) for 20 min or 1 mM 8-Br-cAMP for 40 min. FFA concentrations in the culture medium were determined. n = 4 (Iso) and 3 (8-Br-cAMP). (b) 3T3-L1 adipocytes incubated with or without 2×10^{-8} M insulin for 24 h were stimulated with 1μM isoprotenerol for 20 min. Total cell lysates were subjected to immunoblot analysis using anti perilipin antibody (1st panel), anti phospho-PKA substrate antibody (2nd panel), anti β-actin antibody (3rd panel), anti phospho-HSL antibody (4th panel) and anti HSL antibody (5th panel). (c) The bands corresponding to phosphorylated HSL in (b) were quantitated and expressed relative to the corresponding values with isoprotenerol stimulation without insulin. $*p = 0.08$, calculated isoprotenerol stimulation without insulin. n = 4.

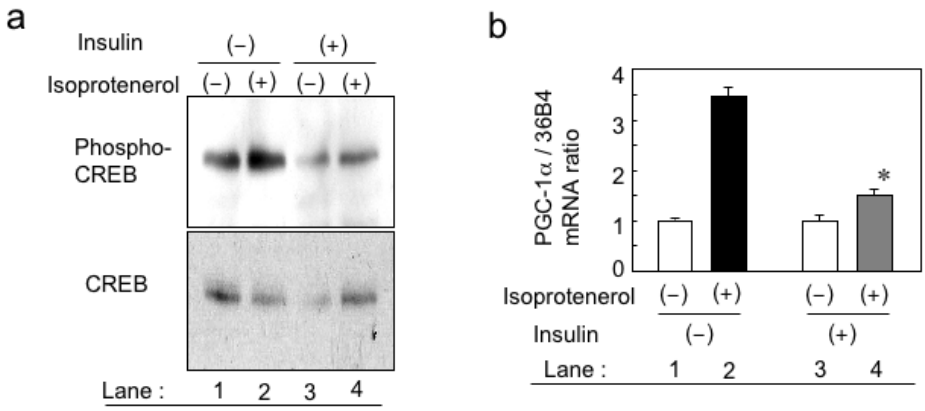

Figure 2. Insulin suppresses β-agonist-induced mRNA expression of PGC-1α.
3T3-L1 adipocytes incubated with or without 2×10^{-8} M insulin for 24 h were stimulated with 1μM isoprotenerol for 20 min (a) or for 4 h (b). (a) Total cell lysates were subjected to immunoblot analysis using anti phospho-CREB antibody (upper panel) and anti CREB antibody (lower panel). (b) Total RNA was extracted and subjected to RT-PCR. The results were expressed relative to the corresponding value of basal state without insulin. ∗p<0.001, calculated versus isoprotenerol stimulation without insulin (*lane 2*). n = 6.

Insulin inhibits β-agonist-induced expression of PGC-1α in 3T3-L1 adipocytes

PKA phosphorylates CREB and induced CRE-mediated gene transcription. Thus, we examined the phosphorylation of CREB at Ser^{133} in 3T3-L1 adipocytes. Immunoblot analysis using anti-phospho-CREB (Ser133) antibody revealed that CREB was phosphorylated in basal state (Fig. 2a, upper panel, *lane 1*). 1 μM isoprotenerol for 20 min further increased the phosphorylation level of CREB (Fig. 2a, upper panel, compare lanes 1 and 2). However, insulin inhibited not only the basal but also isoprotenerol-induced phosphorylation level of CREB (Fig. 2a, upper panel). We next investigated the mRNA expression of PGC-1α in these cells by quantitative real-time RT-PCR. Consistent with the results of CREB phosphorylation, 1 μM isoprotenerol for 4 h augmented the PGC-1α expression (Fig. 2b, compare lanes 1 and 2). However, insulin treatment decreased the isoprotenerol-induced PGC-1α expression significantly (Fig. 2b, compare lanes 2 and 4). Insulin did not affect the basal level of PGC-1α expression without isoprotenerol (Fig. 2b, compare lanes 1 and 3), whereas insulin decreased the phosphorylation level of CREB without isoprotenerol (Fig. 2a, upper panel, compare lanes 1 and 3). This may imply that the factors other than CREB are involved in the regulation of PGC-1α expression in adipocytes. These results can suggest the possibility that insulin can play an inhibitory role in mitochondrial function in 3T3-L1 adipocytes.

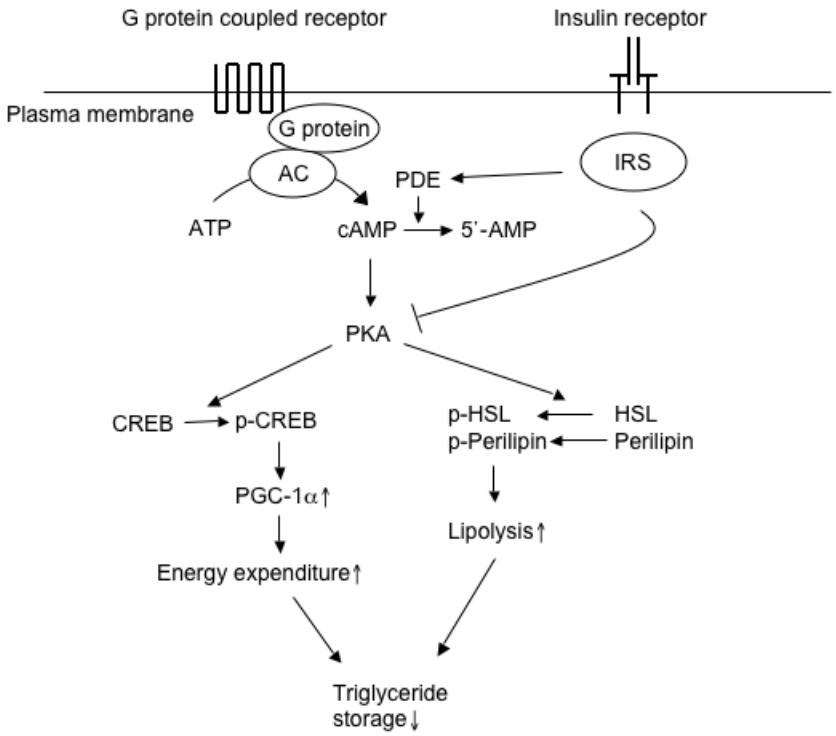

Figure 3. Schema for the hypothesis that insulin regulates triglyceride storage by affecting PKA signaling pathway in 3T3-L1 adipocytes.
Chronic insulin treatment is supposed to inhibit β-agonist-induced PKA activation by repressing PKA or the downstream signals of PKA in addition to cAMP degrardation by increasing PDE3B activity. This leads to the decreased phosphorylation level of HSL and perilipin followed by the inhibition of lipolysis. In addition, insulin inhibits β-agonist-induced increase of PGC-1α expression. These can result in the efficient storage of triglyceride in adipocytes.

DISCUSSION

The major function of adipose tissue is uptake of glucose and FFA followed by storage as triglycerides in post prandial state and supplement of FFA and glycerol by lipolysis of stored triglyceride to other tissues, especially to liver for gluconeogenesis in the fasting. Thus, the regulation of triglyceride synthesis and lipolysis in adipose tissue is very important for maintaining the normal glucose, lipid and energy homeostasis. We showed that insulin inhibited not only β-agonist-induced lipolysis but also β-agonist-induced increase of PGC-1α expression by inhibiting PKA signaling pathway in 3T3-L1 adipocytes. These results suggest that insulin plays an important role in the efficient energy storage in adipocytes.

Detelioration of type 2 diabetes is known to be accompanied with inappropriate increase of lipolysis in adipose tissue due to deficiency of insulin effect, followed by increased serum FFA level and subsequent gluconeogenesis in liver. In addition, increased FFA itself induces further insulin resistance directly in insulin sensitive tissues (15). This also leads to the further hyperglycemia. Thus, it is supposed that inhibition of FFA release from adipose tissue results in amelioration of diabetes. Insulin sensitizing drug, thiazolidinedione (TZD)

induces glycerol kinase in adipose tissue, which re-synthesizes the triglyceride from glycerol and FFA produced by lipolysis (4). As a result, the release of FFA and glycerol from adipose tissue was decreased and in turn leads to the increased insulin sensitivity caused by TZD (4). This clearly shows the importance of lipolysis in adipose tissue in the development of whole body insulin resistance.

It is well known that insulin stimulates the redistribution of GLUT4 from intracellular compartment to plasma membrane in adipocytes (7). As a result, increased postprandial blood glucose was transported into adipose tissue through GLUT4. In addition, insulin increases the activity of lipoprotein lipase on adipose cell surface, leading to the lipolysis of serum lipoprotein and subsequent uptake of FFA into adipose tissue. Insulin-induced increase of glucose and FFA uptake is also important as substrate supply in efficient triglyceride synthesis in adipocytes.

Signaling by β-adrenergic receptors and cAMP triggers mitochondrial biogenesis and energy expenditure in adipose tissue (9). Activation of the catalytic subunit of PKA by cAMP results in the phosphorylation of CREB on serine-133 (5,11), which in turn promotes mitochondrial biogenesis (13,14). Our results suggest the possibility that insulin-mediated inhibition of PKA signaling results in reduction of mitochondrial biogenesis in adipose tissue, leading to the decrease of energy consumption and the subsequent increase of cellular triglyceride content. This can be one of the mechanisms in which insulin efficiently stores energy in adipose tissue. Recently, it is reported that in adipocytes, chronically high insulin levels inhibit β-adrenergic receptors but not other cAMP-elevating stimuli from activating PKA by disrupting the close apposition of β-adrenergic receptors and PKA (17). This finding may elucidate the mechanism in which insulin inhibited isoprotenerol-induced phosphorylation of CREB, perilipin and HSL in 3T3-L1 adipocytes. However, our results show chronic insulin treatment did also inhibit cAMP analogue-induced lipolysis dramatically, suggesting the possibility that insulin inhibits cAMP analogue-induced lipolysis by other mechanism than the inhibition of β-adrenergic receptors and PKA pathway.

Taken together, our data suggest that insulin promotes the efficient energy storage in adipose tissue at least in part by decreasing lipolysis by inhibition of PKA signaling. In addition, insulin can possibly play an inhibitory role in mitochondrial function, leading to decreased energy expenditure and increased triglyceride storage.

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