Insulin-induced Adipocyte Differentiation

ACTIVATION OF CREB RESCUES ADIPOGENESIS FROM THE ARREST CAUSED BY INHIBITION OF PRENYLATION

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Insulin is a potent adipogenic hormone that triggers an induction of a series of transcription factors governing differentiation of pre-adipocytes into mature adipocytes. However, the exact link between the insulin signaling cascade and the intrinsic cascade of adipogenesis remains incompletely understood. Herein we demonstrate that inhibition of prenylation of p21ras and Rho-A arrests insulin-stimulated adipogenesis. Inhibition of farnesylation of p21ras also blocked the ability of insulin to activate mitogen-activated protein (MAP) kinase and cyclic AMP response element-binding (CREB) protein. Expression of two structurally different inducible constitutively active CREB constructs rescued insulin-stimulated adipocyte differentiation from the inhibitory influence of prenylation inhibitors. Constitutively active CREB constructs induced expression of PPARγ2, fatty acid synthase, GLUT-4, and leptin both in control and prenylation inhibitors-treated cells. It appears that insulin-stimulated farnesylation of the Ras family GTPases assures normal phosphorylation and activation of CREB that, in turn, triggers the intrinsic cascade of adipogenesis.

White adipose tissue (WAT) is the major site of regulated energy store and release in response to hormones and nutrients at times of nutritional abundance and deprivation. WAT mass is a reflection of the number of adipocytes and their volume. The latter is dependent upon the amount of fat stored in the individual fat cell, whereas the former increases as a result of new adipocyte differentiation from pre-adipocytes. Because mature adipocytes do not undergo cell division and lose their ability to propagate, any increase in their number reflects the process of differentiation of pre-adipocytes into mature adipocytes (1, 2). This process of differentiation of pre-adipocytes into adipocytes culminates in increased transcription of certain genes and expression of specific proteins such as the insulin-sensitive glucose transporter GLUT-4 (3), fatty acid synthase (FAS) (4), and glycerol-2-phosphate dehydrogenase (5). Understanding the process of adipocyte differentiation becomes vitally important for unraveling the pathogenesis of obesity that is characterized by increased WAT mass.

Several excellent reviews have summarized the current understanding of the mechanism of adipocyte differentiation (6–10). These reviews emphasize that during adipocyte differentiation, regardless of the nature of the triggering event, a series of transcription factors CCAAT/enhancer-binding protein (C/EBP)β, δ, and α, PPARγ, etc. is induced in a specific sequence (6–10). In in vitro studies, a combination of dexamethasone, isobutylmethylxanthine (IBMX), and insulin is commonly used to induce this sequence of events. Understanding the role of insulin, the most potent among the three inducers, appears to be of special importance, because of the potential influence of in vivo hyperinsulinemia on the development of obesity. Although insulin within its physiologic range has been shown to induce lipogenesis and adipocyte differentiation (11), the mechanism of its action on adipogenesis is incompletely understood.

From the point of view of insulin signaling, the presence of the insulin receptor appears to be required for adipocyte differentiation (12, 13). Furthermore, inhibition of phosphatidylinositol 3-kinase (PI 3-kinase) has been shown to block insulin-induced differentiation of 3T3-L1 pre-adipocytes (14–15). Because a variety of other agents also activate PI 3-kinase and its downstream targets without any effect on adipocyte differentiation, it is plausible that insulin engages other branch(es) of its signaling pathways into its action on adipogenesis.

We have recently determined that prenylation of Ras and Rho proteins is regulated by insulin (16, 17). We further demonstrated that prenylation of these proteins is significantly augmented by ambient hyperinsulinemia either present in insulin-resistant humans and animals or induced experimentally in controls (18, 19). Insulin appears to promote the phosphorylation and activation of farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I) by a mechanism independent of PI 3-kinase (20, 21). Availability of prenylated p21ras is required for the activation of MAP kinase (22, 23). The latter has been shown to phosphorylate and activate cyclic AMP response element binding (CREB) protein that is critically important for adipogenesis (24). Conceivably, the ability of insulin to promote prenylation of the Ras family of GTPases may constitute an additional mechanism of the insulin effect on adipogenesis. In this study, we examined whether inhibition of prenylation of p21ras and Rho-A can affect insulin-induced 3T3-L1 adipocyte differentiation.
Insulin-stimulated Adipogenesis

EXPERIMENTAL PROCEDURES

Materials—All standard chemicals were from Sigma and anti-Ras monoclonal antibody was from Transduction Laboratories (Lexington, KY). Antibodies to PPARγ2, GLUT-4, leptin, and Rho-A antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-active MAP kinase antibody, CREB, and phospho-CREB specific antibodies were from Cell Signaling (Beverly, MA), and anti-fatty acid synthetase antibody was purchased from PharMingen (San Diego, CA). All supplies and reagents for SDS-PAGE were from Bio-Rad (Hercules, CA), and the chemiluminescence kit was from Amersham Pharmacia Biotech (Arlington Heights, IL). Cell culture media and supplies were from Life Technologies, Inc. (Beverly, MA), Gemini Bioproducts (Gaithersburg, MD), and Specialty Media, Inc. (Lavalutee, NJ). FTAse inhibitor (FTI), α-hydroxy-farnesylphosphonic acid, was from Biomol (Plymouth Meeting, PA) and GGTase inhibitor-286 (GGTI) was from Calbiochem (San Diego, CA). The Edcysone inducible expression system (pIND, pVgRXR vectors, zoein, and ponasterone A) was from Invitrogen (Madison, WI). An expression vector for the constitutively active CREB-DIEML (25) was provided by Dr. Richard Goodman (Oregon Health Sciences University, Portland, OR).

Cell Culture—3T3-L1 fibroblasts were grown to confluence in fibroblast growth medium (Dulbecco's modified Eagle's medium containing 5.5 mM glucose, 10% fetal calf serum, 0.5 mM glutamine, and 0.5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) fungizone). Differentiation was initiated by addition of medium containing 10% fetal calf serum, 1 mM glutamine, 500 μM isobutylmethylxanthine (IBMX) (or 3 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) fungizone), 1 mM dexamethasone, and 1 mM insulin. After 2 days, cells were transferred to adipocyte growth medium containing 25 mM glucose, 50 μM gentamicin, 0.5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) fungizone, 1 mM glutamine, and 1 mM insulin and refed every 2 days. Differentiation of fibroblasts into mature adipocytes was confirmed by Oil Red O staining (26). FTAse inhibitor (FTI) and GGTase I inhibitor (GGTI) were used in concentrations of 1 μM and 3 μM, respectively.

Transfection Procedures—Plates of 3T3-L1 fibroblasts were grown to 70–80% confluency and transfected with the indicated plasmids with Superfect Reagent (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Cells stably transfected with the plasmid pVgRXR were selected in conventional medium containing 500 μg/ml zeocin, and cells stably transfected with pIND-VP16-CREB, pIND-CREB-DIEDML, or pIND-LacZ plasmids were selected in medium containing 500 μg/ml Geneticin. Large, rapidly growing, well separated colonies were isolated 10–12 days after selection was begun with either antibiotic. Isolated clones were passaged in low glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1 mM t-glutamine, and 500 μg/ml each of zeocin and geneticin. VP16-CREB, CREB-DIEDML, or LacZ expression was induced through the addition of 5 μM ponasterone A to the growth medium as indicated in the figure legends. Differentiation of 3T3-L1 preadipocytes to mature adipocytes was followed by observing the accumulation of triacylglycerol in Oil Red O staining vesicles and by the appearance of adipocyte markers. PPARγ, GLUT-4, fatty acid synthetase, and leptin were visualized by broncho-chloro-indolyl-phosphatase and nitro blue tetrazolium, whereas the remaining proteins were detected by ECL chemiluminescence kit. The results were expressed as ratios of phosphoproteins to the total amount of MAP kinase and CREB (Figs. 3 and 4, lower panels). The inhibitor of FTase significantly decreased the ability of insulin to activate MAP kinase (Fig. 3A) and to phospho-creb (Fig. 4A) without affecting the amounts of either MAP kinase or CREB protein (Figs. 3A and 4A, middle panels). In contrast, inhibition of GGTase I did not affect the ability of insulin to promote the phosphorylation of either MAP kinase or CREB (Figs. 3B and 4B, lower and upper panels), indicating that the geranylgeranylation process is not related to the activation of MAP kinase and CREB.

Recently, Reusch et al. (24) have demonstrated that constitutively active CREB promotes adipocyte differentiation even in the absence of insulin. Because CREB enters the differentiation process downstream of farnesylated and activated Ras, we examined whether two distinct constitutively active CREB constructs (VP16-CREB and CREB-DIEDML) can rescue the differentiation process from an arrest induced by prenylation inhibitors. Both constitutively active constructs were inducible by ponasterone A and both promoted adipocyte differentiation, even in the absence of the differentiation mixture (Fig. 5). As expected, the differentiation mixture induced PPARγ, FAS, leptin, and GLUT-4 proteins, and both constitutive CREB mimicked this effect in the absence of

specific immune complexes for PPARγ, FAS, GLUT-4, or leptin were visualized by bromo-chloro-indolyl-phosphatase and nitro blue tetrazolium, whereas the remaining proteins were detected by ECL chemiluminescence kit. The results were expressed as ratios of phosphoproteins to the total amount of MAP kinase and CREB (Figs. 3 and 4, lower panels). The inhibitor of FTase significantly decreased the ability of insulin to activate MAP kinase (Fig. 3A) and to phospho-creb (Fig. 4A) without affecting the amounts of either MAP kinase or CREB protein (Figs. 3A and 4A, middle panels). In contrast, inhibition of GGTase I did not affect the ability of insulin to promote the phosphorylation of either MAP kinase or CREB (Figs. 3B and 4B, lower and upper panels), indicating that the geranylgeranylation process is not related to the activation of MAP kinase and CREB.

RESULTS

In the initial set of experiments, we examined the effect of the inhibitors of FTase (1 μM) and GGTase I (3 μM) on the insulin-induced differentiation of 3T3-L1 fibroblasts. The inhibitors were added either individually or together at the time of the induction and kept in the medium throughout the differentiation period (days 1–10). The presence of either inhibitor alone or in a combination in the differentiation mixture completely blocked differentiation of these cells into mature adipocytes (Fig. 1).

We then examined the effect of the prenylation inhibitors on the expression of the transcription proteins participating in the pre-adipocyte differentiation process. In control cells, C/EBPβ was expressed on day 2 and C/EBPα appeared on day 4. Both inhibitors, FTI and GGTI, blocked the expression of C/EBPβ and C/EBPα (Fig. 2), thus indicating that farnesylation and geranylgeranylation are necessary for the induction of C/EBPβ and C/EBPα expression.

Because insulin stimulates prenylation of p21 Ras and Rho-A (16, 17, 27, 28), a process that is required for subsequent activation of these GTPases and their downstream targets (30), we examined the effect of inhibitors of FTase and GGTase on the ability of insulin to activate MAP kinase and CREB, two downstream signaling intermediates. In the absence of either FTI or GGTI, insulin induced the phosphorylation of both MAP kinase (Fig. 3, A and B, upper panels) and CREB (Fig. 4, A and B, upper panels). The effect of insulin was clearly evident when the results were expressed as ratios of phosphoproteins to the total amount of MAP kinase and CREB (Figs. 3 and 4, lower panels). The inhibitor of FTase significantly decreased the ability of insulin to activate MAP kinase (Fig. 3A) and to phospho-creb (Fig. 4A) without affecting the amounts of either MAP kinase or CREB protein (Figs. 3A and 4A, middle panels). In contrast, inhibition of GGTase I did not affect the ability of insulin to promote the phosphorylation of either MAP kinase or CREB (Figs. 3B and 4B, lower and upper panels), indicating that the geranylgeranylation process is not related to the activation of MAP kinase and CREB.

FIG. 1. Effect of prenylation inhibitors on adipogenesis. 3T3-L1 fibroblasts were incubated with insulin alone (control) from day 1 to day 10 or in the presence of FTI (1 μM), GGTI (3 μM), or both.
the differentiation mixture (Fig. 6). Moreover, induction of either of the two constructs rescued adipocyte differentiation from the inhibitory influence of prenyltransferase inhibitors (Fig. 7). Induction of constitutively active CREB with ponsas-terone A stimulated expression of PPARγ2, leptin, and FAS in cells treated with FTI and/or GGTI (Fig. 8).

DISCUSSION

Obesity has emerged as a major health care concern in the United States and many other countries. Weight gain and obesity occur when energy intake exceeds energy expenditure, and excess energy is deposited in adipocytes. At the cellular level, obesity is characterized by both adipocyte hypertrophy and increased number of adipocytes (30, 31). New adipocytes largely arise in the process of differentiation of pre-adipocytes. Over the past decade, there has been an explosion of knowledge about intrinsic regulatory mechanisms determining adipocyte differentiation (reviewed in Refs. 6–10). It has been determined that adipocyte differentiation is a highly regulated process that involves sequential activation of several transcription factors, such as C/EBPβ, C/EBPα, and PPARγ, culminating in the removal of adipocytes from the cell cycle and induction of highly specific proteins, such as GLUT-4, FAS, and others (1–10).

Nutritional influence, as well as the presence of insulin and glucocorticosteroids appears to be among the most important triggers of the differentiation process. Insulin, however, occupies a special place among various factors regulating white adipose tissue mass. Fasting and/or glucose-induced hyperinsulinemia are characteristic features of obesity. Whether or not hyperinsulinemia can participate in further augmentation of WAT mass remains to be defined further. Clearly, hyperinsulinemia, induced either by administration of exogenous insulin (intensive therapy with insulin) or increases in production of endogenous insulin (insulinoma), is associated with significant weight gain. It is plausible, but not yet proven, that endoge-aneous hyperinsulinemia elicited by excessive food intake can promote adipogenesis, significantly increases WAT mass, and cause obesity with secondary metabolic resistance to insulin. However, it is obvious that insulin has a strong anti-lipolytic action, stimulates lipogenesis, and triggers and promotes new adipocyte differentiation in vitro.

Even though recent studies have demonstrated a requirement for the insulin receptor and activation of PI 3-kinase and
Akt in the process of adipogenesis (12–15), the precise mechanism connecting insulin signaling with the intrinsic regulatory mechanisms of adipocyte differentiation remains obscure. The

![Fig. 4. Effect of FTI and GGTI on insulin-induced phosphorylation of CREB in 3T3-L1 fibroblasts.](image)

![Fig. 5. Effect of constitutively active CREB constructs (VP16-CREB and CREB-DIEDML) on adipogenesis.](image)

![Fig. 6. Effect of VP16-CREB or CREB-DIEDML on adipogenesis in 3T3-L1 cells as determined by expression of adipocyte-specific genes.](image)
Recently, the phosphorylation of CREB has been found to be necessary and sufficient to induce adipogenesis in 3T3-L1 fibroblasts (24). Inducible expression of a constitutively active VP16-CREB alone was sufficient to initiate adipogenesis as determined by triacylglycerol storage, cell morphology, and the expression of adipocyte marker genes, PPARδ determined by triacylglycerol storage, cell morphology, and the expression of adipocyte marker genes, PPARδ determined by triacylglycerol storage, cell morphology, and the expression of adipocyte marker genes. Inducible expression of a constitutively active CREB constructs from an inhibitory influence of FTI and GGTI. 3T3-L1 preadipocytes inducibly expressing VP16-CREB, CREB-DIEDML, or control LacZ were grown to confluence as described under “Experimental Procedures.” The cells were treated with the reagents indicated above each column of photographs. Cells treated with differentiation mixture received 10 μg/ml insulin, 1 μM dexamethasone, and 3 mM βcAMP for 48 h and then were re-fed every 2 days with conventional medium containing 10 μg/ml insulin. Ponasterone A was added to medium at a final concentration of 5 μM for the entire 10 day differentiation period. FTI (1 μM) and GGTI (3 μM) were also present for the entire 10-day period. After 10 days in culture, the cells were stained with Oil Red O to visualize triacylglycerol vesicles, and counterstained with hematoxylin. The photographs show cells on day 10 of each treatment.

Fig. 7. Rescue of adipogenesis with constitutively active CREB constructs from an inhibitory influence of FTI and GGTI. 3T3-L1 preadipocytes inducibly expressing VP16-CREB, CREB-DIEDML, or control LacZ were grown to confluence as described under “Experimental Procedures.” The cells were treated with the reagents indicated above each column of photographs. Cells treated with differentiation mixture received 10 μg/ml insulin, 1 μM dexamethasone, and 3 mM βcAMP for 48 h and then were re-fed every 2 days with conventional medium containing 10 μg/ml insulin. Ponasterone A was added to medium at a final concentration of 5 μM for the entire 10 day differentiation period. FTI (1 μM) and GGTI (3 μM) were also present for the entire 10-day period. After 10 days in culture, the cells were stained with Oil Red O to visualize triacylglycerol vesicles, and counterstained with hematoxylin. The photographs show cells on day 10 of each treatment.

The role of geranylgeranylation of Rho-A in the process of adipocyte differentiation is via its ability to stimulate prenylation. Inhibitors of prenylation completely blocked insulin-induced differentiation of adipocytes and prevented induction of C/EBPβ and C/EBPδ. Inhibition of FTase also blocked the ability of insulin to activate MAP kinase and CREB.

Recently, the phosphorylation of CREB has been found to be necessary and sufficient to induce adipogenesis in 3T3-L1 fibroblasts (24). Inducible expression of a constitutively active VP16-CREB alone was sufficient to initiate adipogenesis as determined by triacylglycerol storage, cell morphology, and the expression of adipocyte marker genes, PPARγ and FAS (24). Alternatively, expression of a non-DNA binding dominant negative mutant of CREB, KCREB, blocked adipocyte differentiation in cells treated with insulin, dexamethasone, and IBMX (24). Finally, recombinant or endogenous CREB in pre-adipocyte nuclear extracts was shown to bind to putative CRE sequences in the promoters of several adipocyte-specific genes (24), further supporting the role of CREB in adipogenesis.

Because insulin is known to promote the phosphorylation of MAP kinase and CREB (32, 33), and because its effect was blocked by an inhibitor of FTase, we suggest that the connection between the insulin-signaling cascade and the intrinsic cascade of adipocyte differentiation occurs via the insulin-in-duced and MAP kinase-mediated phosphorylation of CREB, downstream of farnesylated p21ras (Fig. 9). Shc, via its SH2 domain, and MAP kinase mediate insulin effect on FTase (20, 21). FTase, in turn, farnesylates additional molecules of p21ras and increases its availability for subsequent stimulation. This leads to the phosphorylation and activation of MAP kinase and CREB, with the latter connecting insulin signaling with the intrinsic cascade of adipogenesis. This pathway does not negate or minimize the importance of the possible parallel insulin signaling via PI 3-kinase and Akt. In fact, our own studies (not shown), in concert with reports from other laboratories (14, 15), demonstrate that inhibition of PI 3-kinase with wortmannin blocked adipocyte differentiation. Furthermore, insulin has been also shown to exert a post-transcriptional control of adipocyte differentiation through an activation of PI 3-kinase (34) that might complement its effect via prenylation. Our present data outline the importance of prenylation and the role of CREB in this process.

The role of geranylgeranylation of Rho-A in the process of adipocyte differentiation is not understood. Clearly, an inhibitor of GGTase I prevented normal insulin-induced differentiation of adipocytes (Figs. 1 and 5). Inhibition of GGTase I had no effect on either the phosphorylation of MAP kinase or CREB (Figs. 3 and 4), indicating that it arrests adipogenesis at a different step that is independent of CREB signaling and possibly downstream of CREB. However, induction of the constitutively active CREB still rescued the cells from an inhibitory influence of GGTI (Fig. 7), suggesting that the CREB- and
Rho-A-dependent steps are independent of one another, and the CREB pathway can overcome inhibition induced by blocked geranylgeranylation. Further studies are needed to determine the role of Rho-A in this process.

Interesting studies comparing insulin with PDGF (35, 36) have found that even though both hormones activate PI 3-kinase, PDGF failed to promote adipocyte differentiation. In our previous studies, we observed that PDGF also failed to stimulate the prenylation process (17). If both PI 3-kinase- and prenylation-dependent pathways are indeed needed for physiological activation of adipogenesis, it becomes clear why insulin and not PDGF stimulates this process. Previous data from our laboratory have demonstrated that only insulin, and not PDGF, augments the process of prenylation (17). If both PI 3-kinase- and

In summary, the present observations outline the role of prenylation in the process of the insulin-induced adipogenesis. The current findings also suggest that both the PI 3-kinase and the prenylation pathways are physiologically important to promote adipogenesis, and that insulin, a potent activator of both pathways, is a major regulator of adipocyte differentiation. Inhibition of prenylation with either specific inhibitors or statins (37, 38) may attenuate the magnitude of insulin-induced adipocyte differentiation.

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REFERENCES
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