Fatty acid synthase (FAS) is a critical enzyme in de novo lipogenesis. It catalyzes the seven steps in the conversion of malonyl-CoA and acetyl-CoA to palmitate. We have shown that the rate of FAS transcription is induced dramatically when fasted animals are refed with a high carbohydrate, fat-free diet or when streptozotocin-diabetic mice are given insulin. The FAS promoter was up-regulated by insulin through the proximal insulin response sequence containing an E-box motif at the −65-base pair position. Binding of upstream stimulatory factors to the −65 E-box is functionally required for insulin regulation of the FAS promoter. In the present study, we characterized signaling pathways in the insulin stimulation of FAS transcription using specific inhibitors for various signaling molecules and transfecting engineered phosphatidylinositol 3-kinase (PI 3-kinase) subunits and protein kinase B (PKB)/Akt. PD98059 and rapamycin, which inhibit MAP kinase and P70 S6 kinase, respectively, had little effect on the insulin-stimulated FAS promoter activity in 3T3-L1 adipocytes. On the other hand, wortmannin and LY294002, which specifically inactivate PI 3-kinase, strongly inhibited the insulin-stimulated FAS promoter activity. As shown in RNase protection assays, LY294002 also inhibited insulin stimulation of the endogenous FAS mRNA levels in 3T3-L1 adipocytes. Cotransfection of expression vectors for the constitutively active P110 subunit of PI 3-kinase resulted in an elevated FAS promoter activity in the absence of insulin and a loss of further insulin stimulation. Transfecting a dominant negative P85 subunit of PI 3-kinase decreased FAS promoter activity and blocked insulin stimulation. Furthermore, cotransfected wild-type PKB/Akt increased FAS promoter activity in the absence of insulin and a loss of insulin responsiveness of the FAS promoter. On the other hand, kinase-dead PKB/Akt acted in a dominant negative manner to decrease the FAS promoter activity and abolished its insulin responsiveness. These results demonstrate that insulin stimulation of fatty acid synthase promoter is mediated by the PI 3-kinase pathway and that PKB/Akt is involved as a downstream effector.
as one of the major downstream mediators of PI 3-kinase (18–22). PI 3-kinase has also been suggested to activate P70 S6 kinase (23), which is thought to be important for stimulation of protein synthesis by insulin. While the Ras/MAP kinase pathway is believed to play an important role in mitogenic effects of insulin (12, 24), PI 3-kinase is being demonstrated as an important mediator in metabolic regulation including GLUT4 translocation (25–29) and activation of glycogen synthase (30). Recently, PI 3-kinase has been shown to mediate insulin inhibition of the transcription of the PEPCK gene, which encodes the rate-limiting enzyme in gluconeogenesis (31–33).

Molecular mechanisms mediating insulin regulation of lipogenesis, especially the signaling pathways involved, is largely unknown. Since FAS is a critical enzyme involved in lipogenesis, we set out to investigate the signaling pathways involved in the regulation of FAS transcription by insulin. In this report, we provide experimental evidence to demonstrate that the PI 3-kinase signaling pathway mediates insulin regulation of FAS transcription. While inhibition of MAP kinase and S6 kinase by PD98059 and rapamycin, respectively, had little effect on the insulin stimulation of FAS promoter activity, wortmannin and LY294002, which inhibit PI 3-kinase, blocked the insulin stimulation of FAS promoter activity. Regulation of the endogenous FAS mRNA by insulin was also blocked by treating 3T3-L1 adipocytes with LY294002. Cotransfection of expression vectors encoding a constitutively active P110 subunit of PI 3-kinase resulted in elevated FAS promoter activity in the absence of insulin and a loss of insulin response of the FAS promoter. On the other hand, a dominant negative P85 subunit of PI 3-kinase inhibited FAS promoter activity and abolished insulin stimulation of the FAS promoter. Moreover, cotransfection of PKB/Akt stimulated FAS promoter activity in the absence of insulin to that comparable to the insulin-stimulated level. Acting in a dominant-inhibitory fashion, kinase-dead PKB/Akt inhibited FAS promoter activity in the presence and the absence of insulin. These data suggest that insulin regulation of FAS transcription is mediated by the PI 3-kinase signaling pathway and that PKB/Akt is involved as a downstream effector.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs and Reagents**—The reporter gene constructs of p2.1kb-LUC and p67-LUC which contain the −2.1 kb and −67 bp of wild-type rat FAS promoter fused with luciferase sequence, respectively, were described previously (8). Expression vectors pFLAG-USF2, which contain full-length mouse USF2 cDNA sequences, were constructed as described previously (10). Expression vectors for the wild-type and kinase-dead PKB/Akt as well as for the catalytic subunit (P110) of PI 3-kinase (19, 34), including the constitutively active P110* and kinase-dead p110-Δ3, were kindly provided by Dr. L. T. Williams and Dr. A. Klippel at the Chiron Corporation. Expression vectors for the P55 regulatory subunit of PI 3-kinase were constructed by inserting the bovine wild-type P55 as well as the ΔP55 for dominant negative P55α cDNA sequences from SRα-Wp85 and SRα-Δp55 vectors (27, 35), kindly provided by Dr. M. Kasuga at Kobe University, Japan, and Dr. S. I. Taylor at the National Institutes of Health, into mammalian expression vectorpcDNA3.1 (Invitrogen). The MAP kinase MEK1/2 inhibitor, PD98059, was from New England Biolabs. PI 3-kinase inhibitor, LY294002, was purchased from Calbiochem. Rapamycin, wortmannin, and okadaic acid were purchased from Sigma.

**Cell Culture and Transfection**—3T3-L1 preadipocytes were cultured in Dulbecco’s modified Engle’s medium containing 10% fetal bovine serum and induced to differentiate into adipocytes by 0.5 mM 3-isobutylxanthine and 0.5% dexamethasone as described previously (8). 3T3-L1 cells stably transfected with p2.1kb-LUC were prepared by cotransfecting p2.1kb-LUC with the Neo-containing pCDNA3.0 into 3T3-L1 cells and selecting G418-resistant cells (10). Transient transfection of 3T3-L1 adipocytes was carried out in triplicate plates for each sample using the calcium phosphate-DNA coprecipitation method (36) as described previously (8). For experiments using the inhibitors for various signaling molecules, the same volume (less than 0.1% of the total volume of culture media) of carrier (Me2SO or H2O) was used as the negative control. Before adding 10 nM of insulin, inhibitors at concentrations specified in the figure legends were added to treat 3T3-L1 adipocytes for 1 h. For transient transfections, a second batch of inhibitors was added after 12 h of insulin treatment. Luciferase activity was assayed after 48 h of transfection in transient transfection experiments and 10 h after insulin treatment in stable transfections.

**RNase Protection Assays**—To prepare the probe, a 120-bp fragment of the mouse FAS cDNA sequence (6) was amplified with primers FAS-Hind (5′-TTTTTTAAATCTTAAGGGGTCGACCTGGTCCTCA-3′) and FAS-Xba (5′-GGCCATGTCTAGGCTGTTATGAAAGATG-3′) using the high fidelity Pfu DNA polymerase (Stratagene). The polymerase chain reaction product was restriction digested with HindIII and XbaI and directionally cloned into the pcDNA3.0 vector to make plasmid pFAS-RPA. Antiense RNA probe (150 bp in length) was generated by **in vitro** transcription of the HindIII-digested pFAS-RPA using [α-32P]UTP and SP6 RNA polymerase (Riboprobe system, Promega). After insulin treatment, total RNA from duplicate plates was prepared from the 3T3-L1 adipocytes cultured in the presence and the absence of various concentrations of the PI 3-kinase inhibitor LY294002 using the Trizol method (Life Technologies, Inc.). Equal amounts (10 μg) of total RNA samples were subjected to the RNase protection assay using the RPAlI kit from Ambion Inc. following the manufacturer’s recommendations. The protected probe (120 bp in length) was separated on a 5% denaturing gel in Tris-borate-EDTA buffer. The gel was dried and exposed to x-ray films at −70 °C.

**RESULTS**

Insulin Stimulation of FAS Promoter Activity Is Dependent on PI 3-Kinase but Not on MAP Kinase or P70 S6 Kinases—Previously, we demonstrated that the insulin regulation of FAS transcription is mediated by the proximal promoter element at −68/−52 and that USF binding to the E-box motif within this element is required for the insulin regulation (8, 10). As we reported earlier, the reporter gene construct p67-LUC contains the minimal FAS promoter region (up to −67) that confers insulin response (8). In addition, USF can further increase insulin-stimulated promoter activity when p67-LUC is employed in transient transfection assays (10). To study the signaling pathways involved in the insulin regulation of FAS transcription, we first utilized inhibitors for various insulin signaling pathways to examine their effects on the insulin stimulation of FAS transcription. The following inhibitors were tested for their effects in 3T3-L1 adipocytes transiently transfected with the p67-LUC construct. Since insulin is a potent activator of several phosphorylation cascades, we first used serine/threonine phosphatase inhibitor okadaic acid (37, 38). As shown in Fig. 1A, insulin stimulated the FAS promoter activity 1.8-fold in the control experiments (lanes 1 and 2), as we reported previously (10). Treating 3T3-L1 adipocytes with 0.1 μM of okadaic acid stimulated the p67-LUC activity, both in the presence (lane 5) and absence (lane 6) of insulin, to a level of about 150% of the insulin stimulated FAS promoter activity in the control experiment (lane 2). This result suggests that phosphorylation of cellular protein(s) probably is involved in the regulation of FAS promoter activity. Next, we used wortmannin and rapamycin to examine the potential roles of PI 3-kinase and P70 S6 kinase, which are two of the well documented insulin-stimulated kinases, in mediating the insulin stimulation. At 0.2 μM, rapamycin, an immunosuppressant that inhibits P70 S6 kinase (39), showed no and a minor inhibitory effect on FAS promoter activity in the absence (lane 3) and...
Insulin stimulation of FAS promoter activity is dependent on the PI 3-kinase but not MAP kinase or P70 S6 kinase. A, effects of rapamycin, okadaic acid, and wortmannin on insulin stimulation of the minimal insulin-responsive FAS promoter. 3T3-L1 adipocytes were transfected with 10 μg of p67-LUC in triplicate plates as described previously (8). Rapamycin, okadaic acid, and wortmannin were added 1 h before addition of 10 nM insulin to the transfected cells to final concentrations of 0.2, 0.1, and 1 μM, respectively. The carrier (MeSO, DMSO) for the inhibitors were added as a control. Second aliquot of the inhibitors (less than 0.1% of volume of the culture medium) was added after 12 h. Cells were harvested, and luciferase activity was assayed after 24 h of insulin addition. The luciferase activities were calculated relative to those in the control (MeSO) group in the absence of insulin. B, effects of okadaic acid, PD98059, rapamycin, and LY294002 on insulin stimulation of the 2.1-kb FAS promoter activity. 3T3-L1 cells were stably transfected with p2.1kb-LUC as described previously (10). Triplicate plates of differentiated 3T3-L1 adipocytes were incubated in serum-free medium for 12 h and then treated with okadaic acid, PD98059, rapamycin, and LY294002 at concentrations of 0.1, 50, 0.2, and 100 μM, respectively. After 1 h, 10 nM insulin was added, and luciferase activity was assayed 8 h after insulin addition. The same volume (0.1% of the volume of culture medium) of carriers (H2O for okadaic acid, MeSO for PD98059, rapamycin, and LY294002) was added as controls. Luciferase activities were calculated relative to the MeSO treatment without insulin (lane 5).
fected FAS promoter (Fig. 2C). This may be due to the longer treatment period necessary for transient transfection assays than that for measurement of the endogenous mRNA levels. Regardless, these results suggest that the PI 3-kinase pathway is required for the insulin stimulation of the FAS transcription.

Constitutively Active PI 3-Kinase Abolishes Insulin Activity and Indeed Mediates the Insulin Stimulation of FAS Stimulation of FAS Promoter Activity—If PI 3-kinase transcription, activation of PI 3-kinase should increase FAS promoter activity. In addition, the increased FAS promoter activity should not be insulin-responsive since PI 3-kinase is a downstream effector of insulin binding to its receptor. To explore this hypothesis, we cotransfected expression vectors for the constitutively active (P110*) and the kinase-dead (P110-ΔK) catalytic subunit with the p2.1kb-LUC reporter construct into 3T3-L1 adipocytes. P110* contains the iSH2 region of P85 at its N terminus so that it no longer requires the interaction/activation by P85, while P110-ΔK mutates the ATP binding site (19, 34). These P110 expression vectors have been successfully used by other investigators to demonstrate involvement of PI 3-kinase in insulin-regulated processes such as glucose transport (19, 34).

As shown in Fig. 3, cotransfection of P110* at increasing concentrations (from 0.2 μg/plate to 2.5 μg/plate) all resulted in elevated FAS promoter activities (lanes 3 to 8), which are comparable to the insulin stimulated activity when p2.1kb-LUC was transfected alone (lane 2). Furthermore, FAS promoter activity (in lanes 3–8) did not further increase upon insulin treatment. On the other hand, cotransfection of the kinase-dead p110-ΔK (lanes 9–14) at the same concentrations showed no significant effect on the insulin stimulation of FAS promoter activity. These results indicate the PI 3-kinase activity is required for insulin stimulation of the FAS promoter. Because only the ATP-binding site is mutated in p110-ΔK, all the other structural domains such as the P85 interacting domain still are intact. This may cause the P110-ΔK to compete with the endogenous P110 subunit for docking on the membrane and may explain the minor decrease of the promoter activity seen in lanes 9–14.

Dominant-Negative P85 Subunit of PI 3-Kinase Abolishes Insulin Responsiveness of the FAS Promoter—In the process of insulin signaling, P85 regulatory subunit of PI 3-kinase recruits the P110 catalytic subunit to the membrane. Dominant negative P85, which lacks the binding site of P110, was shown to inhibit insulin action by blocking the recruitment of P110

3T3-L1 adipocytes treated with LY294002. Duplicate plates of 3T3-L1 adipocytes were cultured in serum-free medium for 12 h and then treated with no drug (lanes 3–6), or 50 μM (lanes 7–10) and 100 μM (lanes 11–14) LY294002. After 1 h, 10 nM of insulin were added (lanes 5, 6, 9, 10, 13, and 14). Total RNA was prepared using the Trizol reagent. 10 μg of total RNA from each sample were hybridized with 10⁶ cpm of 32P-labeled antisense FAS probe (150 bp in length). Ribonuclease protection assays were performed following the RPAII kit procedure of Ambion Inc. The protected probe (120 bp in length) was analyzed on a 5% denaturing polyacrylamide gel and exposed to x-ray film. Lane 1, short exposure of control reaction without RNase A/T1 digestion of the hybridized probe; lane 2, 10 μg of yeast total RNA were used as a negative control. B, quantitation of the protected FAS probe in A by densitometry. The autoradiograph was analyzed by the IS-1000 digital imaging system by Alpha Innotech. Relative densities of the duplicate samples under each condition were averaged and plotted. Filled triangles represent the relative band intensity with insulin treatment. Open squares represent relative band intensities without insulin treatment. C, dose-dependent inhibition of insulin-stimulated 2.1-kb FAS promoter activity by LY294002. Triple plates of 3T3-L1 adipocytes were transiently transfected with 10 μg of p2.1kb-LUC and treated with 0, 20, 50, and 100 μM LY294002 for 1 h before adding 10 nM insulin. Cells were harvested after 8 h of insulin treatment, and luciferase activity was measured. The insulin-stimulated activities were plotted in relative to those with 0 μM LY294002 treatment.

![Fig. 2](image-url)
To provide further evidence that PI 3-kinase mediates the insulin stimulation of the FAS transcription, we cotransfected dominant negative and wild-type P85 subunit with the p2.1kb-LUC reporter construct into 3T3-L1 adipocytes. Cotransfection of pcDNA-P85WT (lanes 3 and 4) did not show any effect on FAS promoter activity nor its responsiveness as shown in Fig. 4. Cotransfection of the dominant negative P85 (pcDNA-DP85) resulted in 65% decrease in FAS basal promoter activity (lane 5). Furthermore, insulin responsiveness of the FAS promoter was completely lost in cells expressing the dominant negative P85 (lane 6). These data strongly suggest that PI 3-kinase activity is required for the insulin stimulation of FAS transcription.

Serine/Threonine Kinase PKB/Akt Is a Downstream Effector of PI 3-Kinase in Insulin Stimulation of FAS Transcription—PKB/Akt was suggested to be a major downstream mediator of the PI 3-kinase in the insulin signaling pathway (21, 22). This serine-threonine kinase was suggested to be involved in the insulin stimulation of glucose transport, glycogen synthesis, protein synthesis, and insulin-like growth factor-binding protein-1 expression (45–47). To investigate whether it is also involved in the insulin stimulation of the FAS transcription, we cotransfected expression vectors for the wild-type (Akt-wt) and kinase-dead PKB/Akt (Akt-KA) with the p2.1kb-LUC into 3T3-L1 adipocytes. The same expression vector with the PKB/Akt coding sequence deleted was used as a control plasmid. As shown in Fig. 5A, cotransfection of the control vector (lanes 1 and 2) showed a 2-fold insulin stimulation of FAS promoter activity. Cotransfection of the wild-type PKB/Akt resulted in stimulation of FAS promoter activity both in the presence (lane 4) and absence (lane 3) of insulin. Insulin treatment did not further increase promoter activity when wild-type PKB/Akt was cotransfected (lanes 3 and 4). The effects observed with wild-type PKB/Akt are similar to those seen when the constitutively active P110 subunit of PI 3-kinase was cotransfected (Fig. 3). Co-transfection of the kinase-dead PKB/Akt inhibited basal FAS promoter activity by 80% (lane 5). Furthermore, co-transfection of Akt (K179A) abolished the insulin stimulation of the FAS promoter activity (lane 6). When cotransfected with the expression vector encoding the hemagglutinin-tagged wild-type PKB/Akt, Akt-KA abolished the insulin stimulation of the wild-type PKB/Akt activity (Fig. 5B, lanes 3 and 4). On the other hand, it seemed that the effect of Akt-KA on wild-type
PKB/Akt is a downstream mediator of PI 3-kinase in insulin stimulation of FAS transcription. A, expression vectors at concentrations of 1 µg/plate for the wild-type PKB/Akt (Akt-WT, lanes 3 and 4), kinase-dead PKB/Akt (Akt(K179A), lanes 5 and 6), and control plasmid (Control, lanes 1 and 2), were transfected into 3T3-L1 adipocytes. Transfected cells were treated with (in lanes with even numbers) or without (in lanes with odd numbers) insulin as described under “Experimental Procedures.” Luciferase activities of each group were plotted relative to that in control group (lane 1). In vitro Akt kinase activity assay. 3T3-L1 adipocytes were transfected with expression vectors of hemagglutinin-tagged wild-type Akt (Akt-WT) at 1 µg/plate and either kinase-dead Akt (Akt(K179A)) (lanes 3 and 4) or control plasmid (lanes 1 and 2) at 10 µg/plate. The transfected cells were incubated with serum-free medium for 18 h and then treated with (lanes 1 and 3) or without (lanes 2 and 4) 20 nM insulin for 10 min. Cell lysates were prepared, and in vitro Akt kinase activity assay was performed as described under “Experimental Procedures” using H2B as substrate.

PKB/Akt is specific because Akt-KA had no effect on the insulin stimulation of the MAP kinase activity, as determined by the in vitro MAP kinase activity assay (data not shown). This kinase-dead Akt containing a single amino acid mutation (K179A) within the ATP-binding domain (35) was reported to be dominant-inhibitory to insulin-stimulated GLUT4 translocation (46). A similar kinase-dead PKB/Akt that mutates the same lysine residue to methionine (K179M) was also reported to be a dominant negative PKB/Akt to block the insulin stimulation of phosphorylation of the eIF4E-binding proteins (48). Overall, these data suggest that Akt is a downstream mediator of PI 3-kinase in the insulin regulation of FAS transcription.

**DISCUSSION**

When circulating insulin is high, there is an increase in lipogenesis in liver and adipose tissue. These processes occur in coordination with the increase in glucose uptake by peripheral tissues such as muscle and adipose tissue, with inhibition of hepatic gluconeogenesis and glycogen synthesis, and so forth. Increase in lipogenesis is impaired when insulin is low, and administration of insulin restores the rate to its normal level. FAS is a key lipogenic enzyme, and insulin increases its activity dramatically, not through allosteric effectors or covalent modification but through changes in transcription (5, 6). Rapid and high level induction of the FAS gene by insulin makes FAS an excellent model for studying the transcriptional activation of lipogenic genes by insulin. Previously, we had defined the FAS insulin response sequence to the proximal promoter region at −68/−52 and had shown that USF binding to the E-box motif within this region is functionally required for insulin stimulation of FAS transcription. However, the signaling pathway(s) that leads to the increase in lipogenesis or the activation of lipogenic genes by insulin is not known. In this report, we provide evidence that the PI 3-kinase signaling pathway, but not the P70 S6 kinase nor the MAP kinase pathways, mediates the insulin effect on FAS transcription. Inhibitors of PI 3-kinase (wortmannin and LY294002) abolished the insulin stimulation of endogenous FAS mRNA (Fig. 2) as well as the transfected FAS promoter-reporter constructs in 3T3-L1 adipocytes (Fig. 1). Expression of the constitutively active P110 catalytic subunit of PI 3-kinase resulted in the loss of insulin responsiveness of the FAS promoter at an elevated activity level (Fig. 3). Expression of the dominant negative P85 regulatory subunit also resulted in loss of insulin responsiveness of the FAS promoter, but at a repressed activity level (Fig. 4). Similar effects on the insulin responsiveness of FAS promoter were also observed when wild-type and kinase-dead (dominant negative) PKB/Akt were expressed, suggesting PKB/Akt is a downstream mediator of the insulin stimulation of the FAS transcription (Fig. 5). This is the first report, to our knowledge, that demonstrates the involvement of the PI 3-kinase and PKB/Akt signaling pathway in the transcriptional regulation of lipogenic genes. Our data, therefore, suggest that PI 3-kinase is not only involved in the stimulation of glucose transportation, glycogen synthesis, and inhibition of gluconeogenesis, but also in the stimulation of lipogenesis. It also suggests that the effect of PI 3-kinase is a major branching point of the insulin signaling pathways that exert the insulin effect at multiple levels of gene expression, including control of transcription (e.g. stimulation of FAS and inhibition of PEPCK), phosphorylation of glycogen synthase, and translocation of the GLUT4 transporter. Since inhibiting MAP kinase activity by PD98059 had no effect on insulin stimulation of the FAS promoter, it appears that the MAP kinase pathway, which plays an important role in mediating the mitogenic-effect of insulin, is not likely to be involved in insulin stimulation of lipogenesis.

From the results presented in Fig. 5, it is suggested that PKB/Akt is the downstream mediator of the insulin stimulation of FAS transcription. However, further targets of this signaling pathway remain unknown. We previously reported that binding of the helix-loop-helix leucine zipper transcription factor USF to the insulin response sequence of the FAS gene is required for insulin regulation (10), but thus far there has been no conclusive report on the regulation of USF by a phosphorylation-dephosphorylation mechanism. It is possible that either USF itself is regulated by phosphorylation status, or likely a USF-interacting protein is regulated by phosphorylation. In
this regard, four proteins including Ets-1 (49), Fra-1 (50), c-Maf (51), and Fos (52), which are known to be phosphorylated, were reported to be able to interact with USF and are likely the candidates for regulation by phosphorylation. However, whether phosphorylation of any of these USF interacting proteins plays a role in affecting stimulation of transcription by USF requires further investigation. Whether any of these USF interacting proteins is required for insulin regulation of FAS promoter remains to be elucidated.

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