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Regulation of CCAAT/Enhancer Binding Proteinα (C/EBPα) Gene Expression by Thiazolidinediones in 3T3-L1 Adipocytes

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Thiazolidinediones are a class of antidiabetic drugs that induce preadipocyte differentiation by binding and activating peroxisome proliferator-activated receptor γ2. Although thiazolidinediones are commonly thought of as insulin-sensitizing agents, these drugs have opposing and antagonistic effects to that of insulin on CCAAT/enhancer binding protein α (C/EBPα) gene expression in fully differentiated 3T3-L1 adipocytes. Thiazolidinediones induce expression of C/EBPα mRNA and protein, while insulin stimulates a rapid decline in C/EBPα mRNA and protein. When added in combination, thiazolidinediones block the suppression of C/EBPα mRNA by insulin; however, thiazolidinediones do not block the insulin-induced decline in GLUT4 mRNA, indicating that repression of C/EBPα mRNA is not required for insulin to suppress expression of a C/EBPα-responsive gene such as GLUT4. Instead, insulin may regulate GLUT4 mRNA by inactivating C/EBPα through dephosphorylation as well as by inducing the expression of the dominant-negative form of C/EBPβ (liver inhibitory protein), since both of these processes occur in the presence of thiazolidinediones.

The 3T3-L1 cell line has been widely used to delineate the process of preadipocyte differentiation. (1–6). When exposed to inducers of differentiation, 3T3-L1 preadipocytes differentiate from a fibroblast-like phenotype to a phenotype characterized by many of the morphological and biochemical characteristics of adipocytes. Our understanding of the sequence of events involved in differentiation remains incomplete; however, substantial progress has been made in our understanding of pathways acting to stimulate or repress differentiation (reviewed in 7–12). Two of the transcription factors identified as playing critical roles in stimulating preadipocyte differentiation are CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ2 (PPARγ2). Although enforced expression of C/EBPα or PPARγ2 is sufficient to induce some preadipocytes to differentiate to adipocytes, coexpression of both transcription factors is required for most cells to differentiate. Considerable evidence has accumulated to indicate that C/EBPα and PPARγ2 reciprocally induce each others' expression during the differentiation process. When coexpressed, these transcription factors act synergistically to induce the expression of downstream adipocyte genes (e.g. PEPCK, 422/aP2) involved in creating and maintaining the adipocyte phenotype.

Although C/EBPα plays a critical role in promoting preadipocyte differentiation, the role of C/EBPα in mature adipocytes is unknown. Expression of C/EBPα remains high in adipocytes in culture and *in vivo,* suggesting that C/EBPα continues to regulate expression of adipocyte genes in these contexts. A growing body of evidence indicates that C/EBPα may play a role in hormonal control of gene expression in adipocytes and other cell types (13-17). For example, the transcription of C/EBPα and C/EBPδ is rapidly and reciprocally regulated by glucocorticoids in 3T3-L1 adipocytes and white adipose tissue (18). In addition, transcription and activity of C/EBPα is regulated by insulin through at least three mechanisms: rapid dephosphorylation of C/EBPα, suppression of C/EBPα gene transcription, and inactivation of C/EBPα through induction of the dominant-negative form of C/EBPβ (liver inhibitory protein, LIP; 15). While the repression of C/EBPα transcription appears to be mediated by activation of the mitogen-activated protein kinase pathway, dephosphorylation of C/EBPα appears to be mediated by activation of phosphatidylinositol 3-kinase and FKBP-rapamycin-associated protein (19). C/EBPα, in turn, may then regulate the expression of adipocyte genes such as GLUT4, the insulin-responsive glucose transporter.

The role of PPARγ2 in inducing preadipocyte differentiation is now well established (20); however, like C/EBPα, the importance of PPARγ2 in regulating gene expression in fully differentiated adipocytes remains

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unknown. It is likely that PPARγ2 continues to regulate adipocyte gene expression, since expression of PPARγ2 is high in adipocytes in culture and *in vivo*. Considerable evidence has accumulated that products of arachidonic acid metabolism (e.g. prostaglandins) may act as natural ligands for PPARγ2, potentially providing a link between fatty acid metabolism and transcriptional regulation (21,22). In addition, a class of antidiabetic drug known as thiazolidinediones bind PPARγ with high affinity and stimulate PPARγ transcriptional activity (22,23). Since PPARγ2 regulates the expression of C/EBPα during preadipocyte differentiation and since both transcription factors are expressed in fully-differentiated adipocytes, we hypothesized that activation of PPARγ2 in 3T3-L1 adipocytes would increase the expression of C/EBPα. Although thiazolidinediones are known as insulin-sensitizing agents, we find that this class of drug has effects that are opposite and antagonistic to those of insulin on C/EBPα mRNA expression in 3T3-L1 adipocytes. Understanding the complex transcriptional and posttranslational control of C/EBPα and PPARγ2 will provide insight into the coordinate regulation of adipocyte gene expression in response to changing hormonal and metabolic signals.

METHODS

Cell culture. 3T3-L1 preadipocytes were maintained and induced to differentiate into adipocytes as described previously (24), except that insulin was withdrawn from the medium on day 4 (18,25). Fully-differentiated 3T3-L1 adipocytes, 11-14 days after induction of differentiation, were switched to fresh media (either serum free or containing 10% fetal bovine serum) 16 h before subjecting cells to treatment. Insulin (Sigma) was dissolved in 0.02 M HCl. Pioglitazone (Upjohn-Pharmacea) and Troglitazone (Parke-Davis) were dissolved in DMSO. The final concentration of vehicle was $\leq$ 0.1%.

Analysis of RNA. Cellular RNA was isolated from 3T3-L1 cells using RNA Stat60 (Tel-Test "B," Inc). The amount of C/EBPα β, and GLUT4 mRNAs was assessed by Northern blot analysis (19,26). Briefly, total RNA (20 μg) was separated by electrophoresis in horizontal 1.2% agarose gels containing 6.5% formaldehyde. RNA was transferred to nylon membranes (Magna; Micron Separations Inc) and covalently bonded to the membrane by exposure to ultraviolet light (1200 J/cm^2). Blots were prehybridized for 30 min (60°C) in ExpressHyb (Clontech) and hybridized for 60 min at 60°C in an identical solution containing $>2\times10^6$ dpm of labeled probe per ml. Hybridized blots were washed three times in a solution containing 0.1x SSC-0.1% SDS at 65°C. Autoradiography was performed at $-$80°C with Kodak X-OMAT AR film (Eastman Kodak Co.) and an intensifying screen. Results were quantified using a phosphorimager (Bio-Rad) and MultiAnalyst Software.

The DNA fragment used as a probe for C/EBPα mRNA was a $\sim$900 bp Sac I/Hind III fragment complementary to the 3′ end of the C/EBPα coding region, as well as part of the 3′ untranslated region (+1175 to +2078 nucleotides relative to transcriptional start site). The cDNA fragment for C/EBPβ is full length and was cloned from a 3T3-L1 adipocyte library as reported previously (27). The cDNA fragment for GLUT4 was described previously (28). Isolated probes were labeled to high specific activity ($\sim1\times10^9$ dpm per μg) by random hexamer priming (29).

Cell lysates and immunoblotting. 3T3-L1 adipocyte monolayers (10 cm plates) were washed once with 10 ml phosphate buffered saline and scraped in 1 ml of a lysis buffer containing 1% SDS, 60 mM Tris-C1; pH 6.8. Alternatively, monolayers from 6 cm petri plates were lysed in 0.4 ml lysis buffer. Lysates were boiled for 3 min, vortexed, then boiled for an additional 7 min prior to storage at $-$21°C. Western analysis was performed as essentially described previously (30). Briefly, proteins were separated by electrophoresis in 11% polyacrylamide gels (26). Protein was transferred to PVDF-Plus (Micron Separations Inc.) at 150 mA overnight in a buffer containing 20% methanol, 25 mM Tris and 0.2 M glycine. Membranes were then blocked in 1% casein in 1X TBS (Pierce) for 1 h at room temperature. After a brief wash in 1xTTBS (25 mM Tris, 150 mM NaCl, 0.05% Tween 20, 0.17% concentrated HCl and 0.001% Thimerosol), filters were incubated with antisera or affinity-purified antibody in a 1% bovine gamma globulin-1xTTBS solution for 2 h at room temperature. After washing three times (10 min each) in 1xTTBS, filters were incubated in 1xTTBS containing 1% non-fat dried milk and a 1:4000 dilution of anti-rabbit IgG peroxidase conjugate (Sigma ImmunoChemicals) for 1 h at room temperature. Filters were washed as above prior to visualization of bound IgG-peroxidase using Super Signal or Super Signal Ultra Chemiluminescence Substrates (Pierce).

Antibodies. Immune sera against synthetic peptides corresponding to an internal amino acid sequence of C/EBPα (present in both p42$^{C/EBP\alpha}$ and p30$^{C/EBP\alpha}$) and C/EBPβ (present in both Liver Activator Protein and Liver Inhibitory Protein) was prepared as described previously (15, 31). Antisera used for immunoblots are specific for each C/EBP transcription factor and interactions with other C/EBP isoforms are not observed.

RESULTS

Regulation of C/EBPα gene expression by thiazolidinediones. Thiazolidinediones such as pioglitazone and troglitazone induce preadipocyte differentiation through activation of PPARγ2 (20,21,32,33). Induction of differentiation of preadipocytes or cells not determined to the adipocyte lineage by enforced expression of PPARγ2 causes the induction of C/EBPα (20), consistent with the hypothesis that transcription of C/EBPα is regulated by PPARγ2 (11). To determine if an activator of PPARγ2 induces the expression of C/EBPα in fully differentiated adipocytes, a state in which high expression of PPARγ2 is observed, 3T3-L1 adipocytes were incubated with 5 μM pioglitazone for 1, 2, 4, or 6 h prior to preparation of whole cell lysates and immunoblot analysis for C/EBPα (Fig. 1A). As expected, fully differentiated 3T3-L1 adipocytes already express high levels of C/EBPα. Addition of either pioglitazone or troglitazone (data not shown) induces C/EBPα protein a further 3-fold within 4 h.

To determine if induction of C/EBPα protein by thiazolidinediones is the result of induction of C/EBPα mRNA, 3T3-L1 adipocytes were incubated with 5 μM pioglitazone for 1, 2, 4, or 6 h prior to preparation of total RNA and northern blot analysis with a probe specific for C/EBPα (Fig. 1B). Quantification of autoradiographic results indicates that pioglitazone induces C/EBPα mRNA about 2.5 fold within 4 h. Thus, the induction of C/EBPα protein by pioglitazone is largely the

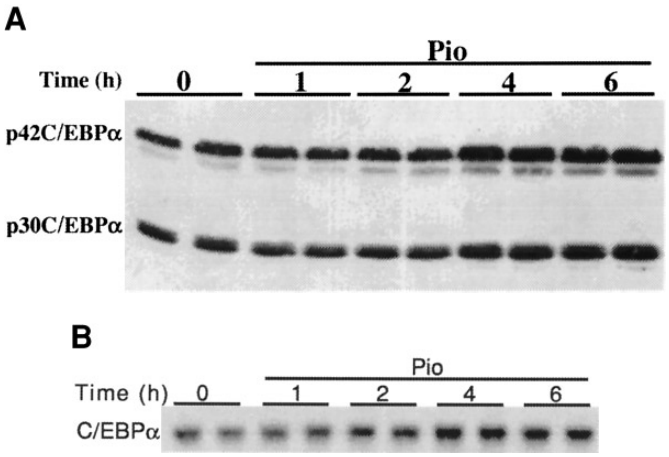

FIG. 1. Pioglitazone rapidly induces the expression of C/EBPα proteins and mRNA. A) 3T3-L1 adipocytes in monolayer culture were treated with 5 μM pioglitazone for 0, 1, 2, 4, or 6 h. Whole cell lysates containing equal cell equivalents (~200 μg protein) were subjected to SDS/PAGE, and immunoblot analysis with antiserum against C/EBPα. Results are representative of three independent experiments. B) Pioglitazone (5 μM) was added to 3T3-L1 adipocytes on day 12 after initiation of differentiation, and total RNA was prepared from 2 independent cell monolayers after 0, 1, 2, 4, or 6 h. Equal amounts of RNA (20 μg) were separated by electrophoresis and analyzed by Northern blot using a DNA fragment complementary to C/EBPα mRNA. Results are representative of three independent experiments.

result of increased C/EBPα gene expression. Since activation of PPARγ by thiazolidinediones in fully-differentiated adipocytes results in an induction of C/EBPα gene expression, endogenous activators of PPARγ2 may also act to induce C/EBPα, and thus signal the cell to maintain adipocyte gene expression and the adipocyte phenotype.

Thiazolidinediones block the suppression of C/EBPα mRNA by insulin. Members of the thiazolidinedione family are known as insulin-sensitizing agents since they decrease the amount of insulin required to reduce hyperglycemia in animal models of type II diabetes (34). Previous studies (15,19) have shown that insulin rapidly suppresses C/EBPα transcription, mRNA, protein and DNA-binding activity in 3T3-L1 adipocytes through activation of the mitogen-activated protein kinase (ERK1/2) pathway. To ascertain whether pioglitazone influences the ability of insulin to suppress C/EBPα mRNA, 3T3-L1 adipocytes were treated with 5 μM pioglitazone, 167 nM insulin, or pioglitazone and insulin for 0, 2, 4, or 7 h prior to cell lysis, purification of total RNA and northern blot analysis (Fig 2). As expected, pioglitazone induces expression of C/EBPα mRNA, while insulin causes a dramatic decline in C/EBPα mRNA. Surprisingly, pioglitazone largely blocks the insulin-induced decline in C/EBPα mRNA (Fig. 2) and proteins (data not shown), suggesting that although thiazolidinediones sensitize animals to the effects of insulin on blood glucose and other whole body variables, thiazolidinediones can have effects on adipocyte gene expression that oppose and antagonize those of insulin.

Suppression of C/EBPα mRNA is not necessary for suppression of GLUT4 mRNA by insulin. Because of the considerable evidence indicating that C/EBPα regulates the insulin-sensitive glucose transporter, GLUT4 (35,36), we have hypothesized that insulin represses GLUT4 gene expression in part through causing a decline in C/EBPα mRNA and protein (15). If suppression of C/EBPα mRNA is necessary for suppression of GLUT4 by insulin, we would predict that, since thiazolidinediones maintain the expression of C/EBPα mRNA in the presence of insulin, thiazolidinediones would also maintain the expression of GLUT 4. In contrast to this hypothesis, pioglitazone rapidly suppresses expression of GLUT4 mRNA even in the presence of pioglitazone (Fig. 2). Northern blot analysis also indicates that pioglitazone alone has no effect on GLUT4 expression. These data indicate that factors other than the decline of C/EBPα mRNA are required for the regulation of GLUT4 mRNA by insulin.

Induction of C/EBPβ mRNA by thiazolidinediones. To ascertain whether C/EBPβ mRNA is regulated by pioglitazone, 3T3-L1 adipocytes were incubated for 0, 2, 4, or 7 h with 167 nM insulin, 5 μM pioglitazone, or both pioglitazone and insulin prior to preparation of total RNA for northern blot analysis. As observed previously (19), insulin rapidly and transiently induces the expression of C/EBPβ (Fig. 2). While addition of pioglitazone causes a small and transient increase in C/EBPβ, addition of pioglitazone and insulin results in an additive increase in the transient expression, consistent with insulin and thiazolidinediones working through independent mechanisms. To determine if regulation of C/EBPβ mRNA by pioglitazone is reflected in the expression of C/EBPβ proteins, immunoblot analysis was performed with whole cell lysate from adipocytes treated for 2 h with insulin, pioglitazone, or insulin and pioglitazone (Fig. 3). As observed previously (19), the expression of the dominant-negative form of C/EBPβ (LIP) predominates over the active form of C/EBPβ (liver activator protein; LAP). Consistent with the regulation of C/EBPβ mRNA observed in Fig. 2, both insulin and pioglitazone induce the expression of LAP and LIP, with the combination of pioglitazone and insulin inducing LAP and LIP more than either agent alone. The expression of LIP exceeds that of LAP in all cases. Thus, the induction of LIP by insulin is consistent with insulin-induced suppression of GLUT4 gene expression. In addition, although thiazolidinediones and insulin have opposite effects on C/EBPα gene expression, these agents both transiently stimulate expression of C/EBPβ mRNA and proteins.

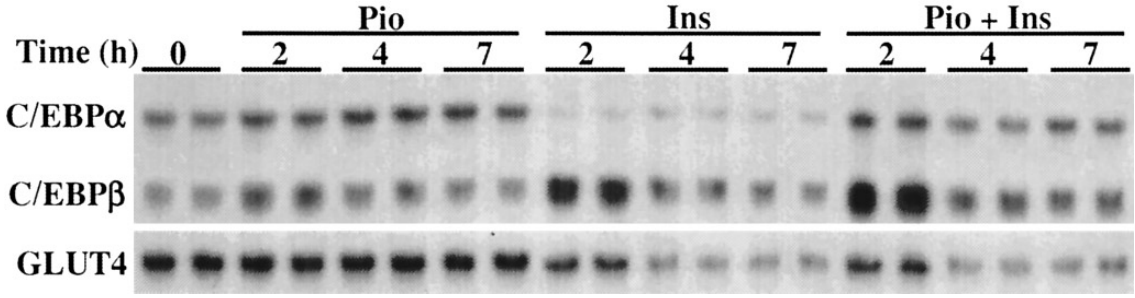

FIG. 2. Effect of pioglitazone on the regulation of C/EBPα, C/EBPβ, and GLUT4 mRNAs by insulin. Fully-differentiated 3T3-L1 adipocytes (14 days after induction of differentiation) were incubated for 2, 4 or 7 h with 5 μM pioglitazone (Pio), 167 nM insulin (Ins), or 5 μM pioglitazone and 167 nM insulin (Pio + Ins). Total RNA was prepared from nontreated adipocytes as well as from cells incubated for the indicated times. Northern blot analysis was performed with probes specific for C/EBPα, C/EBPβ, and GLUT4. Similar results were observed in 3 independent experiments.

Dephosphorylation of C/EBPα correlates with suppression of GLUT4 mRNA by insulin. We reported previously that the suppression of GLUT4 gene expression by insulin may be mediated in part by dephosphorylation and inactivation of C/EBPα (15,19). To ascertain whether dephosphorylation of C/EBPα by insulin occurs in the presence of thiazolidinediones, fully differentiated 3T3-L1 adipocytes were incubated for 45 min with 167 nM insulin, 5 μM troglitazone, or both agents prior to whole cell lysis and immunoblot analysis (Fig. 4). The phosphorylation status of C/EBPα is most easily visualized by the mobility of p30C/EBPα, which corresponds to translation from the third in-frame methionine (31). Similar changes occur in p42C/EBPα, except they are more difficult to resolve because the N-terminal 12 kDa is highly phosphorylated and because the protein doesn't migrate as far as p30C/EBPα upon SDS-PAGE (19). In lysates from nontreated adipocytes, p30C/EBPα migrates as three bands (Cont; Fig. 4), designated bands a - c. Forty five min of insulin treatment causes dephosphorylation of C/EBPα with a decrease in bands a and b, and an increase in band c (Ins; Fig. 4)), and insulin-induced dephosphorylation occurs in the presence of troglitazone (Trog + Ins; Fig. 4) or pioglitazone (not shown). Forty five min of troglitazone (Trog; Fig. 4) or pioglitazone (data not shown) alone does not appear to affect the phosphorylation of C/EBPα. Therefore, the repression of insulin-responsive adipocyte genes such as GLUT4 may be due to insulin-stimulated dephosphorylation of C/EBPα, in conjunction with inactivation of C/EBPα by insulin-dependent induction of LIP.

DISCUSSION

In addition to causing dephosphorylation of C/EBPα, insulin also stimulates a decline in C/EBPα mRNA and protein, a process largely blocked by coincubation with thiazolidinediones (Fig. 2). Because the decline in

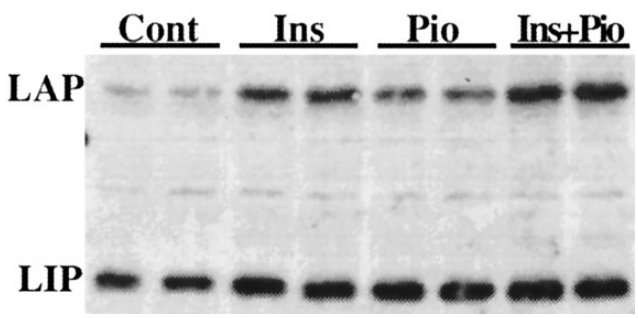

FIG. 3. Pioglitazone and insulin induce expression of C/EBPβ proteins. 3T3-L1 adipocytes were incubated for 2 h with vehicle (Cont), 167 nM insulin (Ins), 5 μM pioglitazone (Pio), or 167 nM insulin and 5 μM pioglitazone (Ins+Pio). Whole cell lysates were prepared and subjected to immunoblot analysis with antiserum against C/EBPβ. The migration of C/EBPβ (LAP and LIP) proteins are indicated. Similar results were observed in 2 independent experiments.

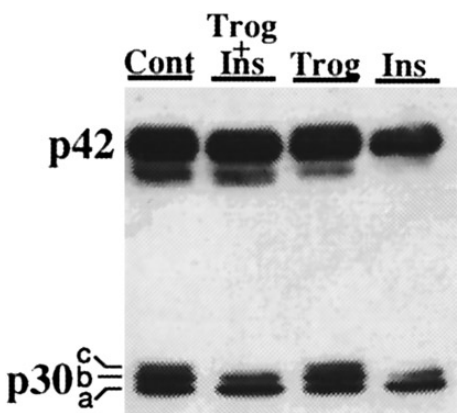

FIG. 4. Troglitazone does not influence insulin-dependent dephosphorylation of C/EBPα. 3T3-L1 adipocytes were incubated for 45 min with vehicle (Cont), 5 μM troglitazone and 167 nM insulin (Trog + Ins), 5 μM Troglitazone (Trog) or 167 nM insulin (Ins). Whole cell lysates were prepared and subjected to immunoblot analysis for C/EBPα. Similar results were observed in 3 independent experiments.

GLUT4 mRNA following insulin treatment occurs in the presence of thiazolidinediones (Fig. 2), repression of C/EBPα mRNA does not appear to be necessary for the regulation of GLUT4 by insulin. In contrast, insulin-induced dephosphorylation of C/EBPα correlates well with the suppression of GLUT4 mRNA levels since both occur in the presence of thiazolidinediones (Fig. 4). Similarly, the transient induction of C/EBPβ by insulin, which results in a large increase in LIP, also correlates well with repression of GLUT4 since the induction of LIP by insulin is potentiated in the presence of thiazolidinediones (Fig. 3). These results are reminiscent to those obtained with an inhibitor of mitogen-activated kinase kinase (MEK), which blocks the insulin-induced decline in C/EBPα mRNA, but not the dephosphorylation of C/EBPα or induction of LIP observed after treatment with insulin (19).

Although PPARγ2 is known to play an important role in stimulating preadipocyte differentiation (20), regulation of gene expression by PPARγ2 in fully-differentiated adipocytes remains poorly studied. Since thiazolidinediones induce expression of C/EBPα and C/EBPβ in 3T3-L1 adipocytes, it appears likely that PPARγ2 retains control over the transcription of at least a subset of adipocyte genes. It also appears likely that hormonal and metabolic signals are processed through C/EBPα and PPARγ2 in order to affect large scale changes in adipocyte gene transcription and metabolism. This regulation is likely to be extremely complex since both PPARγ2 and C/EBPα are regulated at the transcriptional (10,18,37-39) and posttranslational levels (15,19,40-42). Moreover, a positive feedback mechanism exists by which C/EBPα induces transcription of the PPARγ2 gene (43), and PPARγ2 induces transcription of the C/EBPα gene (20). In addition, it also appears that binding of both PPARγ and C/EBPα to the 5′-flanking region of adipocyte genes is required to fully activate transcription of certain adipocyte genes (e.g. 422/aP2, PEPCK; 20,44). Finally, further complexity is added by the observation that PPARγ acts to repress transcription of the leptin promoter by antagonizing the effects of C/EBPα (45). Understanding the elaborate interplay between these two important transcription factors will provide insight into the mechanism by which extracellular (e.g. hormones) and intracellular (e.g. fatty acid metabolites) signals regulate adipocyte gene expression and metabolism.

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