Insulin- and Mitogen-activated Protein Kinase-mediated Phosphorylation and Activation of Peroxisome Proliferator-activated Receptor γ⁎

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Peroxisome proliferator-activated receptor (PPAR) γ plays an important role in adipocyte differentiation and the regulation of adipocyte gene expression. Insulin also serves to promote adipogenesis. We report that insulin and a PPARγ ligand (thiazolidinedione (TZD)) stimulate in a synergistic manner the expression of an adipocyte-specific gene (aP2) in rat adipocytes and 3T3-L1 cells. Potential cross-talk between insulin signaling and PPARγ was studied in Chinese hamster ovary cells expressing insulin receptors (CHO.T), PPARγ, and reporter genes. Both TZD and insulin independently stimulated PPARγ-mediated transactivation of aP2 promoter-luciferase reporter genes; both agents combined resulted in a synergistic effect. Co-transfection of CHO.T cells with dominant-negative mitogen-activated protein (MAP) kinase-kinase (MKK1) abrogated both insulin- and TZD-mediated activation of PPARγ transactivation was markedly increased in cells co-transfected with constitutively active MKK1. Both insulin and constitutively active MKK1 also stimulated 32P incorporation into PPARγ in vivo. The conclusions are: 1) Insulin synergizes with a PPARγ ligand and can activate the receptor in a ligand-independent fashion. 2) PPARγ is phosphorylated in vivo by insulin stimulation or activation of the MAP kinase pathway. 3) MAP kinase is an important mediator of cross-talk between insulin signal transduction pathways and PPARγ function.

Adipogenesis is a complex process regulated by a variety of hormones and molecules. Upon the induction of differentiation of fibroblast-like preadipocytes to mature adipocytes, a cascade of gene transcription events occurs, leading to the expression of a constellation of genes specific for adipocytes (1). One of the earliest genes to be induced during differentiation encodes peroxisome proliferator-activated receptor (PPAR)γ, a member of the nuclear receptor superfamily that includes ligand-activated transcription factors such as steroid, retinoid, and thyroid hormone receptors (2, 3). Two related isoforms (γ1 and γ2), which differ by only 30 N-terminal amino acids are expressed as a result of alternative promoter usage and mRNA splicing (4). Both isoforms are expressed predominantly in brown and white adipose tissue (5, 6). Ligands for PPARγ, such as 15-deoxy-Δ12,14-prostaglandin J3 and anti-diabetic thiazolidinedione (TZD) compounds, are potent inducers of adipocyte differentiation (7, 8). Thus, PPARγ functions as a dominant activator of adipocyte differentiation through transactivation of adipose-specific genes (5, 7–9).

Insulin is the major anabolic hormone that counters lipolysis and promotes in vivo accumulation of adipose tissue. Either insulin (10) or insulin-like growth factor 1 (IGF-1) (11) have an important role in potentiating the differentiation of 3T3-L1 preadipocytes. Because both insulin (or IGF-1) and PPARγ regulate adipocyte differentiation, cross-talk between the two signaling pathways may occur. Insulin elicits its biological effects through activation of its receptor tyrosine kinase, resulting in phosphorylation of insulin receptor substrate-1 and other immediate substrates (12). These proximal tyrosyl phosphorylation events lead to activation of several distinct signal transduction pathways including the mitogen-activated protein (MAP) kinase cascade. This involves activation of p21 Ras, which in turn activates Raf-1 leading to the activation of MAP kinase kinase (M KK) and the activation of p44MAPK (ERK1) and p42MAPK (ERK2) MAP kinases (13). MAP kinase has been implicated as an important signaling mechanism involved in the differentiation of preadipocyte fibroblasts into adipocytes (14–16).

The present study was initiated to gain insight into the potential for cross-talk between insulin-stimulated signal transduction pathways and PPARγ. Here, we show that insulin and a PPARγ-specific ligand (TZD) synergize to transactivate gene expression mediated by PPARγ. Evidence is presented that suggests that insulin’s effects are mediated by MAP kinase, which results in phosphorylation of PPARγ.

EXPERIMENTAL PROCEDURES

Materials—CHO cell line expressing human insulin receptor (CHO.T) was a gift from Dr. Richard Roth (Stanford University). PPARγ1 and PPARγ2 cDNAs were cloned as described previously (17). The following cDNA constructs were provided as gifts: aP2 cDNA (Dr. David Bernlohr, University of Minnesota); mouse PPARγ2 cDNA (3) and aP2 promoter fragments (18) (Dr. Bruce Spiegelman, Dana Farber Cancer Institute); M KK-1 constructs (19) (Drs. Sally Cowley and Chris Marshall, Institute of Cancer Research, London); a chimeric receptor construct (pSG5-GR/PPARγ-LBD) consisting of the N-terminal portion of the glucocorticoid receptor (GR) and the C-terminal 300 amino acids (ligand binding domain) of PPARγ, a GR-responsive reporter construct (M MMV-LUC), and h-NUC (PPARδ) (Dr. Azriel Schmidt, Merck); murine PPARα and a PPRE-reporter (PPRE-LUC) (Dr. Tom Rushmore, Merck); pMT2-RA-RSK1 and pMT2-RA-ERK1 (Dr. Joseph Avruch, Massachusetts General Hospital). A synthetic inhibitor of MKK (FPD98059) (20) was purchased from New England Biolabs. A high affinity PPARγ ligand, 5-(4-[2-(5-methyl-2-phenyl-4-oxazolyl)-2-hydroxyethoxy]-benzyl)-2,4-thiazolidinedione (TZD), was provided by G.

MAP, mitogen-activated protein; M KK, MAP kinase kinase; CHO, Chinese hamster ovary; GR, glucocorticoid receptor; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PPRE, peroxisome proliferator response element.

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† The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; IGF, insulin growth factor;
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Kieczykowski, P., Eckola, C., Santini, J., Leone, and P. Cicala (Merck). Transactivation of Rat Adipocytes and 3T3-L1 Cells—Adipocytes were isolated from male Wistar rats using a previously described procedure (21) with modifications. Packed adipocytes (4 ml) were added to 20 ml of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium containing 2% fetal bovine serum (Hyclone), and the incubation was carried out at 37 °C in 5% CO2 for 16 h. Insulin (100 nm) or TZD (1 μM) were added to the culture medium, and the incubation was continued for 24 h. Confluent 3T3-L1 cells were incubated in medium containing 10% fetal bovine serum, 1 μM dexamethasone with insulin (100 nm), TZD (1 μM), or both agents for 72 h.

Isolation of RNA and Northern Blot Analysis—Total RNA preparation, Northern, and slot blot procedures were performed as described (22). The hybridization signals were analyzed with a PhosphorImager (Molecular Dynamics) or autoradiography using XAR-5 film (Eastman Kodak).

Plasmids and Transfections—The entire coding region of PPARγ1 and PPARγ2 were subcloned into pSG5 vector or pMT2-HA. aP2 reporter plasmids were constructed by ligating promoter fragments (~5.4 kilobases) into PGL3 basic (Promega) or by ligating the luciferase gene into a reporter plasmid adjacent to the aP2 518-base pair enhancer and minimal promoter. A mutant PPARγ receptor with Ser182 to Ala mutation was prepared from pMT2-HA-PPARγ2 using the Transformer® site-directed mutagenesis kit (Clontech). The mutation was verified by DNA sequencing.

CHO.T cells (600,000 cells/well, 6-well plate) were transiently transfected with various expression plasmids (total, 2–3 μg of DNA) and pCH110 (1 μg) using Lipofectamine (Life Technologies, Inc.) reagent following the manufacturer’s instruction. Lysates were prepared followed by the determination of luciferase and β-galactosidase activities as described (17).

In Vivo Labeling and Analysis of 32P-Labeled PPARγ—CHO.T cells (100-mm plates) were transfected with pMT2-HA-PPARγ2 with or without MKK1 expression vectors. 5 h after transfection, cells were refed and incubated in medium containing 10% fetal calf serum for 16 h. Cells were then serum-starved for 24 h, followed by incubation with phosphate-free medium for 4 h and then with medium containing [32Porthophosphate (1 mCi/3 ml) and okadaic acid (300 nm)]. Cells were subsequently incubated for 30 min with or without insulin (500 nm). Cells were then washed and lysed. HA-tagged PPARγ was immunoprecipitated using anti-HA monoclonal antibody (Babco) and separated by electrophoresis on 4–20% SDS-polyacrylamide gels. The proteins were transferred to polyvinylidene difluoride membranes and the 32P-labeled proteins were detected by PhosphorImager or autoradiography. Separate sets of membranes were incubated with anti-HA antibody. Protein bands were detected using the ECL system (Amersham Corp.) and quantitated using IMAGEQUANT™ (Molecular Dynamics).

In Vitro Phosphorylation of PPARγ—GST-PPARγ and GST-PPARγ-LBD proteins were expressed in Escherichia coli and purified using glutathione-Sepharose 4B as described by the manufacturer (Pharmacia Biotech Inc.). PPARγ proteins (3 μg) were incubated in 30 μl of buffer containing 18 mM HEPES (pH 7.4), 10 mM MgCl2, 1 mM vanadate, 5 mM NaF, 1 mM β-glycerophosphate, 1 mM EDTA, 20 μM ATP (5 μCi), and 0.2 μg of purified activated GST-p42 MAP kinase (USB) at 30 °C for 30 min. The proteins were separated on 4–20% SDS-polyacrylamide gels, and the phosophoproteins were visualized with a PhosphorImager or by autoradiography.

RESULTS

Synergy between Insulin and a PPARγ Ligand for Activation of aP2 mRNA Expression in Rat Adipocytes and 3T3-L1 Cells—The effects of insulin and TZD, a high affinity PPARγ ligand, on the expression of a PPARγ-regulated gene, adipocyte-specific fatty acid binding protein (aP2), were assessed. In cultured primary rat adipocytes, incubation with insulin did not greatly affect the expression level of aP2, whereas incubation with TZD resulted in a slight increase in mean aP2 mRNA levels (Fig. 1A). However, when cells were incubated with both agents, aP2 mRNA levels were increased in a synergistic manner (Fig. 1A). To verify these observations using a different cell system, confluent 3T3-L1 preadipocytes were incubated for 72 h in the presence of TZD, insulin, or a combination of the two, followed by subsequent measurement of aP2 mRNA levels. Insulin alone did not cause significant changes in aP2 mRNA expression, whereas TZD induced a 30-fold increase in mean mRNA levels. Coincubation with insulin and TZD caused a further increase (50-fold) in aP2 expression (Fig. 1B).

Effects of Insulin on PPARγ-mediated Gene Transcription—To determine whether insulin has an effect to augment the transcriptional activity of PPARγ, transactivation experiments were performed using transfected CHO cells that express insulin receptor, PPARγ, and PPARγ-responsive reporter genes. Following transfection, the cells were treated with TZD, insulin, or the combination of the two agents. As shown in Fig. 2, TZD caused a ~2-fold increase in −5.4 kilobases aP2 promoter-luciferase activity, which was mediated by co-transfected PPARγ2. In the same cells, insulin alone stimulated PPARγ-mediated aP2 transcription by 10-fold. Even greater stimulation (60-fold) was observed when cells were incubated simultaneously with TZD and insulin. Lessor degrees of stimulation by insulin or insulin plus TZD were observed using cells without co-transfected PPARγ, indicating the presence of low levels of the endogenous protein (confirmed by Northern analysis; not shown). A similar pattern of PPARγ-mediated transactivation that was stimulated by insulin and (synergistically) by TZD plus insulin was also observed using alternative reporter constructs containing the distal 518-base pair enhancer of the aP2 promoter or a generic PPRE (data not shown). These results suggest that the effects of insulin and TZD on aP2 expression are mediated through PPARγ and PPRE. Furthermore, similar effects were observed when PPARγ1 rather than γ2 was used in the transactivation assay (data not shown). Thus, insulin stimulation can modulate the activity of PPARγ, independently of exogenous ligand as well as synergistically when combined with a known PPARγ agonist.

In addition, we studied the effects of insulin and TZD in CHO.T cells that were co-transfected with pSG5-GR/PPARγ-LBD and the MMTV-LUC reporter gene. Although TZD stimulation resulted in a 10-fold increase in the luciferase activity, insulin had no effect (not shown). This suggests that the amino acid residue(s) of PPARγ, which are modulated in response to insulin signaling, reside outside of the receptor’s ligand binding domain.

The MAP Kinase Pathway Is Necessary and Sufficient for Insulin-stimulated PPARγ Activation—In order to assess whether the MAP kinase pathway was required for insulin-mediated activation of PPARγ, CHO.T cells co-transfected with PPARγ and aP2 (5.4)-LUC were treated with or without PD98059, a specific inhibitor of MKK1 (20). The effect of insulin on PPARγ activation was attenuated by this agent (50% inhibition at 10 μM, data not shown). To further explore this hypothesis, CHO.T cells were co-transfected with PPARγ and aP2 (5.4)-LUC, with or without the addition of alternative MKK1 constructs. As shown in Fig. 3, co-transfection with
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Fig. 2. Effect of insulin and TZD on transactivation of aP2 promoter by PPARγ. CHO.T cells were transfected with vector control or pSG5-PPARγ-2, and cotransfected with aP2 (5.4)-LUC and pCH110. 24 h after transfection, the cells were incubated with TZD (100 nM), insulin (100 nM), or both agents for 24 h. Luciferase activity was determined in lysates and normalized against β-galactosidase activity for transfection efficiency. Shown are means ± S.E. for triplicates. The experiment was repeated three times with similar results.

Fig. 3. Insulin and MAP-kinase mediated activation of PPARγ. CHO.T cells were transfected with pSG5-PPARγ-2 and cotransfected with aP2 (5.4)-LUC as well as M KK vector control or expression plasmids encoding one of the following: wild-type M KK1, mutant M KK1 with constitutive activity (CA), dominant-negative M KK1 (DN), RSK1, or ERK-1. Cells were treated and analyzed as described in legend to Fig. 2. Shown are means ± S.E. for triplicates. The experiment was repeated three times with similar results.

Dominant-negative M KK1 abolished the effect of insulin and TZD to stimulate transactivation. In contrast, co-transfection with a constitutively active M KK1 mutant resulted in a 3- to 5-fold increase in basal transactivation and an even more marked increase in TZD-mediated transactivation; the responses to insulin and insulin plus TZD were also augmented (~3-fold).

Co-transfection with wild-type M KK1 or RSK1, a kinase that lies downstream of MAP kinase, had no effects. Co-transfection with wild-type MAP kinase (ERK-1) resulted in a modest increase in the effect of insulin and insulin plus TZD, suggesting amplification of the insulin-induced signal. The above results suggest that MAP kinase is both necessary and sufficient for the insulin-mediated effect on PPARγ function.

Examination of the primary sequence of PPARγ indicates the presence of a potential MAP kinase phosphorylation site at Ser112. This sequence (PASP) matches the consensus sequence for MAP kinase (PX[S/T]P; where X is a neutral or basic amino acid and n = 1 or 2) as defined by Gonzalez et al. (29). As shown in Fig. 4, the GST fusion protein containing full-length PPARγ-2 was phosphorylated by purified P42MAP kinase in vitro. In contrast, protein containing the 300-residue PPARγ ligand binding domain (and lacking Ser112) was a poor substrate for MAP kinase. In addition, a PPARγ site-directed mutant (Ser112 → Ala) was not phosphorylated in vitro by p42 MAP kinase (not shown).

To determine whether MAP kinase could modulate the phosphorylation state of PPARγ in intact cells, CHO.T cells were transfected with epitope-tagged PPARγ with or without M KK1 mutants, labeled with [32P]orthophosphate, and then treated acutely with insulin. [32P]-Labeled HA-PPARγ was subsequently immunoprecipitated from the lysates and analyzed by SDSPAGE (Fig. 5). Stimulation with insulin resulted in a significant (4-fold) increase in [32P] incorporation into the HA-PPARγ protein. The addition of co-transfected constitutively active M KK1 was associated with a substantial increase (10-fold over basal) in PPARγ phosphorylation, whereas dominant-negative M KK1 inhibited the insulin-stimulated incorporation of [32P] into HA-PPARγ. Phosphoamino acid analysis indicated that the [32P] incorporation occurred on serine residue (data not shown).

DISCUSSION

In the present study, we have demonstrated that insulin stimulates the phosphorylation and transcriptional activity of PPARγ. These observations provide a link between two important elements involved in the regulation of adipogenesis and the expression of adipocyte-specific genes. Several lines of evidence were provided to show that the effects of insulin are mediated by MAP kinase. MAP kinase activation was shown to be both necessary and sufficient for in vivo phosphorylation of PPARγ and for mediating transcriptional activation in response to insulin. The results of these studies also suggest that insulin, via MAP kinase, can activate PPARγ in a ligand-independent fashion, as demonstrated in Figs. 2 and 3 using CHO cells expressing insulin receptors. Data using rat adipocytes or 3T3-L1 cells clearly showed that insulin can substantially augment ligand-stimulated expression of a classic PPARγ-responsive gene (aP2). However, the extent to which insulin may independently promote the transcriptional activity of PPARγ in adipocytes may be more limited because insulin alone did not significantly affect expression of aP2 mRNA (Fig. 1).

These results also imply that the function of PPARγ to regulate adipogenesis and adipocyte gene expression may involve critical control by a variety of stimuli (in addition to insulin or IGF-1) that modulate the activity of MAP kinase. In this re-
In conclusion, the results of this study demonstrate that PPARγ is a phosphoprotein and can be activated independently by insulin and TZD. MAP kinase-mediated cross-talk between insulin signaling and PPARγ results in a synergistic effect that has important implications for our understanding of the regulation and function of PPARγ in vivo.

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