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Activation of Lipoprotein Lipase by Glucose-dependent Insulinotropic Polypeptide in Adipocytes

A ROLE FOR A PROTEIN KINASE B, LKB1, AND AMP-ACTIVATED PROTEIN KINASE CASCADE[*] [S]

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Glucose-dependent insulinotropic polypeptide (GIP) has been mainly studied because of its glucose-dependent insulinotropic action and its ability to regulate β-cell proliferation and survival. Considerably less is known about the effects of GIP on fat metabolism, and the present study was directed at identifying the mechanisms underlying its stimulatory action on lipoprotein lipase (LPL). In differentiated 3T3-L1 adipocytes, GIP, in the presence of insulin, increased LPL activity and triglyceride accumulation through a pathway involving increased phosphorylation of protein kinase B (PKB) and reductions in phosphorylated LKB1 and AMP-activated protein kinase (AMPK). Knockdown of AMPK using RNA interference and application of the AMPK inhibitor, Compound C, supported this conclusion. In contrast, the other major incretin hormone, glucagon-like peptide-1, exhibited no significant effects on LPL activity or PKB, LKB1, or AMPK phosphorylation. Cultured subcutaneous human adipocytes showed similar responses to GIP but with greater sensitivity. Chronic elevation of circulating GIP levels in the Vancouver diabetic fatty Zucker rat *in vivo* resulted in increased LPL activity and elevated triglyceride accumulation in epididymal fat tissue, combined with a modulation of PKB, LKB1, and AMPK phosphorylation similar to that observed *in vitro*. This appears to be the first demonstration of a GIP-stimulated signal transduction pathway involved in increasing fat storage in adipocytes.

Glucose-dependent insulinotropic polypeptide (GIP)[2] is a pleiotropic hormone that is released from gut endocrine cells in response to nutrient ingestion (1–3). There is strong evidence that GIP and glucagon-like peptide-1 (GLP-1) are the two most important gut-derived insulinotropic hormones, or incretins (1–4). Both incretins also exert powerful positive effects on pancreatic β-cell growth, development, and survival (5, 6). A number of studies have demonstrated that GIP plays an important role in the regulation of fat metabolism (7–9). GIP is released in response to administration of triglycerides (TG) (1, 2), with long chain fatty acids (FAs) being responsible for stimulating secretion (1). In dogs, GIP has been shown to promote clearance of chylomicron-associated TG from blood (10), and in rats, it has been shown to promote infusion of GIP-lowered plasma TG responses to intraduodenal fat (11). GIP enhanced FA synthesis from acetate in adipose tissue explants (12) as well as potentiating insulin-stimulated FA incorporation into adipose tissue (13) and stimulating lipoprotein lipase (LPL) activity in cultured preadipocytes (14) and mature adipocytes (15). These studies pointed to a significant role for GIP in the regulation of adipogenesis, and its physiological importance was emphasized by the demonstration by Miyawaki *et al.* (16) that GIP receptor knock-out mice exhibited reduced adipose tissue accretion on a high fat diet.

The GIP receptor is a member of the class B seven-transmembrane G protein-coupled family to which the receptors for glucagon, GLP-1, and secretin belong (17, 18). The majority of studies on the mode of action of GIP have been performed in islets, dissociated β-cells, or β-cell lines, and these have shown that receptor activation results in the stimulation of adenylyl cyclase (17) and phospholipase A_2 (19). Downstream signaling involves a number of enzyme modules, including protein kinase A/cAMP-response element-binding protein, Rap1/Raf-A/Mek/Erk1/2 (20), and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB)/Foxo1 (21, 22). The mechanism by which GIP acts on adipocytes is largely unknown. GIP receptor expression has been demonstrated in rat adipocytes (23) and differentiated 3T3-L1 cells (8), and, in the absence of insulin, activation results in the stimulation of adenylyl cyclase and lipolysis (8). However, since this lipolytic action is inhibited by insulin (8), it was considered likely that the lipogenic effects of GIP are mediated through alternative pathways.

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that acts as an intracellular energy sensor (24, 25) or "fuel gauge" (26). AMPK exists as a heterotrimeric protein complex consisting of a catalytic subunit (α) and two regulatory subunits

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[2] The abbreviations used are: GIP, glucose-dependent insulinotropic polypeptide; LPL, lipoprotein lipase; PKB, protein kinase B; AMPK, AMP-activated protein kinase; GLP-1, glucagon-like peptide-1; FA, fatty acid; VDF, Vancouver diabetic fatty; PI3K, phosphatidylinositol 3-kinase; CA, constitutively active; DN, dominant negative; TG, triglyceride(s); DMEM, Dulbecco's modified Eagle's medium; OGTT, oral glucose tolerance test; ANOVA, analysis of variance; siRNA, small interfering RNA.

(β and γ) (24, 25). Two α isoforms exist, and they are both found in 3T3-L1 adipocytes (27). In keeping with its energy sensor role, starvation activates AMPK in adipose tissue (28, 29), and AMPK exerts antilipolytic effects (28–30), as well as inhibiting adipocyte fatty acid synthesis, by phosphorylating acetyl-CoA-carboxylase-1 (29) and inhibiting insulin-induced glucose uptake (30). The overall effect of AMPK is to convert adipocytes into lipid oxidizing cells with suppressed lipolysis and lipogenesis (29).

LPL catalyzes the hydrolysis of TG associated with chylomicrons and very low density lipoproteins in the circulation, thus generating 2-monoacylglycerol and fatty acids, that undergo re-esterification in adipocytes (31–34). The regulation of LPL expression and action is complex and involves transcriptional, post-transcriptional, and translocation events (31–34). Food deprivation results in down-regulation of adipose tissue LPL, whereas insulin increases overall activity, possibly by acting at several levels (31–35). Since GIP increases adipocyte LPL activity (14, 15), we postulated that it might act by suppressing AMPK levels, thus promoting fatty acid and 2-monoacylglycerol delivery to the cell and contributing to increased adipogenesis (6, 7, 13). Using differentiated 3T3-L1 cells and human subcutaneous adipocytes, we have demonstrated that GIP increases phosphorylation of PKB and decreases LKB1 and AMPK phosphorylation in the presence of insulin, resulting in activation of LPL and TG accumulation. Knockdown of AMPK using RNA interference and application of the AMPK inhibitor Compound C supported this conclusion. Chronic elevation of circulating GIP levels in the Vancouver diabetic fatty (VDF) Zucker rat *in vivo* resulted in activation of LPL in epididymal fat tissue by a similar pathway. This appears to be the first description of a signaling pathway by which GIP stimulates FA storage in adipocytes.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation of 3T3-L1 Adipocytes—3T3-L1 cells (American Type Culture Collection; ATCC) were cultured in DMEM containing high glucose and supplemented with 5% newborn calf serum plus penicillin/streptomycin (standard medium) in 6-well culture plates. Cells were induced to differentiate into the adipocyte phenotype as previously described (8). In brief, 2 days after cells were confluent, medium was supplemented with dexamethasone (0.6 μM), 3-isobutyl-1-methylxanthine (0.1 mM), and insulin (16 μM) for 72 h, after which cells were cultured in DMEM high glucose medium plus 10% fetal calf serum. Differentiation was complete in 7 days. Differentiated cells were confirmed by Oil Red O staining, and fully differentiated cells (>85% adipose cells) from passages 3–8 were used in all experiments.

Cell Culture and Differentiation of Human Adipocytes—Subcutaneous human preadipocytes were from Zen-Bio Inc. (Research Triangle Park, NC). They were obtained from healthy, nondiabetic women ($n = 7$; average body mass index, 25.17 kg/m^2 (range 22.5–28.2); average age, 41 years (range 27–51)) and differentiated into adipocytes according to the supplier's protocol. Institutional review board approval and informed consent for use of the adipose tissue were obtained from the patients by Zen-Bio Inc.

Western Blot Analysis—For studies on the effect of GIP on PKB, LKB1, and AMPK phosphorylation, 3T3-L1 adipocytes or human adipocytes were incubated with GIP in the presence of 1 nM insulin, as indicated in the figure legends. Where appropriate, the AMPK inhibitor, Compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine) (Calbiochem), was added at a final concentration of 40 μM. Total cellular extracts from each sample were separated on a 13% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad). Probing of the membranes was performed with phospho-PKB (serine 473), PKB, phospho-LKB1 (serine 428), phospho-AMPK (threonine 172), AMPK (Cell Signaling Technology, Beverly, MA), and β-tubulin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences) using horseradish peroxidase-conjugated IgG secondary antibodies.

Generation of Stable Cell Lines—3T3-L1 preadipocytes were grown in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (50 IU/ml, 50 μg/ml; Invitrogen) and transfected with constitutively active AMPK (CA-AMPK) and dominant negative AMPK (DN-AMPK) cDNAs, expressing the constitutively active and dominant negative forms of AMPK, respectively. For CA-AMPK, the cDNA encoded residues 1–312 of AMPK subunit α1, containing a mutation resulting in a change of threonine 172 to aspartic acid. A cDNA encoding subunit α1 containing a mutation altering an aspartic acid residue 157 to alanine was used. CA- and DN-AMPK constructs were kindly provided by Dr. David Carling (Imperial College School of Medicine, London, UK). Transfections were performed using Lipofectamine 2000™ transfection reagent (Invitrogen) for 4 h according to the manufacturer's instructions. Stably transfected cells were selected with G418 (Invitrogen), and cell clones were combined, differentiated into adipocytes, and analyzed by Western blotting to confirm AMPK protein expression levels.

Knockdown of AMPKα by RNA Interference—To reduce levels of endogenous AMPKα, 3T3-L1 adipocytes were transfected with a pool of three siRNAs for AMPKα (sc-45313; Santa Cruz Biotechnology) using Lipofectamine 2000™ transfection reagent and incubated for 72 h. The specific interference of AMPK protein expression was confirmed by Western blot hybridization using antibody against phospho-AMPK, phospho-LKB1, and phospho-PKB.

LPL Enzyme Activity Assays—The LPL activity assay kit (Roar Biomedical Inc.) was used to measure enzyme activity, according to the manufacturer's protocol. Enzyme activity is presented as relative activity normalized to protein concentration.

GIP Infusion—Obese VDF rats and their lean littermate controls (12 weeks old) were subjected to a 2-week continuous infusion of GIP (10 pmol/kg·min). The infusion was performed using an Alzet miniosmotic pump (Alzet Corp., Minneapolis, MN) implanted in the intraperitoneal region under pentobarbital (40 mg/kg) anesthesia. Rats were sacrificed at the end of the infusion, and epididymal fat tissues were harvested for Western blotting. Experiments were conducted in accordance

with guidelines of the University of British Columbia Animal Care Committee and Canadian Council on Animal Care.

Oral Glucose Tolerance Tests (OGTTs) and Measurements of Blood Glucose and Plasma Insulin Levels—Blood glucose levels were measured using a SureStep Glucose analyzer (LifeScan Canada, Burnaby, Canada). Following an approximate 16-h overnight fast, OGTTs (2 g/kg) were performed, with blood glucose levels following the glucose challenge measured at the time points indicated in Fig. 8*E*. Plasma insulin levels were determined using a radioimmunoassay kit (Linco Research Inc., St. Charles, MO).

Oil Red O Staining—After overnight serum starvation, human adipocytes were treated for 24 h with GIP (100 nM) or GLP-1 (100 nM) in the presence of insulin (1 nM). Cells were then fixed and stained for 2 h by complete immersion in a working solution of Oil Red O. The method of Ramirez-Zacarias *et al.* (36) was used to determine the level of staining. Isopropyl alcohol was added to the stained culture dish and dye-extracted by gentle pipetting, and the absorbance at 490 nm was measured spectrophotometically.

Determination of Intracellular TG Content—A TG assay kit (Zen-Bio Inc.) was used to measure intracellular TG content of human adipocytes and epididymal fat tissues, according to the manufacturer's protocol.

Statistical Analysis—Data are expressed as means ± S.E. with the number of individual experiments presented in the figure legend. Data were analyzed using the nonlinear regression analysis program PRISM (GraphPad, San Diego, CA), and significance was tested using analysis of variance (ANOVA) with Newman-Keuls *post hoc* test ($p < 0.05$) as indicated in the figure legends.

RESULTS

GIP, but Not GLP-1, Strongly Increases LPL Activity in 3T3-L1 Adipocytes—The effect of the incretins, GIP and GLP-1, on LPL activity was first studied in 3T3-L1 adipocytes. Treatment with GIP (100 nM) in the presence of insulin (1 nM) for 24 h resulted in ~2.6-fold increases in LPL activity, compared with basal. In contrast, treatment of 3T3-L1 adipocytes with GLP-1 (100 nM) under identical conditions resulted in only small increases in mean LPL activity that did not reach significance (Fig. 1*A*). Concentration-dependent effects of GIP on LPL activity were observed with EC_{50} values of 15.3 ± 0.1 nM (Fig. 1*B*).

GIP Treatment of 3T3-L1 Adipocytes Results in Increased PKB Phosphorylation and Decreased Phosphorylation of AMPK and LKB1—The mechanisms involved in the activation of LPL by GIP treatment were next studied. Phosphorylation of AMPK at Thr^{172} by upstream kinase AMPK kinases is essential for its activation (24, 25), resulting in increases in activity of at least 50-fold. The major upstream kinase for activation of AMPK in most tissues, including adipose (25), has recently been identified as LKB1 (37, 38). Treatment of 3T3-L1 adipocytes with GIP (100 nM) in the presence of insulin (1 nM) resulted in profound decreases in phosphorylation of AMPK at Thr^{172} (Fig. 2*A*) and LKB1 at Ser^{428} (Fig. 2*C*). GIP-induced responses were concentration-dependent, with EC_{50} values of 34.7 ± 0.2 nM for AMPK (Fig. 2*B*) and 25.5 ± 0.2 nM for LKB1 (Fig. 2*D*). In parallel experiments, GIP stimulated phosphorylation of PKB at Ser^{473} (Fig. 2*E*) with an EC_{50} of 35.6 ± 0.2 nM (Fig. 2*F*). Decreased phosphorylation of both AMPK and LKB1 (Fig. 2, *A* and *C*) and increased phosphorylation of PKB (Fig. 2*E*) were evident by 6 h following GIP treatment and sustained for 24 h. Treatment of 3T3-L1 adipocytes with GLP-1 (100 nM) in the presence of 1 nM insulin resulted in no significant changes in phosphorylation of PKB, LKB1, or AMPK (Fig. 3). These results correlated well with the lack of effect of GLP-1 on LPL (Fig. 1).

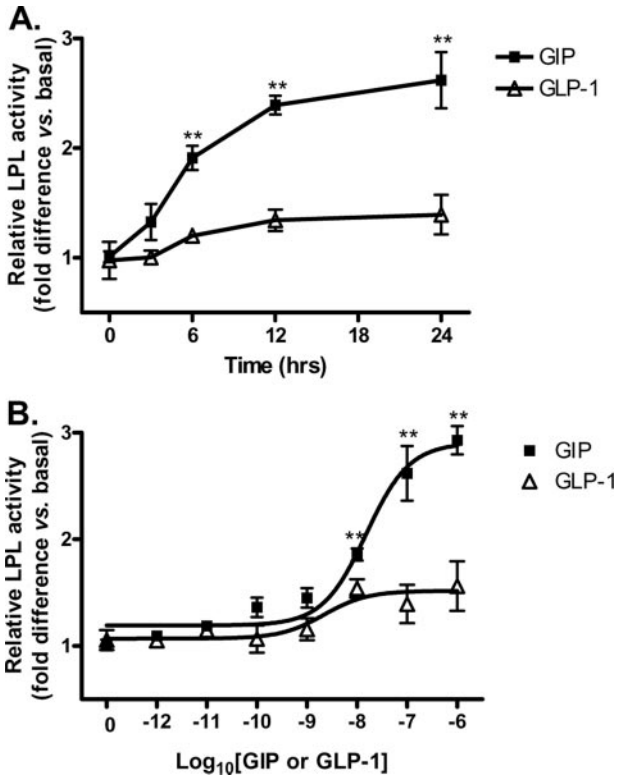
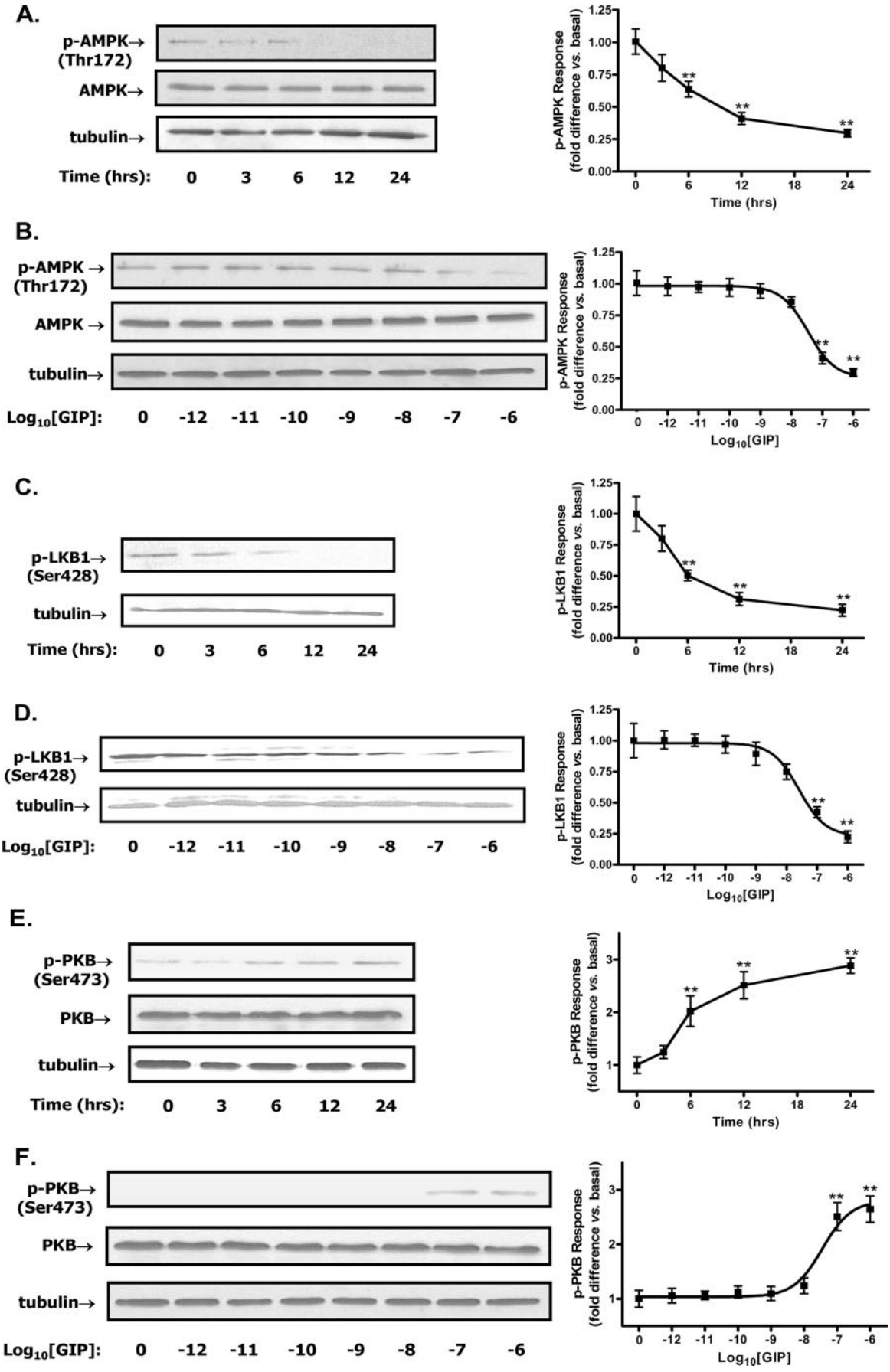


FIGURE 1. **GIP, but not GLP-1, strongly increases LPL activity in 3T3-L1 adipocytes.** *A*, time course of GIP/GLP-1-stimulated LPL activity. 3T3-L1 adipocytes were serum-starved in 3 mM glucose DMEM containing 0.1% bovine serum albumin overnight and treated for the indicated periods of time with GIP or GLP-1 (100 nM) in the presence of insulin (1 nM). LPL activity was determined as described under "Experimental Procedures." *B*, concentration-response effect of GIP and GLP-1 on LPL activity. 3T3-L1 adipocytes were treated for 24 h with the indicated concentrations of GIP or GLP-1 in the presence of insulin (1 nM), and LPL activity was determined. All data represent three independent experiments, each carried out in triplicate. Significance was tested using ANOVA with Newman-Keuls *post hoc* test. **, $p < 0.05$ *versus* control.

PI3K Is Involved in GIP Regulation of the PKB/LKB1/AMPK/LPL Signaling Pathway—Since phosphorylation of both Thr^{308} and Ser^{473} by PI3K is essential for PKB activation (39), the relationship between PI3K and the PKB/LKB1/AMPK signaling modules was next studied using the selective pharmacological inhibitors of PI3K, LY294002 and wortmannin. Inhibition of PI3K greatly reduced basal levels of phospho-PKB and ablated PKB responses to GIP (Fig. 4*A*). On the other hand, inhibition of PI3K slightly increased basal levels of both phosphorylated LKB1 and AMPK, and GIP treatment had no significant effect on levels of either phosphorylated kinase (Fig. 4, *B* and *C*). Under the same conditions of PI3K inhibition, basal LPL activity was decreased,



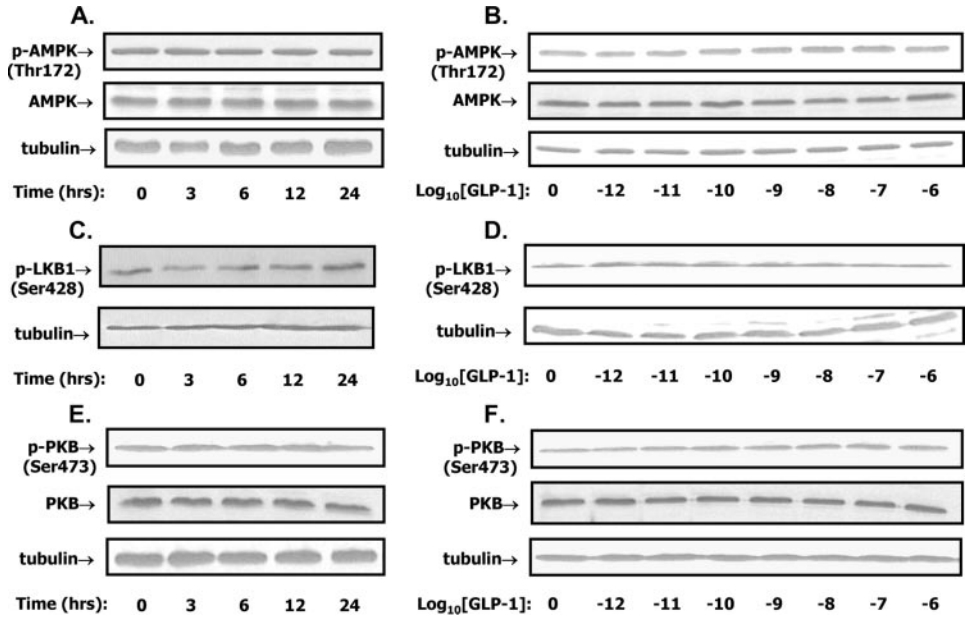


FIGURE 3. **GLP-1 had no significant effects on phosphorylation of AMPK, LKB1, or PKB in 3T3-L1 adipocytes.** Protocols for treatment of 3T3-L1 adipocytes with GIP were as described in the legend to Fig. 2. Shown is the time course of phosphorylation of AMPK (*A*), LKB1 (*C*), and PKB (*E*) in the presence of GIP. Shown are concentration-response effects of GIP on AMPK (*B*), LKB1 (*D*), and PKB (*F*). Western blots were quantified using densitometric analysis and are representative of $n = 3$. Significance was tested using ANOVA with Newman-Keuls *post hoc* test. **, $p < 0.05$ *versus* control.

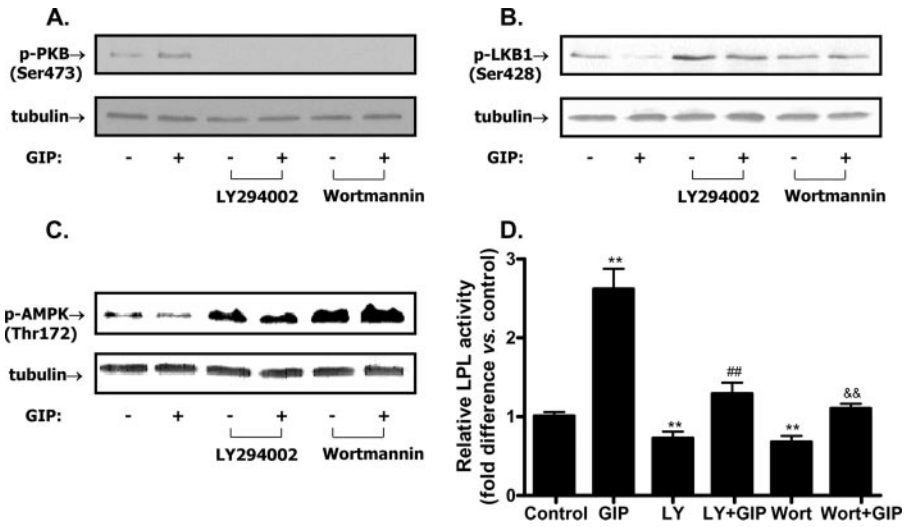


FIGURE 4. **PI3K is a component of the GIP-induced PKB/LKB1/AMPK/LPL signaling pathway.** 3T3-L1 adipocytes were treated with GIP (100 nM) plus insulin (1 nM) in the presence or absence of the PI3K inhibitors, LY294002 (*LY*; 40 μM) or wortmannin (*Wort*; 400 nM) for 24 h. Inhibitors were added to cells during the 1-h preincubation and during GIP stimulation. To test the effect of GIP on kinase phosphorylation, Western blot analyses were performed using antibodies against phospho-Ser^{473}-PKB (*A*), phospho-Ser^{428}-LKB1 (*B*), phospho-Thr^{172}-AMPK (*C*), and β-tubulin. *D*, effects of PI3K inhibitors on LPL activity. Significance was tested using ANOVA with Newman-Keuls *post hoc* test. **, $p < 0.05$ *versus* control; ##, $p < 0.05$ *versus* LY294002; &&, $p < 0.05$ *versus* wortmannin.

and responses to GIP were severely attenuated (Fig. 4*D*). These results suggest that PI3K is an upstream component of the GIP-stimulated PKB/AMPK/LPL signaling pathways.

PKB and LKB1 Act Upstream of AMPK and LPL—To define further the relationship between PKB/LKB1 and AMPK signaling modules in the regulation of LPL activity, 3T3-L1 adipocytes stably expressing CA or DN forms of AMPK were generated. 3T3-L1 adipocytes stably expressing CA-AMPK demonstrated increased basal phospho-AMPK levels that were reduced by treatment with GIP or high concentrations of insulin (100 nM) (Fig. 5*A*). In contrast, levels of phospho-AMPK were greatly reduced in 3T3-L1 adipocytes expressing DN-AMPK (Fig. 5*A*). Surprisingly, the residual phospho-AMPK was ablated by GIP or insulin (100 nM). There were no significant changes in the phosphorylation levels of PKB and LKB1 with the expression of CA- or DN-AMPK, when compared with 3T3-L1 adipocytes transfected with empty vector (Fig. 5, *B* and *C*). In parallel experiments, 3T3-L1 adipocytes expressing CA-AMPK had decreased basal LPL that was still responsive to GIP stimulation. Expression of DN-AMPK increased basal LPL activity, and GIP treatment resulted in a further increase (Fig. 5*D*). Taken together, these results strongly suggest that PKB/LKB1 is an upstream signaling module of AMPK. However, since LPL activity of CA- and DN-AMPK cells was still responsive to GIP, alternative approaches were applied to establish that phospho-AMPK was involved in GIP-mediated LPL activation. First, pretreatment of cells with an AMPK inhibitor, Compound C, resulted in a marked decrease in phospho-AMPK levels (Fig. 6*A*) without changes in phosphorylation levels of PKB and LKB1 (Fig. 6, *B* and *C*). Phospho-AMPK levels were too low to assess effects of GIP. Under the same conditions, AMPK inhibitor increased LPL activity, but it was not further increased by GIP treatment

FIGURE 2. **GIP decreases phosphorylation of LKB1 and AMPK and increases phosphorylation of PKB in 3T3-L1 adipocytes.** 3T3-L1 adipocytes were serum-starved in 3 mM glucose DMEM containing 0.1% bovine serum albumin overnight and treated with GIP (100 nM) for the indicated time periods or with GIP for 24 h at the concentrations indicated in the presence of insulin (1 nM). Total cellular extracts were isolated, and Western blot analyses was performed with antibodies against phosphorylated Thr^{172}-AMPK, AMPK, Ser^{428}-LKB1, Ser^{473}-PKB, or PKB and β-tubulin. Shown are the time courses of phosphorylation of AMPK (*A*), LKB1 (*C*), and PKB (*E*) in the presence of GIP and concentration-response effects of GIP on AMPK (*B*), LKB1 (*D*), and PKB (*F*). Western blots were quantified using densitometric analysis and are representative of $n = 3$. Significance was tested using ANOVA with Newman-Keuls *post hoc* test. **, $p < 0.05$ *versus* control.

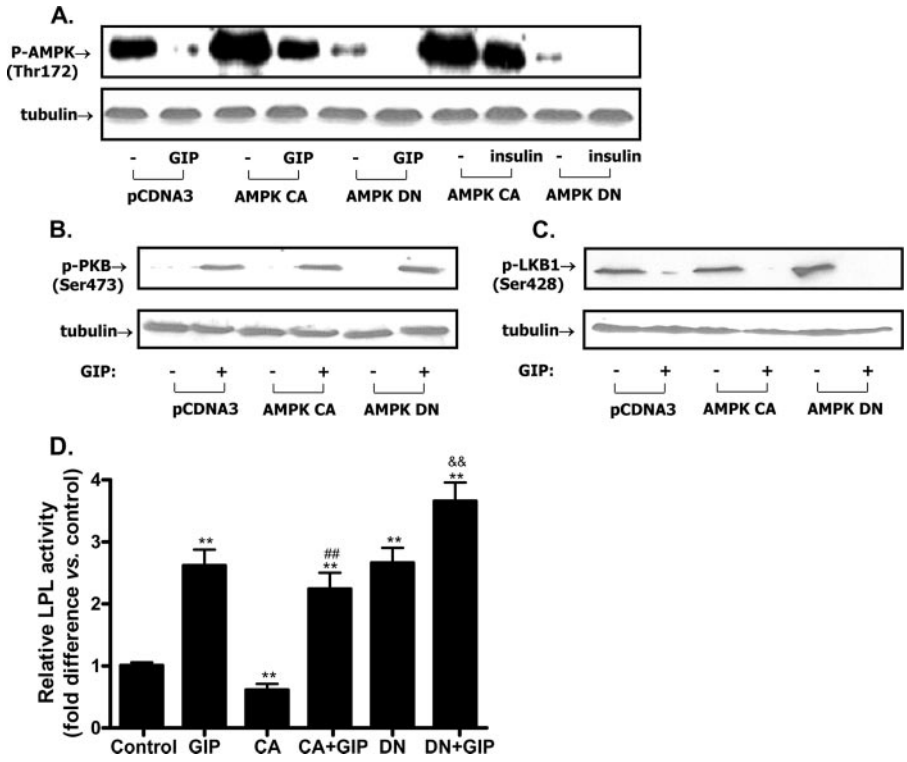


FIGURE 5. **PKB/LKB1 is an upstream signaling module of AMPK/LPL.** *A*, effects of AMPK constructs on AMPK phosphorylation. 3T3-L1 preadipocyte were transfected with DN or CA forms of AMPK constructs or empty vector (pCDNA3), and selected stable cell lines were differentiated into adipocytes as described under "Experimental Procedures." Cells were treated with GIP (100 nM) for 24 h in the presence of insulin (1 nM). To test the effect of AMPK constructs on kinase phosphorylation, Western blot analyses were performed using antibodies against phospho-Thr^{172}-AMPK (*A*), phospho-Ser^{473}-PKB (*B*), or phospho-Ser^{428}-LKB1 (*C*) and β-tubulin. In *A*, stable cell lines expressing CA- or DN-AMPK were treated with insulin (100 nM) as controls. *D*, effects of AMPK constructs on LPL activity. Cells were treated with GIP (100 nM) as described above, and LPL enzyme activity assays were performed. Significance was tested using ANOVA with Newman-Keuls *post hoc* test. **, $p < 0.05$ *versus* control; ##, $p < 0.05$ *versus* CA; &&, $p < 0.05$ *versus* DN.

(Fig. 6*D*). RNA interference-mediated knockdown of AMPKα resulted in similar results: a substantial decrease in phospho-AMPK levels (Fig. 6*E*), no changes in PKB and LKB1 phosphorylation (Fig. 6, *F* and *G*), and increased LPL activity that was not responsive to GIP (Fig. 6*H*). Following treatment with scrambled (control) siRNA, GIP increased LPL activity. Together, these results strongly support a role for AMPK in GIP-mediated LPL activation, with PKB/LKB1 as an upstream signaling module.

Concentration-dependent Effects of GIP and GLP-1 on Human Adipocytes—Although GIP was demonstrated to regulate the levels of PKB/LKB1/AMPK phosphorylation and LPL activity in 3T3-L1 adipocytes, the EC_{50} values were in the 15–35 nM range. This might be due to low GIP receptor expression (8) compared with normal adipocytes. Consequently, we examined the responses of human adipocytes incubated under similar conditions to the 3T3-L1 adipocytes. Treatment with GIP (100 nM) in the presence of insulin (1 nM) for 24 h resulted in a ~3.7-fold increase in LPL activity, compared with basal. Concentration-dependent effects of GIP on LPL activity were observed with EC_{50} values of 115.3 ± 0.1 pM, whereas GLP-1 did not exert any significant effect on LPL activity (Fig. 7*A*). In parallel experiments, suppression of AMPK and LKB1 phosphorylation and increases in PKB phosphorylation occurred with GIP concentrations as low as 10–100 pM (Fig. 7, *B–D*). Conversely, GLP-1 exerted no significant effect on PKB/LKB1/AMPK phosphorylation. To examine whether GIP-induced LPL activation was associated with increased lipogenesis, intracellular TG content was determined. As shown in Fig. 7*E*, treatment with GIP (100 nM) in the presence of insulin (1 nM) for 24 h resulted in a ~1.5-fold increase in TG accumulation compared with control. GLP-1 exerted no significant effect (Fig. 7*E*). When cells were stained with Oil Red O, levels of extracted dye in the GIP-treated group were increased compared with the control or GLP-1-treated group (Fig. 7*F*), providing further support for GIP-enhanced lipogenesis. Taken together, these results indicate that GIP modulation of the PI3K/PKB/LKB1/AMPK signaling module results in increased LPL activity and lipogenesis.

Effect of GIP on Epididymal Fat in Vivo—To determine whether GIP can regulate LPL *in vivo*, GIP was systemically administrated to lean (*Fa/?*) or obese (*fa/fa*) VDF Zucker rats, the latter being an animal model of mild Type 2 diabetes (40). As shown in Table 1, fasting blood glucose levels in VDF fatty rats were slightly increased compared with lean littermates, and they demonstrated clear glucose intolerance (Fig. 8*E*). GIP administration had no significant effects on body weight, fasting or nonfasting glucose levels, or fasting insulin in either lean or obese animals. However, glucose intolerance in obese VDF rats showed a small improvement with the GIP infusion (Fig. 8*E*) when compared with controls, and this was associated with slightly increased mean peak insulin in the OGTTs, although not reaching significance. There were no such GIP-induced changes in the lean rats.

LPL activity was significantly elevated in epididymal fat from obese VDF rats when compared with lean (Fig. 8*A*). Following 2 weeks of treatment with GIP, epididymal fat LPL activity was increased in both lean and obese animals when compared with controls (Fig. 8*A*). GIP infusion resulted in increased epididymal phospho-PKB (Fig. 8*D*) and reduced phospho-AMPK and -LKB1 (Fig. 8, *B* and *C*) in obese VDF rats as well as their lean littermates. These results correlate well with the *in vitro* results in 3T3-L1 and human adipocytes. Intracellular TG content was significantly elevated in epididymal fat from obese VDF rats, compared with lean controls, and following 2 weeks of treatment with GIP, it was increased in both lean and obese animals (Fig. 8*F*). These results support a role for GIP in the regulation of adipose tissue LPL and subsequent TG accumulation *in vivo*, acting through a PI3K/PKB/LKB1/AMPK signaling module.

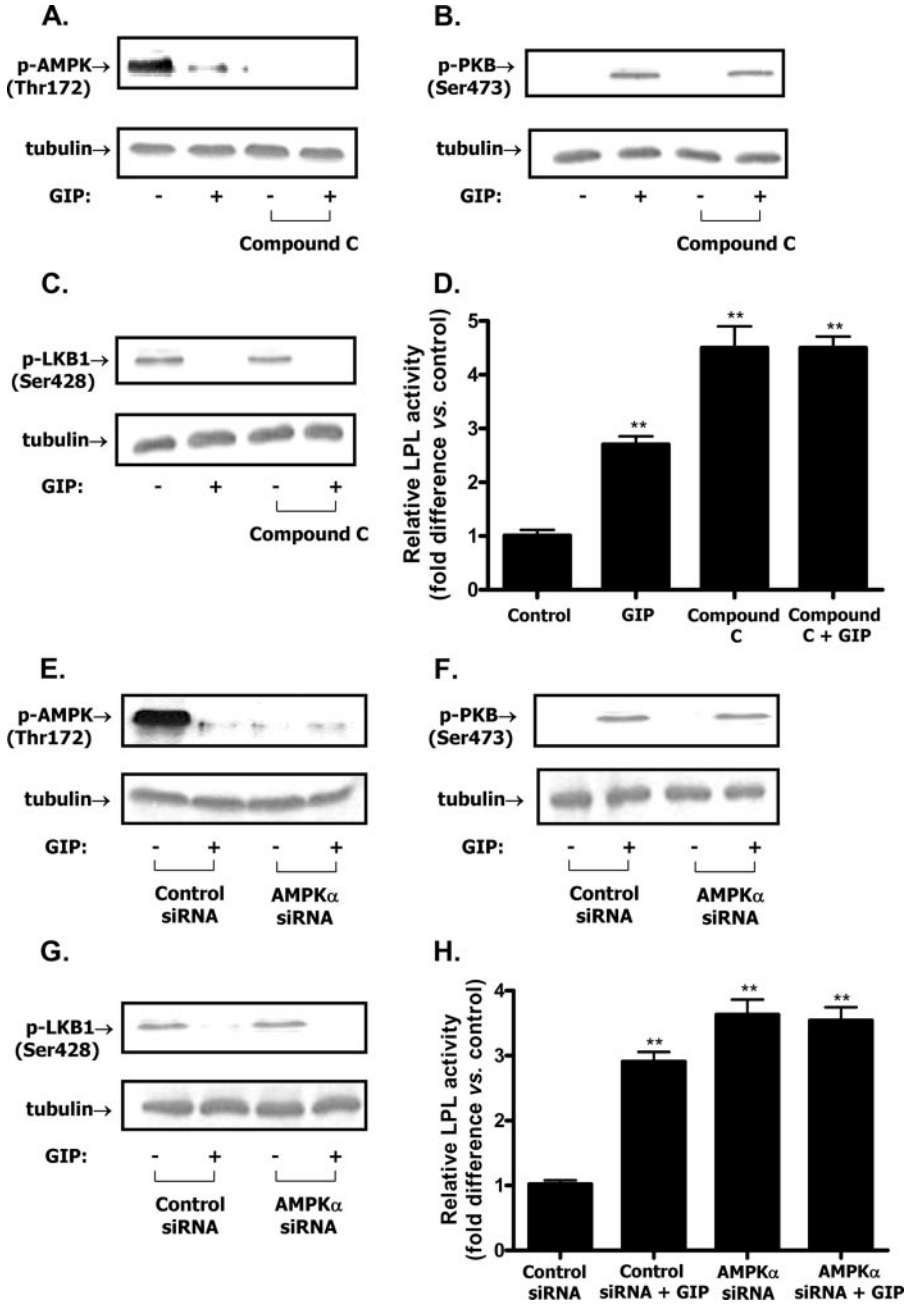


FIGURE 6. **AMPK is involved for the regulation of GIP-mediated LPL activation.** Effect of AMPK inhibitor on kinase phosphorylation. 3T3-L1 adipocytes were treated with GIP (100 nM) plus insulin (1 nM) in the presence or absence of AMPK inhibitor (Compound C; 40 μM) for 24 h. Inhibitors were added to cells during a 1-h preincubation and GIP stimulation. Western blot analyses were performed using antibodies against phospho-Thr-172-AMPK (*A*), phospho-Ser^{473}-PKB (*B*) or phospho-Ser^{428}-LKB1 (*C*) and β-tubulin. *D*, effect of AMPK inhibitor on LPL activity. Shown is the effect of AMPKα knockdown using siRNA on kinase phosphorylation. 3T3-L1 adipocytes were transfected with AMPKα or control scrambled siRNAs (100 nM) and treated with GIP (100 nM) plus insulin (1 nM). Western blot analyses were performed using antibodies against phospho-Thr^{172}-AMPK (*E*), phospho-Ser^{473}-PKB (*F*), or phospho-Ser^{428}-LKB1 (*G*) and β-tubulin. *H*, effect of AMPKα siRNA on LPL activity. For more detailed dose-dependent responsiveness of RNA interference-mediated knockdown of AMPKα, see supplemental Fig. 1. Significance was tested using ANOVA with Newman-Keuls *post hoc* test. **, $p < 0.05$ *versus* control.

DISCUSSION

Considerable recent advances have been made in our understanding of the physiological roles of the two major incretin hormones, GIP and GLP-1, and their modes of action. There is controversy over the effects of GIP on fat metabolism, since it exhibits stimulatory actions on both lipolysis (8, 23) and lipogenesis. Triglyceride is a stronger stimulator of GIP secretion than glucose (1, 2), and GIP infusion has been shown to promote clearance of chylomicron-associated TG from blood (10) and to reduce plasma TG responses to intraduodenal fat (11). GIP also stimulated adipose tissue synthesis of FA from acetate (12) as well as potentiating insulin-stimulated FA incorporation into fat (13) and enhancing LPL activity in cultured preadipocytes (14) and mature adipocytes (15). The physiological importance of the lipogenic actions was emphasized by the demonstration by Miyawaki *et al.* (16) that fat deposition in GIP receptor knock-out mice fed a high fat diet was greatly reduced when compared with wild type mice. However, the underlying mechanisms by which GIP promotes fatty acid incorporation into adipose tissue remain unclear.

LPL is regulated by hormones and nutrients in a tissue-specific manner (31, 34). In adipose tissue, the main storage site for TG, LPL activity is induced by feeding and suppressed by fasting, whereas in muscle, which utilizes fatty acids for energy production in the postabsorptive state, LPL activity is induced by fasting and suppressed by feeding (31, 34). Insulin increases LPL activity in 3T3-L1 adipocytes (41) and adipose tissue (31) but reduces its activity in the heart (42). In the current study, GIP was shown to increase LPL activity in the presence of insulin in differentiated 3T3-L1 cells and human adipocytes through modulation of a PKB/LKB1/AMPK signaling module. The most parsimonious explanation of the results is outlined in Fig. 9 and includes GIP-mediated activation of PKB through increased phosphorylation of Ser^{473}, decreased LKB1/AMPK phosphorylation, and increased activity of LPL. LKB1, TAK1, and Ca^{2+}/calmodulin-dependent protein kinase kinases have all been implicated in the phosphorylation and regulation of AMPK (38), but current evidence suggests that LKB1 is predominantly responsible in adipose tissue (29). In the heart, it has been demonstrated that there is cross-talk between PKB and AMPK pathways, with PKB acti-

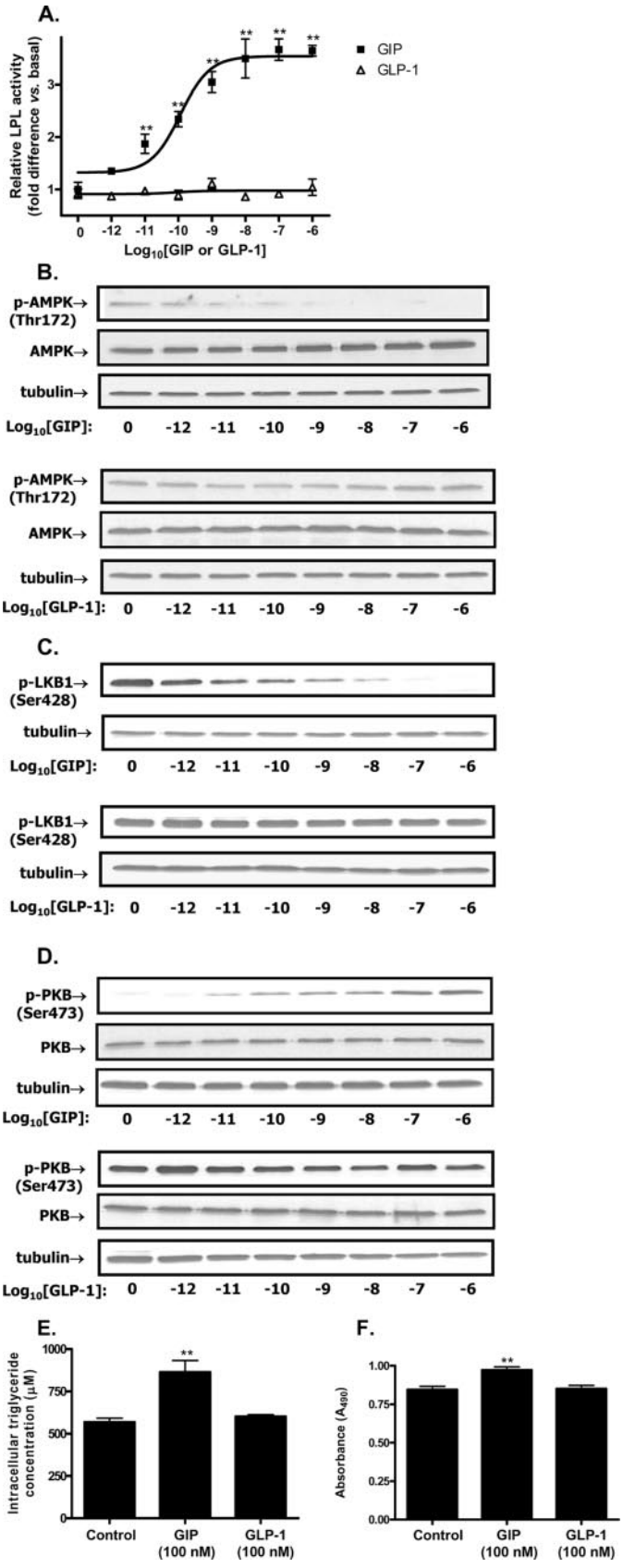


FIGURE 7. **Concentration-dependent effects of GIP and GLP-1 on human adipocytes.** Human adipocytes were serum-starved in DMEM/Ham's F-12 medium (1:1, v/v) containing 0.1% bovine serum albumin overnight and treated for 24 h with the indicated concentrations of GIP or GLP-1 in the presence of insulin (1 nM). *A*, effects of GIP and GLP-1 on LPL activity. LPL activity was determined as described under "Experimental Procedures." Shown are the effects of GIP and GLP-1 on kinase phosphorylation. Western blot analyses were performed using antibodies against phospho-Thr^{172}-AMPK, AMPK (*B*), phospho-Ser^{428}-LKB1 (*C*), or phospho-Ser^{473}-PKB, PKB (*D*), and β-tubulin. *E*, effects of GIP and GLP-1 on TG content. Human adipocytes were treated with GIP (100 nM) or GLP-1 (100 nM) for 24 h in the presence of insulin (1 nM), and intracellular TG content was determined. *F*, quantification of Oil Red O staining of 3T3-L1 adipocytes was performed as described under "Experimental Procedures." Dye was extracted, and the absorbance at 490 nm was determined. Significance was tested using ANOVA with Newman-Keuls *post hoc* test. **, $p < 0.05$ *versus* control.

vation leading to decreased AMPK activity (43). A similar pattern was seen in the adipocyte in response to GIP. The decrease in LKB1 phosphorylation in response to GIP treatment demonstrated a temporal pattern similar to that of AMPK (Fig. 2, *A–D*). Expression of a CA form of AMPK increased basal phospho-Thr^{172}-AMPK levels and decreased LPL activity below control values (Fig. 5, *A* and *D*). Unexpectedly, LPL activity of 3T3-L1 adipocytes expressing CA-AMPK was still responsive to GIP stimulation. There are several potential explanations for this observation. The activity of the CA form of AMPK utilized has been shown to be much lower than that of the endogenous heterotrimeric complexes (44). Relatively low CA-AMPK expression and the presence of cells that only weakly expressed or failed to express CA-AMPK cDNA could also have contributed to a lower total activity. The suppression of LPL activity therefore may have been mainly a result of the increased levels of endogenous phospho-Thr^{172}-AMPK. Upon GIP stimulation, suppression of the endogenous activity would be the predominant effect, thus resulting in increased LPL activity. The responsiveness of CA-AMPK cells to insulin (Fig. 5*A*) supports this possibility. Expression of a DN form of AMPK reduced phospho-Thr^{172}-AMPK, and this was associated with increases in LPL activity to levels identical to those observed following GIP stimulation in control cells (Fig. 5, *A* and *D*). The addition of GIP to DN-AMPK-expressing cells produced a further increase in LPL activity. This may be explained by the fact that, although phospho-Thr^{172}-AMPK levels were very low (Fig. 5*A*), there may have been residual AMPK activity appropriately situated intracellularly for stimulating LPL activity. Previous studies with this DN form have shown that even adenoviral expression does not produce protein levels sufficient to completely sequester the β and γ subunits that are necessary for expression of activity of the endogenous enzyme (45). Since the DN-AMPK cells were still responsive to insulin (Fig. 5*A*), incomplete removal of activity is a distinct possibility. The involvement of AMPK in the regulation of GIP-mediated LPL activation was further assessed using pretreatment with an AMPK inhibitor and AMPKα siRNA knockdown. Both treatments resulted in reduced levels of phospho-AMPK, increased basal LPL activity, and ablated responsiveness to GIP (Fig. 6, *D* and *H*). Compound C acts as a competitive inhibitor at the ATP binding site of AMPK (46), and the associated reduction in phospho-AMPK levels is in agreement with studies on a neuroblastoma cell line (47), skeletal muscle cells (48), and pancreatic β-cell line (49).

Studies on potential upstream pathways revealed that inhibition of PKB phosphorylation at Ser^{473}, by treatment with PI3K inhibitors, resulted in increased phosphorylation of LKB1 at Ser^{428} and AMPK at Thr^{172}, implying that PKB is a negative

TABLE 1

Key metabolic parameters in GIP-infused VDF rats

Obese VDF rats or their lean littermates (8–12 weeks old) were subjected to a 2-week continuous infusion of GIP (10 pmol/kg·min) or vehicle using an Alzet miniosmotic pump, implanted in the intraperitoneal region. Body weight, blood glucose levels, and plasma insulin levels were determined at the end of the infusion. Significance was tested using ANOVA with Newman-Keuls *post hoc* test.

	VDF fatty ($n = 3$)	Fatty + GIP ($n = 3$)	VDF lean ($n = 3$)	Lean + GIP ($n = 3$)
Body weight (g)	480.2 ± 33.1[a]	492.4 ± 52.2[a]	288.2 ± 40.6	349.9 ± 57.1
Nonfasting blood glucose levels (mM)	7.0 ± 1.1	6.5 ± 1.2	5.1 ± 0.7	5.0 ± 0.5
Fasting blood glucose levels (mM)	7.7 ± 0.4[a]	7.1 ± 1.2	5.4 ± 0.4	6.2 ± 1.1
Area under the curve	1303	1166	867.8	800.5
Peak insulin levels (ng/ml)	7.3 ± 0.4[a]	8.9 ± 0.6[a]	2.6 ± 0.8	2.5 ± 0.6
Fasting insulin levels (ng/ml)	4.2 ± 0.7[a]	6.0 ± 1.3[a]	0.4 ± 0.1	0.7 ± 0.2

[a] $p < 0.05$ *versus* VDF lean control.

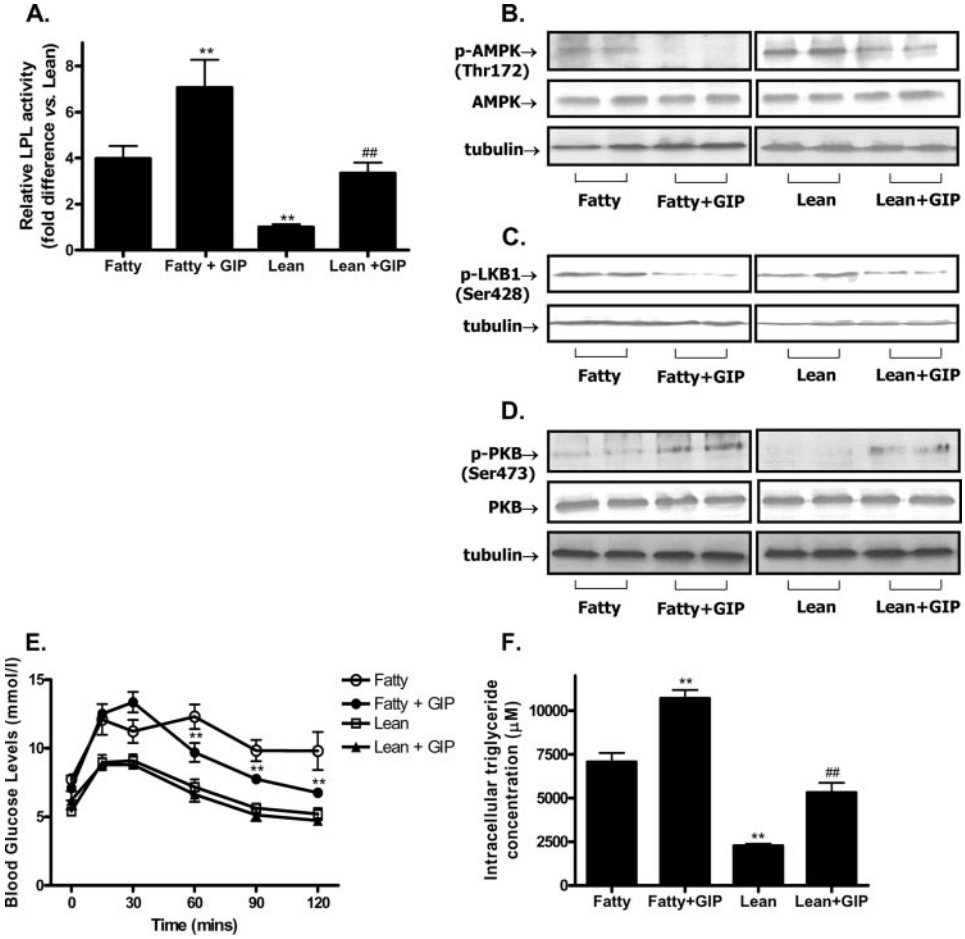


FIGURE 8. **Effects of GIP on adipose tissue *in vivo*.** VDF rats and their lean littermates received a 2-week continuous infusion of GIP (10 pmol/kg·min), and epididymal fat tissues were harvested. *A*, effect of GIP on LPL activity. LPL enzyme activity assays were performed as described under "Experimental Procedures." Shown is the effect of GIP on kinase phosphorylation. Total cellular extracts were prepared from epididymal fat tissues, and Western blot analyses were performed using antibodies against phospho-Thr^{172}-AMPK (*B*), phospho-Ser^{428}-LKB1 (*C*), or phospho-Ser^{473}-PKB, PKB (*D*), and β-tubulin. *E*, OGTT. Following an approximate 16-h fast, OGTTs (2 g/kg) were performed on each group, with blood glucose levels measured at the indicated time points following the glucose challenge. *F*, effect of GIP on TG accumulation in epididymal fat tissue. TG content was determined in epididymal fat tissues. Western blots are representative of $n = 3$, and all data represent three independent experiments, each carried out in triplicate. Significance was tested using ANOVA with Newman-Keuls *post hoc* test. **, $p < 0.05$ *versus* VDF rats; ##, $p < 0.05$ *versus* lean control.

regulator in this pathway, similar to the heart. The underlying mechanisms are not clear, although PKB activation of a phosphatase for LKB1 could be involved. Alternatively, the ability of insulin to prevent AMPK activation in the heart has recently been shown to involve a mechanism whereby PKB-mediated Ser^{485}/Ser^{491} phosphorylation of AMPK results in a subsequent reduction in LKB1-mediated Thr^{172} phosphorylation (50). Although we have presented evidence implicating LKB1 in the lipogenic action of GIP on the adipocyte, it has been shown recently in an INS-1 β-cell line that agents capable of increasing intracellular cAMP levels, such as GIP, can also reduce AMPK activity via inhibition of Ca^{2+}/calmodulin-dependent protein kinase kinase through activation of protein kinase A (51). It will clearly be of interest to establish whether this also occurs in adipose tissue.

The association between increased LPL activity and reduced AMPK phosphorylation contrasts with the situation in the heart (52), where AMPK stimulation results in increased heparin-releasable LPL activity, although this is compatible with the role of LPL in delivering fatty acids to the adipocyte during a meal and postprandially for storage. Importantly, in both *in vitro* studies on human adipocytes and *in vivo* studies on VDF rats, GIP administration could be shown to increase adipocyte TG content, a functional correlate with the increased LPL activity. The link between reduced AMPK phosphorylation and increased LPL activity is currently unclear and, in view of the complex regulation of LPL in different tissues (32–34), could involve transcription, translation, and transport of the enzyme.

Two characteristics of the *in vitro* responses to GIP in differentiated 3T3-L1 adipocytes need further comment. All responses were studied in the presence of insulin, because in its absence GIP acts mainly as a lipolytic hormone (8, 9), and its stimulatory action on PKB is also insulin-dependent. The responses to GIP under these conditions are relatively slow. The reason for this is unclear, and the basic

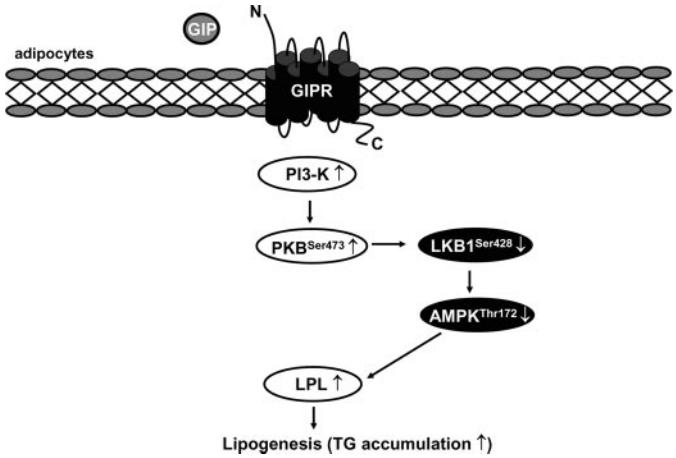


FIGURE 9. **Proposed pathway by which GIP increases LPL activity in adipocytes.** GIP activation of PI3K results in increased phosphorylation of PKB, reduced phosphorylation of Ser^{428} in LKB1 and Thr^{172} in AMPK, and increased LPL activity. The mode of activation of PI3K and origin of the increase in LPL activity (transcription, posttranscriptional modification, and/or translocation) are unknown.

model presented (Fig. 9) does not exclude the involvement of factors upstream of PI3K that require an extended time of action (*e.g.* growth factors, gene expression). At present, we have no evidence for the involvement of such factors. Additionally, with differentiated 3T3-L1 cells, the concentrations of GIP utilized were supraphysiological, with EC_{50} values in the range of 15–35 nM. Two possible explanations for this low sensitivity were considered. First, since it was earlier speculated that GIP may function on adipocytes mainly via competitive binding to glucagon receptors (53), we examined the effect on GIP-mediated actions of the specific glucagon receptor antagonist des-His^1-[Glu^9]glucagon amide (54) at concentrations up to 1 μM but found no significant effects (data not shown). Second, we considered that it might be due to low expression of receptor or intracellular signaling molecules in 3T3-L1 adipocytes, due to the passage number, since lipolytic responses to GIP were found earlier to occur with lower concentrations of GIP (8), and it has been shown that cell phenotype can change with passage number (55). We therefore examined the responsiveness of human subcutaneous adipocytes and found that they were exquisitely responsive to GIP at concentrations as low as 10 pM, well within the physiological range (Fig. 7). Furthermore, a 2-week continuous infusion of low concentrations of GIP (10 pmol/kg·min) in the obese VDF Zucker rat led to a modulation of PKB, LKB1, AMPK, and LPL similar to that observed in 3T3-L1 adipocytes (Fig. 8). These *in vivo* studies are complicated by the fact that GIP stimulates insulin secretion, and long term administration could therefore potentially induce indirect effects. As seen from their metabolic profiles, with the low dose infusion utilized, the rats demonstrated no major changes in circulating glucose or insulin levels when compared with controls (Table 1). The obese animals showed a small but significant improvement in glucose tolerance, whereas there was no change in the lean animals. However, both groups of animals showed increased epididymal fat TG content and changes in enzyme profiles similar to those observed with the *in vitro* studies (Fig. 8), suggesting that the responses observed were due to GIP rather than insulin or blood glucose levels.

In many tissues, GIP shares similar activity with the second major incretin hormone, GLP-1, and both elevate cAMP levels and activate protein kinase A and PKB following interaction with their cognate receptors. The GLP-1 receptor is also expressed in adipocytes (56), but GLP-1 demonstrated only minor, nonsignificant effects on PKB, LKB1, AMPK, and LPL in both human and 3T3-L1 adipocytes. These results imply either very low receptor expression or differences from GIP in adipocyte signaling.

In summary, GIP is likely to play an important role in fat storage, by increasing LPL activity in adipocytes, and a PKB/LKB1/AMPK signaling module is involved in this process. This appears to be the first report describing the mechanisms underlying the action of GIP on adipocyte fat storage. GIP probably acts on adipose tissue synergistically with insulin *in vivo*, and, since circulating GIP levels may be increased by hypernutrition, its effects would be more evident in obesity, with its accompanying hyperinsulinemia. Such an action supports the search for GIP receptor antagonists, potentially with an adipocyte specificity.

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