Insulin Regulates Lipoprotein Lipase Activity in Rat Adipose Cells Via Wortmannin- and Rapamycin-Sensitive Pathways

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Lipoprotein lipase (LPL) hydrolyzes the triacylglycerol component of circulating lipoprotein particles, mediating the uptake of fatty acids into adipose tissue and muscle. Insulin is the principal factor responsible for regulating LPL activity in adipose tissue, yet the mechanisms whereby insulin controls LPL expression are unknown. The current studies used wortmannin, a specific inhibitor of phosphatidylinositol (PI) 3-kinase, and rapamycin, a specific inhibitor of activation of phosphoprotein 70 ribosomal protein S6 kinase ($p70^{s6k}$), to explore some of the components of the insulin signaling pathway controlling LPL activity in adipose cells. Preincubation of isolated rat adipose cells with wortmannin completely abrogated the stimulation of LPL activity by insulin, while preincubation with rapamycin caused approximately a 60% inhibition of insulin-stimulated LPL activity. Thus, the current studies show that the regulation of adipose tissue LPL by insulin is mediated via a wortmannin-sensitive pathway, most likely PI 3-kinase, and that a rapamycin-sensitive pathway, most likely $p70^{s6k}$, constitutes an important downstream component in the insulin signaling pathway through which LPL is regulated.

LIPOPROTEIN LIPASE (LPL) has its physiological site of action at the luminal surface of capillary endothelial cells, where the enzyme hydrolyzes the triacylglycerol component of circulating lipoprotein particles, chylomicrons, and very–low-density lipoproteins to provide free fatty acids and 2-monoacylglycerol for tissue utilization.[1,2] LPL plays an important role in triacylglycerol metabolism and is widely distributed in adipose tissue, heart, skeletal muscle, mammary gland, lung, and kidney, with adipose tissue, heart, and lactating mammary gland having the highest transcriptional and catalytic activity for LPL.[3,4] On the basis of numerous studies both in humans and in various experimental models, it is clear that insulin is the major hormone regulating LPL activity in adipose tissue.[5,6] Plasma insulin concentrations show the highest correlation with adipose tissue LPL activity, and administration of insulin under conditions normally associated with low adipose tissue LPL activity leads to a pronounced increase in LPL activity.[7] Insulin has well-documented direct effects on LPL in adipose tissue in vitro. Insulin increases immunodetectable LPL on the cell surface of adipocytes and enhances the rate of LPL spontaneous release into the medium.[8] Insulin also increases cellular LPL activity, LPL synthetic rates, and LPL mRNA levels in adipocytes.[9] Insulin did not stimulate LPL gene transcription in nuclear run-on assays, suggesting that insulin-induced increases in steady-state LPL mRNA levels must have been due to changes in mRNA stability. The regulation of adipose tissue LPL by insulin is complex and appears to act at multiple levels (transcriptional, translational, and posttranslational), yet the pathways through which insulin effects these changes are unknown. The mechanisms underlying the cellular actions of insulin are not fully understood, but some important key steps in the signaling pathways have been identified, supporting the view that insulin action is mediated by a complex cascade of phosphorylation/dephosphorylation events.[10] The current experiments were conducted to explore the portions of the insulin signaling cascade that are involved in the regulation of LPL by insulin.

MATERIALS AND METHODS

Chemicals

The following reagents were obtained: collagenase (Worthington Biochemical, Freehold, NJ); wortmannin (Sigma Chemical, St Louis, MO); medium 199 (Life Sciences Technology, Gaithersburg, MD); bovine serum albumin ([BSA] Intergen, Purchase, NY); D-[U-^{14}C]glucose, glycerol tri[9,10(*n*)-^{3}H]oleate, and [γ^{32}P] adenosine triphosphate (Amersham Life Sciences, Arlington Heights, IL); rapamycin (a kind gift from Dr G. Crabtree, Stanford University); antibodies against phosphatidylinositol (PI) 3-kinase p110 (Santa Cruz Biotechnology, Santa Cruz, CA); and human insulin (Eli Lilly and Co, Indianapolis, IN). All other chemicals were obtained from standard commercial sources.

Adipose Cell Isolation

Male Sprague-Dawley rats (180 to 240 g; B and K, Fremont, CA) were obtained and maintained on ad libitum rat chow and tap water with a 12-hour light/dark cycle according to Stanford University guidelines. Animals were killed in the morning by decapitation, and epididymal fat pads were quickly removed from each rat and washed with phosphate-buffered saline (pH 7.4) for isolation of adipocytes. Adipose cells were isolated by collagenase digestion with sterile technique as previously described.[11] Collagenase digestion was performed in medium 199 (pH 7.4) containing 3% BSA and 200 nmol/L adenosine. After washing, the isolated adipose cells were incubated in medium 199 with BSA and adenosine in 12 × 75-mm polypropylene tubes in an atmosphere of 95% air/5% CO_2 in a shaking (60 cycles/min) water bath at 37°C in the presence or absence of insulin and various inhibitors. To document that the isolation of adipocytes with collagenase did not disturb the responsiveness of the cells to insulin, aliquots of each preparation of isolated adipose cells were incubated with [U-^{14}C]glucose in the presence or absence of insulin for 1 hour. After incubation, the cells were layered under 0.5 mL silicone oil and centrifuged at 12,000× *g* for 1 minute, and radioactivity in the concentrated adipose cells was counted in a liquid scintillation counter. Preparations of adipocytes that displayed less than a fivefold stimulation of glucose uptake by insulin

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were discarded. A 100-μL aliquot of diluted cells was fixed in a solution of 2% osmium tetraoxide in collidine buffer and then counted in a Coulter counter (Coulter Electronics, Hialeah, FL) to determine cell number.

Measurement of LPL Activity

For LPL determination, after 4 hours of incubation (or other times as indicated), heparin (10 U) was added to the incubation for 10 minutes. A time course for heparin stimulation of LPL release demonstrated the greatest LPL activity at 10 minutes, with a slight decline with incubation to 60 minutes (data not shown). Following the incubations, the cells were centrifuged at 500× *g* for 1 minute, and the infranatant under the adipose cells was removed for LPL assay. The remaining adipose cells were layered under 0.5 mL silicone oil and centrifuged at 12,000 × *g* for 1 minute. The concentrated adipose cells were homogenized in Tris hydrochloride (pH 8.3) containing 1% BSA, heparin (10 U/mL), 0.5% deoxycholate, and 0.02% Nonidet P-40.[12] After centrifuging the homogenates at 12,000 × *g* for 15 minutes, the infranatants under the fat cake were carefully removed and aliquots were assayed for extractable LPL activity and protein. LPL activity was measured using a radioenzymatic assay with a glycerol-stabilized triolein substrate emulsion as described previously.[13] Specific LPL activity was defined as the difference in activity measured in the presence and absence of serum during the incubation.

PI 3-Kinase Activity

At the end of the incubations, cells were rapidly separated from the medium by centrifugation in a microfuge through 0.5 mL silicone oil. Packed cells were collected and solubilized in buffer containing 1% Nonidet P-40, 25 mmol/L HEPES (pH 7.5), 50 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L EDTA, 10 mmol/L okadaic acid, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL antipain, aprotonin, and leupeptin. After centrifugation at 12,000 × *g* for 15 minutes, the cell lysates were incubated with antibody against the p110a subunit of PI 3-kinase (2 μg/mg protein). The immune complexes were collected, resuspended in 20 mmol/L Tris hydrochloride, pH 7.5, 100 mmol/L NaCl, 0.5 mmol/L EGTA, and 200 μg/mL phosphatidylinositol (PI), and assayed for PI 3-kinase activity as described by Hu et al.[14]

Statistical Analysis

The data are expressed as the mean ± SEM. Statistical analyses were performed by ANOVA, and comparisons among groups were made by the Bonferroni/Dunn test or unpaired Student's *t* test using StatView software (Abacus Concepts, Berkeley, CA) on a Power Macintosh (Apple Computer, Cupertino, CA) computer. For all analyses, a *P* value less than .05 was considered significant.

RESULTS AND DISCUSSION

To determine the optimum time for examining insulin stimulation of LPL activity, the effects of insulin on LPL activity in isolated rat adipose cells were examined at varying times (Fig 1). No effects of insulin on LPL activity were observed at early time points; however, there was a substantial increase in heparin-releasable LPL activity after 4 hours of incubation with insulin ($P < .005$). A slow decline in total LPL activity was observed in incubations longer than 4 hours as cell viability diminished (data not shown). No statistically significant effects of insulin were observed on LPL activity extractable from the cells following LPL release by heparin (data not shown). These results are consistent with previous observations, where most investigators have documented an increase in

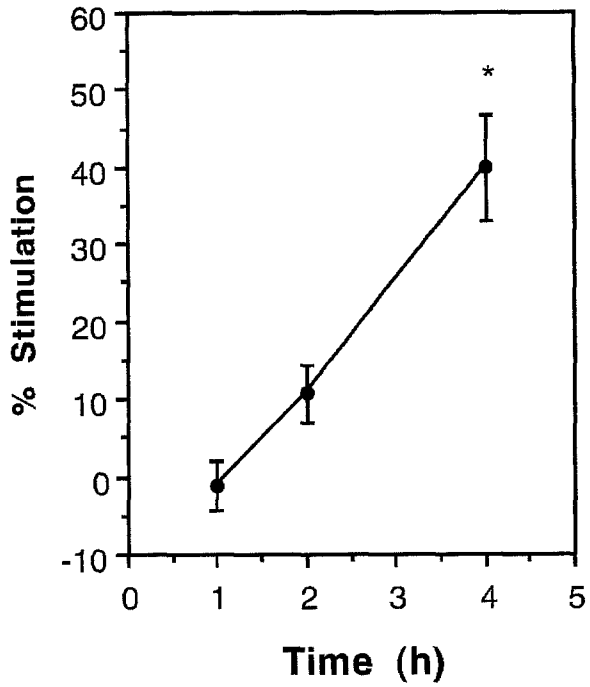

Fig 1. Time course of insulin action on LPL activity. Isolated adipose cells were incubated in the presence or absence of insulin for the indicated times. Heparin-releasable LPL activity was measured in the media. Results are the mean ± SEM of triplicate incubations. *$P <$ .005.

heparin-releasable LPL activity and/or spontaneously released LPL when isolated adipose cells are incubated with insulin; however, significant effects of insulin on cell-extractable LPL activity have not been consistently observed.[8,15-18] Thus, subsequent experiments to explore the mechanism of insulin regulation of LPL were conducted after 4-hour incubations with insulin when heparin-releasable activity was generally stimulated twofold to threefold.

The cellular effects of insulin are broad and include modulation of the transport of molecules across the plasma membrane, decreased levels of cyclic nucleotides, modulation of the activity of key enzymes in intermediary metabolism, changes in the rate of protein synthesis and degradation, alteration of the rate of DNA and RNA synthesis, and alterations in cellular growth and differentiation. Although the exact mechanisms through which insulin exerts these effects are currently incompletely understood, insulin first binds to its cell surface receptor, thereby activating the intrinsic tyrosine kinase activity of the receptor that mediates the phosphorylation of tyrosine residues in signaling or adaptor proteins such as insulin receptor substrate-1 (IRS-1), IRS-2, and Shc.[19] Once the adaptor proteins are tyrosyl-phosphorylated, a number of different proteins possessing phosphatase or serine/threonine kinase activity associate with these adaptor proteins and become activated, thereby propagating a phosphorylation/dephosphorylation cascade. Among the proteins that associate with IRS-1 is PI 3-kinase.[20] PI 3-kinase is a heterodimeric protein, composed of 110-kd catalytic and 85-kd regulatory subunits, that mediates phosphorylation of the D-3 position of the inositol ring of PI, PI-4-P, and PI-4,5-P_2.[21] These D-3-phosphorylated phospholipids can then

act as second messengers in the signaling cascade. Insulin stimulation of PI 3-kinase occurs rapidly at physiological concentrations of insulin, and can be detected in vivo in ^{32}P-labeled cells.[22,23] The role of PI-3 kinase in signaling pathways has been explored using specific inhibitors such as wortmannin, a fungal metabolite that has been shown to directly inhibit PI 3-kinase.[24] Blockade of PI 3-kinase activity in isolated adipocytes is associated with an inhibition of the ability of insulin to stimulate glucose uptake,[24] glucose transporter translocation,[25] glycogen synthesis,[26] and DNA synthesis[25] and a loss of the ability of insulin to inhibit isoproterenol-stimulated lipolysis.[27] This inhibition of some insulin actions occurred without affecting the insulin-mediated activation of pyruvate dehydrogenase or the mitogen-activated protein kinase cascade.[25,26,28] To examine whether PI 3-kinase activity was important in mediating the effects of insulin on LPL, isolated adipose cells were preincubated with wortmannin before exposure to insulin (Fig 2). In the absence of wortmannin, insulin increased heparin-releasable LPL activity about threefold ($P < .0001$). Wortmannin had no consistent effects on basal LPL activity, but completely blocked the ability of insulin to stimulate LPL ($P < .0001$). As described previously,[24] the stimulatory effects of insulin on PI 3-kinase were also inhibited by wortmannin (data not shown). Although wortmannin is a fairly specific inhibitor of PI 3-kinase, it has been reported that wortmannin can also inhibit bombesin-stimulated phospholipase A_2.[29] Thus, a wortmannin-sensitive signaling pathway that most likely involves PI 3-kinase is responsible for mediating the effects of insulin on LPL.

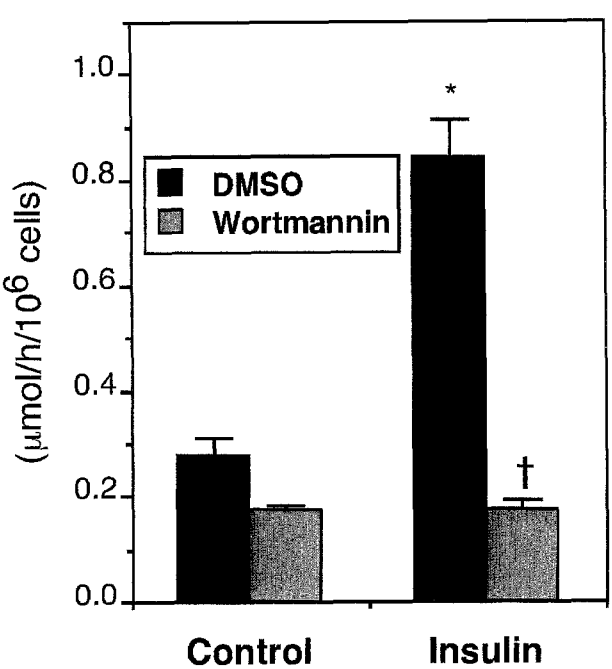

Fig 2. Effects of wortmannin on insulin-stimulated LPL activity. Isolated adipose cells were incubated in the presence of carrier (dimethyl sulfoxide [DMSO]) or wortmannin (1 μmol/L) for 30 minutes prior to addition of insulin (10^{-8} mol/L) or buffer for 4 hours. Heparin-releasable LPL activity was measured in the media. Results are the mean ± SEM of triplicate incubations and are representative of 3 separate experiments. *$P < .0001$ *v* control cells; †$P < .0001$ *v* insulin-treated DMSO cells.

Under most experimental conditions, PI 3-kinase appears to be a relatively proximal, upstream effector in the insulin signaling cascade. Among the downstream targets of PI 3-kinase activity is phosphoprotein 70 ribosomal protein S6 kinase ($p70^{s6k}$). $p70^{s6k}$ appears to be the physiologically relevant serine/threonine kinase that phosphorylates the S6 protein of the 40 S ribosomal subunit.[30,31] Although the actual function of phosphorylated S6 is unclear, S6 phosphorylation is associated with increases in protein synthesis and appears to be related to changes in the efficiency of initiation/translation of mRNAs in general or of selective mRNAs.[30] Studies using specific inhibitors of PI 3-kinase or expression of mutant proteins have established that the activation of $p70^{s6k}$ is mediated via PI 3-kinase.[25,32] Since blockade of PI 3-kinase with wortmannin completely inhibited the stimulatory effect of insulin on LPL, we next examined the role of $p70^{s6k}$ in signaling the effects of insulin on LPL. Establishing the involvement of $p70^{s6k}$ in mediating some of insulin's actions has been facilitated by the observation that the macrolide immunosuppressant, rapamycin, specifically inhibits the activation of and signaling by $p70^{s6k}$ without affecting other S6 kinases such as $p90^{rsk}$.[33] In the absence of rapamycin, insulin increased heparin-releasable LPL activity about threefold ($P < .0001$; Fig 3A). When isolated adipose cells were preincubated with 400 nmol/L rapamycin before exposure to insulin, there were no effects of rapamycin on basal LPL activity; however, rapamycin inhibited insulin-stimulated LPL activity 50% to 60% ($P < .0001$). To ensure that this was the maximal effect of rapamycin, a dose-response for rapamycin was determined (Fig 3B). Rapamycin inhibited insulin-stimulated LPL activity in a complex concentration-dependent manner. First, there was a small increase in insulin-stimulated LPL activity observed at very low concentrations of rapamycin, followed by a dose-dependent decrease in insulin-stimulated LPL activity with an IC_{50} of about 3 nmol/L, similar to the IC_{50} of rapamycin for inhibition of $p70^{s6k}$ activation.[33] A partial loss of the ability of rapamycin to inhibit insulin-stimulated LPL activity was observed at concentrations of rapamycin above 100 nmol/L. While the complex dose-response of rapamycin suggests that it may be affecting steps in addition to $p70^{s6k}$, it appears that $p70^{s6k}$ mediates a portion of the effects of insulin on LPL.

These findings highlight the fact that PI 3-kinase and $p70^{s6k}$ constitute points of divergence in the signal transduction cascade of insulin action. For instance, the ability of insulin to stimulate glucose transport and the translocation of GLUT4 glucose transporters is unaffected by rapamycin,[34] yet rapamycin has been shown to completely inhibit the stimulation of GLUT1 glucose transporter protein induced by chronic insulin exposure.[35] Since rapamycin has no acute effects on insulin-mediated glucose transport, our results suggest that the majority of insulin's effects on LPL are not mediated via insulin-induced changes in cellular glucose transport, but via specific insulin signaling pathways. $p70^{s6k}$ has been shown to be important in the induction of hexokinase II,[36] but not phosphoenolpyruvate carboxykinase,[37] gene expression by insulin. In addition, $p70^{s6k}$ is involved in the insulin signaling pathway leading to the control of eukaryotic translation initiation factor 4E and its inhibitor, PHAS-1.[38,39] Given the potential role of $p70^{s6k}$ in the signaling pathway for gene expression and protein synthesis, it

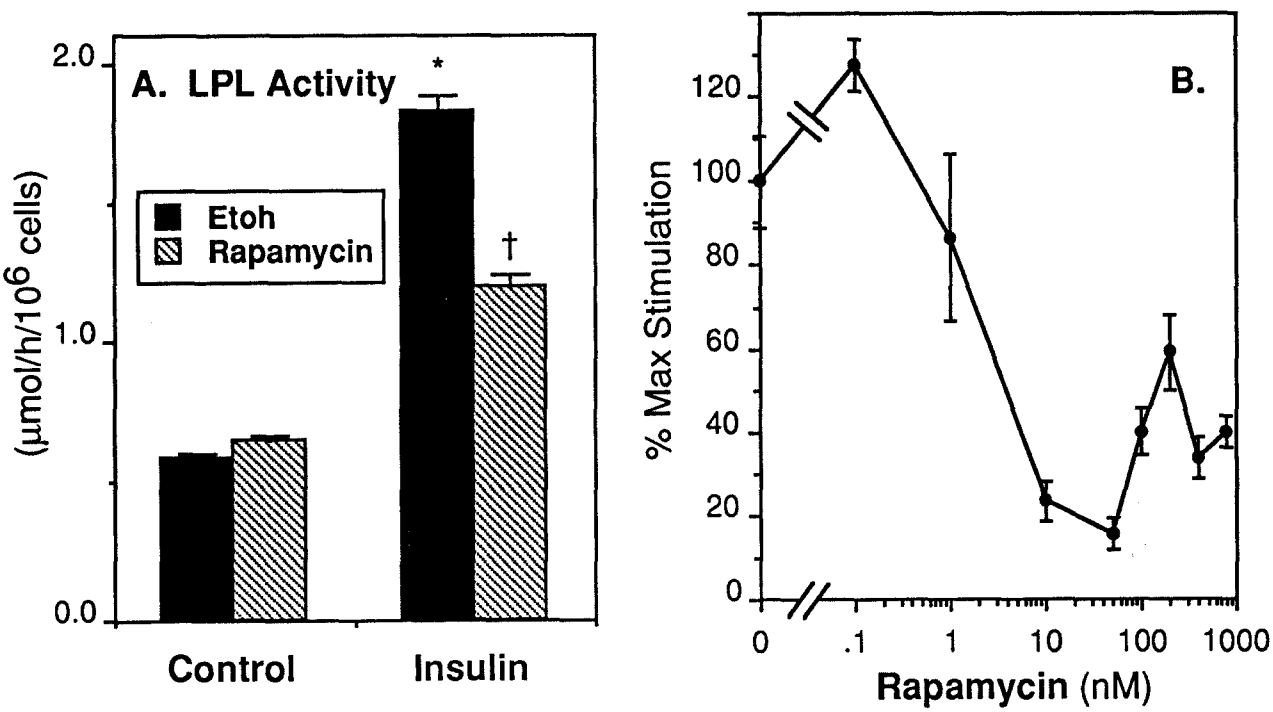

Fig 3. Effects of rapamycin on insulin-stimulated LPL activity. (A) Isolated adipose cells were incubated in the presence of ethanol carrier (Etoh) or rapamycin (400 nmol/L) for 30 minutes prior to addition of insulin (10^{-8} mol/L) or buffer for 4 hours. Heparin-releasable LPL activity was measured in the media. (B) Isolated adipose cells were incubated in the presence of carrier or the indicated concentration of rapamycin for 30 minutes prior to addition of insulin (10^{-8} mol/L) or buffer for 4 hours. Heparin-releasable LPL activity was measured in the media. Results are the mean × SEM of triplicate incubations and are representative of 3 separate experiments. *$P < .0001$ *v* control cells; †$P < .0001$ *v* insulin-treated Etoh cells.

is tempting to speculate as to how it functions in the regulation of LPL. LPL has been shown to be regulated by insulin in adipose tissue via transcriptional,[5,9] posttranscriptional,[6,9] translational,[5,9,40] and posttranslational[6,40] events. Thus, $p70^{s6k}$ might be involved in the control of LPL synthesis and perhaps control of LPL mRNA expression by insulin. Because $p70^{s6k}$ was responsible for mediating most but not all of the effects of insulin on LPL, other components of the insulin signaling pathway downstream from PI 3-kinase must also be involved. Future studies will explore these possibilities. In summary, the current studies show that the regulation of adipose tissue LPL by insulin is mediated via a wortmannin-sensitive pathway, most likely PI 3-kinase, and that a rapamycin-sensitive pathway, most likely $p70^{s6k}$, constitutes an important downstream component in the insulin signaling pathway through which LPL is regulated.

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