Insulin Stimulates Mitogen-activated Protein Kinase by a Ras-independent Pathway in 3T3-L1 Adipocytes*

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To characterize tissue-specific differences in insulin signaling, we compared the mechanisms of mitogen-activated protein (MAP) kinase activation by insulin in the mitogenically active 3T3-L1 fibroblasts with the metabolically active 3T3-L1 adipocytes. In both cell lines, insulin significantly increased p21ras-GTP loading (1.5-2-fold) and MAP kinase activity (5-8-fold). Inhibition of Ras farnesylation with lovastatin, insulin continued to stimulate MAP kinase predominantly via a Ras-independent pathway. In contrast, in 3T3-L1 adipocytes, despite an inhibition of activation of p21ras and Raf-1 by lovastatin, insulin continued to stimulate MAP kinase activity. Fractionation of the cell lysates on the FPLC Mono-Q column revealed that lovastatin inhibited insulin stimulation of ERK2 (and, to a lesser extent, ERK1) in 3T3-L1 fibroblasts and had no effect on the insulin-stimulated ERK2 in 3T3-L1 adipocytes. These results demonstrate an important distinction between the mechanism of insulin signaling in the metabolically and mitogenically active cells. Insulin activates MAP kinase by the Ras-dependent pathway in the 3T3-L1 fibroblasts and by the Ras-independent pathway in the 3T3-L1 adipocytes.

Insulin’s interaction with its cell surface receptor triggers both metabolic and mitogenic cellular responses. Insulin binding activates the tyrosine kinase of the β-subunit of the insulin receptor, which immediately phosphorylates insulin receptor substrate-1 and Shc. Phosphorylated sites of insulin receptor substrate-1 and Shc bind Src homology-2 domain-containing intermediates such as phosphatidylinositol 3-kinase (PI 3-kinase) and Grb-2, which, in turn, propagate insulin signaling downstream. PI 3-kinase appears to be involved in regulation of glucose transport and protein synthesis, while Grb-2 associates with Sos appears to be an important step in activating the Ras-Raf-MEK-MAP kinase pathway (reviewed in Ref. 1).

Although many intermediates of the insulin signaling have been identified, numerous questions remain unanswered. In particular, most of the signaling molecules activated by insulin are also significantly stimulated by other growth factors, such as platelet-derived growth factor and EGF (2). However, in contrast to these growth factors, only insulin elicits defined metabolic responses, suggesting that additional steps must be involved in the mechanism of insulin action.

Recent studies from our laboratory have identified significant differences between the regulation of certain aspects of insulin signaling in metabolically responsive 3T3-L1 adipocytes and mitogenically responsive 3T3-L1 fibroblasts (3, 4). For example, in 3T3-L1 adipocytes, PI 3-kinase and PKC exert a constitutively inhibitory influence on GAP-activating protein (GAP), allowing insulin signaling to proceed through Sos and p21ras. Removal of this inhibitory influence results in activation of GAP and inhibition of the p21ras-GTP loading. The inhibitory influence of PI 3-kinase and PKC on GAP activation was not observed in 3T3-L1 fibroblasts (3, 4). Since activation of p21ras is believed to be required for the activation of MAP kinase by growth factors (reviewed in Refs. 5–8), we compared the mechanism of MAP kinase activation by insulin in 3T3-L1 adipocytes and 3T3-L1 fibroblasts. We found that, while in fibroblasts, insulin activates MAP kinase exclusively by a Ras-dependent pathway, in adipocytes, insulin stimulates MAP kinase predominantly via a Ras-independent pathway.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and supplies were from Life Technologies, Inc. and Gemini Bioproducts (Calabasas, CA); radioisotopes from DuPont NEN. All standard chemicals were from Sigma; insulin was a gift from Eli Lilly and Co. Anti-Ras antibodies were from Transduction Laboratories (Lexington, KY); anti-ERK1/2 antibody and Raf-1 antibody were from Upstate Biotechnology Inc. (Lake Placid, NY); lovastatin and the farnesyl transferase inhibitor, α-hydroxysterifarnesylphosphonic acid, were from Merck and Co. MEK was kindly provided by Dr. R. Nemenoff (University of Colorado Health Sciences Center, Denver, CO), the MEK inhibitor (PD98059) was a gift from Dr. Alan Saltiel (Parke-Davis, Ann Arbor, MI), and Raf-1 fibroblasts transfected with a dominant negative mutant of p21ras (N17) were courtesy of Dr. Jerrold Olefsky (University of California, San Diego).

Cell Culture—3T3-L1 fibroblasts were grown to confluence in fibroblast growth media (Dulbecco’s modified Eagle’s medium (DMEM) containing 5.5 mM glucose with 10% fetal calf serum (FCS), 50 μg/ml gentamicin, 1 μM l-glutamine). Differentiation of adipocytes was induced via the following protocol. Cells were refed with fibroblast growth media when 80% confluent. Two days later, cells were fed differentiation media (DMEM containing 5 mM glucose, 10% FCS, 50 μg/ml gentamicin, 1 mM l-glutamine, 2.5 ml of 10 × PBS, 0.055 g of isobutyl-methylxanthene, 20 ml of deionized water, 250 μl of 49 mM dexamethasone, and 2.5 mg of insulin). On day 4, cells were fed adipocyte growth media (DMEM containing 25 mM glucose with 10% FCS, 50 μg/ml gentamicin, 1 mM l-glutamine, and 1 μg/ml insulin). Cells were refed...
with three 1-ml aliquots of 50 mM Sephacel (Pharmacia Biotech, Uppsala, Sweden) columns (prewashed for 15 min in 10 mM starved overnight and stimulated with 100 nM insulin for 10 min. Cells were amped on gels for 60 min at 100 V. Gels were dried and analyzed by sample buffer. Samples were boiled and run on 10% SDS-polyacrylamide gels.

**Results**

Results represent mean ± S.E. of three to five independent experiments.

### MAP Kinase Assay on FPLC Mono-Q Column Fractions—

Confluent cells were serum-starved overnight and stimulated with 100 nM insulin for 10 min. Cells were pelleted and lysed in 1 ml of lysis buffer (50 mM EGTA, 2 mM MgCl$_2$, 1 mM DTT, 1 mM EGTA, 50 mM Na$_3$VO$_4$, 2 mM EDTA, 1 mM DTT). Twenty microliters of each collected fraction was incubated for 10 min at 30°C with 20 μl of kinase buffer (0.5% Triton X-100, 1 mM Na$_2$HPO$_4$·7H$_2$O, 5 mM NaH$_2$PO$_4$·H$_2$O, 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 50 mM NaF, 200 μM sodium orthovanadate (Na$_3$VO$_4$), 0.1% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 100 units/ml apro tin) was added to samples and incubated on ice for 30 min, vortexed frequently. Samples were centrifuged for 5 min at 3000 rpm. Pellets were resuspended in RIPA buffer, RIPA without aprotinin, and two times in PAN buffer (100 mM NaCl, pH 7.0) plus 0.5% Nonidet P-40. Pellets were resuspended in PAN buffer, centrifuged at 5000 rpm for 1 min, resuspended in 20 μl of PAN buffer, and incubated at 30°C for 15 min in 10 μl of 2% PAN buffer plus 1000 units/ml aprotinin, 4 μl of 100 mM MnCl$_2$, 1 μl of MEK, 2 μl of 0.1 mM ATP, 20 μCi of [γ-32P]ATP. Reactions were stopped by adding 10 μl of 5 × Laemmli sample buffer. Samples were boiled and run on 10% SDS-polyacrylamide gels for 60 min at 100 V. Gels were dried and analyzed by autoradiography.

### MAP Kinase Assay on Cell Lysates—

Confluent cells were serum-starved overnight and stimulated with 100 nM insulin for 10 min. Cells were placed on ice, rinsed with PBS, and lysed in 1 ml of cell lysis buffer (0.5% Triton X-100, 50 mM β-glycerophosphate, 0.1 mM Na$_2$VO$_4$, 2 mM MgCl$_2$, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.2). Lysates were quickly vortexed, and then centrifuged (5 min at 10,000 × g at 4°C). Supernatants were absorbed to 0.5-ml DEAE-Sephacel (Pharmacia Biotech, Upsalla, Sweden) columns (preshaved with three 1-ml aliquots of 50 mM β-glycerophosphate, 0.1 mM Na$_2$VO$_4$, 1 mM EDTA, 1 mM DTT, 1 mM EGTA, pH 7.2, at 4°C). Columns were washed with three 1-ml aliquots of the above buffer and eluted with 1 ml of the above buffer containing 1 mM sodium chloride. Twenty microliters of the collected eluent were incubated for 10 min at 30°C with 20 μl of kinase buffer (50 mM β-glycerophosphate, 100 μM Na$_2$VO$_4$, 20 mM MgCl$_2$, 200 μM ATP, 0.45 μCi/ml [γ-32P]ATP, 400 μg EGFR-662–681 peptide (Macromolecular Resources, Ft. Collins, CO), 50 μg/ml cAMP-dependent protein kinase inhibitor, PKI 5–24 (Bachem, Torrance, CA), and 1 mM EGTA). The reaction was terminated with 10 μl of 25% trichloracetic acid, and 45 μl of the mixture was spotted onto Whatman P-81 phosphocellulose squares. The squares were washed four times in 75 mM phosphoric acid, washed once in 100% acetone, and counted in a scintillation counter. In several experiments, cell lysates were immunoprecipitated with anti-ERK1/2 antibody and the precipitates used in the MAP kinase assay.

**MAP Kinase Assay on Cell Lysates—**

Confluent 3T3-L1 fibroblasts and adipocytes were serum- and phosphate-starved for 24 h and labeled with [32P]orthophosphate (0.25 mM/ml overnight. Cells were

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**Fig. 1. Effect of lovastatin on insulin-induced p21ras-GTP loading.** 3T3-L1 fibroblasts and adipocytes were pretreated with lovastatin (2 μg/ml) for 18 h prior to a 10-min incubation with or without insulin (100 nM). Percent p21$^{ras}$-GTP was determined as described under “Experimental Procedures.” Insulin significantly increased the amount of p21$^{ras}$-GTP in control (p < 0.05), but not in the lovastatin-treated cells. Results represent mean ± S.E. of three to five independent experiments.

**Fig. 2. Basal and insulin-stimulated MAP kinase activity in control and lovastatin-treated 3T3-L1 fibroblasts and adipocytes.** Cells were pretreated with lovastatin (2 μg/ml) for 18 h prior to a 10-min incubation with (shaded bars) or without (open bars) insulin (100 nM). MAP kinase activity was determined as described under “Experimental Procedures” using EGF receptor peptide as a substrate. Results represent mean ± S.E. of five independent experiments. *p < 0.05; **p < 0.01.
To investigate the role of p21ras in mediating insulin activation of MAP kinase, we initially employed the hydroxymethylglutaryl-CoA-reductase inhibitor lovastatin to block the activation of p21ras by insulin in 3T3-L1 fibroblasts and 3T3-L1 adipocytes. Lovastatin blocks farnesylation of p21ras, thus decreasing the pool of the intracellular Ras available for subsequent activation by growth factors, including insulin (9). Lovastatin (2 µg/ml for 18 h) significantly inhibited the ability of insulin to stimulate p21ras-GTP loading in both 3T3-L1 fibroblasts and 3T3-L1 adipocytes (Fig. 1). However, insulin-stimulated MAP kinase activity was inhibited by lovastatin in the 3T3-L1 fibroblasts (by 85%), but not in 3T3-L1 adipocytes (Fig. 2). Despite complete inhibition of Ras stimulation, insulin continued to activate MAP kinase (5–8-fold) in 3T3-L1 adipocytes.

In the next series of experiments, activation of p21ras was inhibited by 1 µM α-hydroxyfarnesylphosphonic acid, a specific inhibitor of farnesyltransferase. This enzyme catalyzes farnesylation of p21ras, which is necessary for its subsequent activation. The presence of the farnesyltransferase inhibitor completely blocked the ability of insulin to stimulate MAP kinase in 3T3-L1 fibroblasts, but had no effect in 3T3-L1 adipocytes (Fig. 3).

We have recently observed that, in 3T3-L1 adipocytes, depletion of PKC with prolonged exposure to the phorbol ester, TPA, results in activation of GAP and inhibition of the insulin-induced p21ras-GTP loading (4). Therefore, we used this paradigm to further examine the role of p21ras-GTP in mediating insulin’s effect on MAP kinase in 3T3-L1 adipocytes. Cells treated with TPA (100 nM for 18 h) were unable to increase p21ras-GTP formation in response to insulin (4), but responded fully to activation of MAP kinase by insulin (Fig. 4), suggesting that the latter is activated by a Ras-independent pathway.

Assuming a linear progression of the insulin signal from Ras to Raf to MEK to ERK1/ERK2, we compared the involvement of Raf and MEK kinases in the activation of MAP kinase in 3T3-L1 fibroblasts and adipocytes. Activation of Raf-I kinase activity by insulin was clearly Ras-dependent in both cell lines, as lovastatin inhibited insulin-stimulated Raf-1 activity in fibroblasts and adipocytes (Fig. 5). Similarly in both cell lines, the MEK inhibitor, PD98059, was equipotent in inhibiting the activation of MAP kinase by insulin in both cell lines (Fig. 6), implying the importance of MEK as the upstream activator of MAP kinase in both fibroblasts and adipocytes.

In order to confirm that in fibroblasts insulin stimulates MAP kinase activity via the Ras-dependent pathway, we examined the effect of insulin on MAP kinase in the Rat-1 fibroblasts transfected with the dominant negative mutant (N17) of Ras. As shown in Fig. 7, insulin failed to stimulate MAP kinase activity in these cells.

In the experiments described above, MAP kinase activity reflected the total cellular kinase activity assayed using the EGF receptor peptide as a substrate. Conceivably, differential expression of various members of the MAP kinase family (11–14) may explain the differences in response to insulin between the adipocytes and fibroblasts. To examine this possibility, we fractionated cell lysates from 3T3-L1 fibroblasts and 3T3-L1 adipocytes on FFPLC Mono-Q column and measured MAP kinase activity in each fraction. Two major peaks of MAP kinase activity were detected in both cell types. The first peak corresponded to ERK2 and the second smaller peak to ERK1. Insu-
activated ERK2 and ERK1 in both cell types. Inhibition of p21ras activation with lovastatin resulted in a decreased ability of insulin to stimulate ERK2 in fibroblasts (Fig. 8A), but had no effect on the activation of ERK2 by insulin in adipocytes (Fig. 8B).

Finally, to verify the effect of insulin on ERK1/2, we determined MAP kinase activity in the ERK1/2 immunoprecipitates of control and lovastatin-treated cells. Insulin’s effect on MAP kinase was completely blocked in 3T3-L1 fibroblasts, but remained unaffected in the adipocytes (Fig. 9).

DISCUSSION

It is well known that insulin stimulates both p21ras-GTP loading and MAP kinase in most cells expressing insulin receptors (15, 16). It has been assumed that these two events are obligatorily coupled in these cells. The main point of the present study is that, at least in 3T3-L1 adipocytes, these events are parallel but independent of each other.

Insulin stimulates both p21ras and MAP kinase in 3T3-L1 adipocytes, but inhibition of p21ras by lovastatin, farnesyltransferase inhibitor, or PKC depletion does not affect insulin’s ability to stimulate MAP kinase in these cells (Figs. 2–4). This is in direct contrast to 3T3-L1 fibroblasts, where activation of p21ras is an obligatory step for MAP kinase activation (Figs. 2–4). Despite differential regulation, the Mono-Q pattern of the MAP kinases is similar in both cell lines (Fig. 8), indicating that the same MAP kinase family members are differentially regulated in 3T3-L1 fibroblasts and 3T3-L1 adipocytes: one via a Ras-dependent and the other via a Ras-independent pathway.

Several MAP kinases (ERK1/2, JNK/SAP, p38/HOG1, p57 MAP), MEKs (MEK1, -2, -3, -5, and -6), Rafs (A-Raf, c-Raf-1, B-Raf), and MEK kinases (MEKK-1, -2, and –3) have been identified and cloned (11–14, 17–25). Various members of these families may be either differentially expressed in various tissues, differentially localized intracellularly, or differentially regulated within the same tissue. Any one of these possibilities can explain the existence of Ras- and Raf-dependent and Ras- and Raf-independent activation of MAP kinase observed previously (6, 26–28) and in this investigation. Regulation of different MAP kinases involves preferential influence of different MEKs, MEKKs, and Raf kinases (5–8). Recent comparison of various MEKKs indicated that greater degree of selectivity for ERK1/2 or JNK pathways was apparent only at modest levels of expression of MEK1, -2, or -3 (19). Moreover, activities of MEKKs have been found to be quite distinct from the activities of Raf-1 and B-Raf kinases (6–8). The present data support the existence of diverse signaling pathways and for the first time suggest that these diverse pathways can be activated by the same ligand, depending upon the differentiated phenotype. It appears that in 3T3-L1 fibroblasts, insulin activates ERK1/2 via the traditional Ras-dependent pathway. In contrast, in the 3T3-L1 adipocytes, insulin stimulates ERK1/2 via a Ras- and Raf-independent pathway.

The signaling molecules within the Ras-independent pathway are unknown. ERK1/2 is believed to be activated by its own kinase, MEK1/2 (5–8), and MEK1/2 activation appears to be essential for insulin signaling in both adipocytes and fibroblasts, since the MEK inhibitor blocked insulin-stimulated MAP kinase activation in both cell lines (Fig. 6). MEK itself is a dual specificity kinase, which is activated by the upstream
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serine/threonine kinases Raf and MEKK. The present data suggest that activation of MAP kinase by MEK in 3T3-L1 adipocytes may occur in the absence of Ras and Raf activation, suggesting that MEK is activated by another MEK kinase. Haystead et al. (29) have recently identified a novel insulin-responsive MEK kinase (I-MEKK) in Wistar rat adipocytes. This kinase was distinct from Raf and showed rapid phasic kinetics in response to insulin but not phorbol ester. They have proposed that in adipocytes, I-MEKK may represent a divergence point between the insulin- and PKC-mediated signal transduction pathways. It remains unknown whether or not this new insulin-responsive MEK kinase is Ras-dependent.

Although 3T3-L1 adipocytes are derived from 3T3-L1 fibroblasts as a result of differentiation, there are numerous distinctions between the two lines. The major difference, however, is that 3T3-L1 adipocytes are terminally differentiated, metabolically active, and non-dividing cells, whereas the 3T3-L1 fibroblasts are mitogenically active, growing, and propagating cells. Conceivably, with differentiation, the influence of insulin shifts to a different signaling cascade. It would be of great importance to investigate whether or not insulin activates

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