Three Mitogen-Activated Protein Kinases Inhibit Insulin Signaling by Different Mechanisms in 3T3-L1 Adipocytes

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TNF-α, which activates three different MAPKs [ERK, p38, and jun amino terminal kinase (JNK)], also induces insulin resistance. To better understand the respective roles of these three MAPK pathways in insulin signaling and their contribution to insulin resistance, constitutively active MAPK/ERK kinase (MEK)1, MAPK kinase (MKK6), and MKK7 mutants were overexpressed in 3T3-L1 adipocytes using an adenovirus-mediated transfection procedure. The MEK1 mutant, which activates ERK, markedly down-regulated expression of the insulin receptor (IR) and its major substrates, IRS-1 and IRS-2, mRNA and protein, and in turn reduced tyrosine phosphorylation of IR as well as IRS-1 and IRS-2 and their associated phosphatidylinositol 3-kinase (PI3K) activity. The MKK6 mutant, which activates p38, moderately inhibited IRS-1 and IRS-2 expressions and IRS-1-associated PI3K activity without exerting a significant effect on the IR. Finally, the MKK7 mutant, which activates JNK, reduced tyrosine phosphorylation of IRS-1 and IRS-2 and IRS-associated PI3K activity without affecting expression of the IR, IRS-1, or IRS-2. In the context of our earlier report showing down-regulation of glucose transporter 4 by MEK1-ERK and MKK6/3-p38, the present findings suggest that chronic activation of ERK, p38, or JNK can induce insulin resistance by affecting glucose transporter expression and insulin signaling, though via distinctly different mechanisms. The contribution of ERK is, however, the strongest. (Molecular Endocrinology 17: 487–497, 2003)

Glucone UPTAKE IS dependent on the activity of glucose transporters (GLUTs) located on the cell surface (1, 2). Expressed exclusively by muscle and fat cells, GLUT4 resides in an intracellular compartment under basal conditions but then moves to the cell surface in response to insulin stimulation (3). One element known to be essential for the translocation of GLUT4 to the plasma membrane is the activation of phosphatidylinositol 3-kinase (PI3K), through association with tyrosine-phosphorylated insulin receptor substrate (IRS) proteins, via insulin receptor (IR) binding (4–7). PI3K in turn phosphorylates serine/threonine protein kinase B (also known as Akt) on two regulatory sites: Thr-308 in the activation loop and Ser-473 in the hydrophobic C-terminal regulatory domain (8–10). Akt activation has not been shown to directly mediate insulin-induced GLUT4 translocation, impairment of PI3K and Akt activation has been demonstrated in several insulin-resistant diabetic models (11–15). Thus, decreased GLUT4 expression and/or impaired insulin signaling could be the molecular mechanism underlying insulin resistance in muscle and fat cells. TNF-α is known to activate three different MAPKs—ERK, p38 MAPK (p38), and jun amino terminal kinase (JNK); Refs. 16–18)—and to contribute to insulin resistance under such pathophysiological conditions as obesity, cancer-induced cachexia, and bacterial infections (19, 20). For example, TNF-α reduces IRS-1 expression and insulin-induced IRS-1 tyrosine phosphorylation, effects that should each reduce insulin sensitivity (19, 21–24). However, we previously demonstrated the effects of chronic ERK, p38 or JNK activation on glucose transporter expression by selectively overexpressing the kinase situated immediately upstream from each MAPK. We found that chronic activation of either the MAPK/ERK kinase (MEK) 1-ERK...
or MAPK kinase (MKK) 6/3-p38 pathway leads to up-regulation of GLUT1 expression and down-regulation of GLUT4 expression, resulting in significantly increased basal glucose transport but decreased insulin-induced transport (25). Consistent with that finding, PD98059, a specific ERK inhibitor, restores insulin sensitivity in the presence of TNFα (21, 26). On the other hand, recent evidence suggests that activated JNK phosphorylates a serine residue on IRS-1, thereby inhibiting its tyrosine phosphorylation (27). Thus, the precise roles played by ERK, p38, and JNK in mediating TNFα-induced insulin resistance remain unclear.

In the present study, we used an adenovirus-mediated transfection procedure to overexpress three constitutively active MKK mutants in 3T3-L1 adipocytes and then evaluated the effects of the resultant MAPK activation (18, 28–31) on the expressions of IR, IRS-1, and IRS-2, the degree of their tyrosine phosphorylations, and their associated PI3K activations. Our results indicate that, by acting via IR, IRS-1, and IRS-2, ERK has the strongest inhibitory effect on insulin signaling.

RESULTS

Effects of Stress-Inducing Stimuli or Overexpression of Constitutively Active MKK Mutants on Phosphorylation of ERK in 3T3-L1 Adipocytes

Constitutively active forms of MEK1 (LASDSE-MEK1), MKK6 (EE-MKK6), and MKK7 (DED-MKK7) were overexpressed in 3T3-L1 adipocytes, after which relative levels of ERK phosphorylation were measured by immunoblot analysis using anti-phospho-ERK (Thr202/Tyr204) antibody as a probe (Fig. 1A). We found that LASDSE-MEK1 significantly increased the level of ERK phosphorylation, whereas EE-MKK6 and DED-MKK7 had little effect. Similarly, exposure to stressful stimuli—epidermal growth factor (EGF), platelet-derived growth factor (PDGF)-B chain/B chain dimer (BB), and TNFα—also increased ERK phosphorylation (Fig. 1A), although only TNFα-induced increases persisted for at least 24 h. The effects of EGF and PDGF-BB were more transient, with levels of ERK phosphorylation returning to baseline within 8–24 h after stimulation (Fig. 1B).

Effects of EE-MKK6 and DED-MKK7

Overexpression of EE-MKK6 up-regulated phosphorylation of p38 in 3T3-L1 adipocytes (Fig. 2A). EGF and PDGF-BB evoked transient phosphorylation of p38 that was detected within 10 min of their application (Fig. 2B). TNFα again had a more pronounced and prolonged effect than EGF or PDGF-BB, evoking an elevation in p38 phosphorylation that persisted for at least 24 h.

DED-MKK7 overexpression and TNFα stimulation each induced prolonged phosphorylation of JNK, whereas EGF and PDGF-BB evoked transient and minimal JNK phosphorylation (Fig. 3).

Effects of TNFα, EGF, and PDGF on Insulin-Induced Increases in Glucose Transport

Figure 4 shows basal and insulin-stimulated 2-deoxy-[3H]glucose uptakes into 3T3-L1 adipocytes exposed to various stress-inducing stimuli for 24 h. Exposure to TNFα for 24 h significantly elevated basal glucose transport in 3T3-L1 adipocytes but reduced the insulin-induced increase in glucose uptake. On the other hand, consistent with earlier reports (32–34), exposure to EGF or PDGF-BB had no significant effect on either basal or insulin-stimulated glucose uptake.

![Fig. 1. Effects of Stress-Inducing Stimuli and Constitutively Active MKK Mutants on Phosphorylation of ERK](https://academic.oup.com/mend/article-abstract/17/3/487/2747358/488-Mol-Endocrinol-March-2003-17-3-487-497-Fujishiro-et-al.-Three-MAPKs-that-Impair-Insulin-Signaling)
Effects of Constitutively Active MKK Mutants or TNFα on Expressions of IR and IRS

As a first step in evaluating the effects of MAPK activation on insulin signaling and its contribution to insulin resistance in 3T3-L1 adipocytes, the expressions of IR and IRS proteins were investigated in cells stimulated with TNFα or overexpressing a constitutively active MKK mutant. We found that overexpression of LASDSE-MEK1 markedly reduced expression of the IR and its major substrates, IRS-1 and IRS-2 proteins, by 82%, 97%, and 90%, respectively (Fig. 3, A–C); overexpression of EE-MKK6 did not significantly affect IR protein expression but induced a more modest reduction than MEK1 in the expressions of IRS-1 and IRS-2 proteins, by 38% and 21%, respectively. Furthermore, DED-MKK7 overexpression had no significant effect on the expression of IR, IRS-1, or IRS-2 protein. Incubating cells with TNFα for 24 h reduced the expressions of IR, IRS-1, and IRS-2 proteins by 63%, 72%, and 48%, respectively. To confirm that these changes are specific to each pathway, we employed two established inhibitors; PD98059, a specific ERK inhibitor, and SB203580, a specific p38 MAPK inhibitor. Expressions of IR, IRS-1, and IRS-2 proteins, reduced by MEK1, were restored upon incubation with PD98059. Those reduced by MKK6 were restored by incubation with SB203580. Furthermore, PD98059 normalized expressions of IR, IRS-1, and IRS-2 reduced by TNFα, whereas SB203580 had no significant effect.

To better understand the mechanism underlying the diminished expression of IR and IRS proteins, levels of IR, IRS-1, and IRS-2 mRNA were assessed using ribonuclease (RNase) protection assays (Figs. 5 and 6, A–C). We found the IR mRNA level to be reduced, in the same manner as expression of its protein, by either MEK1 overexpression or TNFα stimulation. We also found that, as compared with control LacZ, overexp-
pression of either LASDSE-MEK1 or EE-MKK6 significantly reduced expression of both IRS-1 and IRS-2 mRNA, with the effect on IRS-1 being especially pronounced. Exposing cells to TNFα reduced levels of both transcripts by approximately 50%. Apparently then, the observed declines in IRS-2 and IRS-2 proteins reflected the reduced availability of their respective mRNAs.

![Fig. 4. Effects of Stress-Inducing Stimuli on Insulin-Induced Increases in Glucose Transport](image)

3T3-L1 adipocytes were incubated for 24 h with 1 nM EGF, PDGF, or TNFα, after which some were exposed to 100 nM insulin for 15 min, and 2-deoxy-o-[3H]glucose transport was measured as described in Materials and Methods. Bars represent means ± SE from three independent experiments; *, P < 0.05 vs. the indicated control.

![Fig. 5. Effects of Constitutively Active MKK Mutants or TNFα on Expression of IR and IRS Proteins](image)

3T3-L1 adipocytes were infected with recombinant adenoviruses containing LacZ (control), LASDSE-MEK1, EE-MKK6, or DED-MKK7 with or without incubation with 50 μM PD98059 and 10 μM SB203580 for 48 h, or exposed to 10 ng/ml TNFα for 24 h with or without incubation with these two inhibitors for 24 h. The adipocytes were then subjected to SDS-PAGE and immunobotted using anti-IR (A), anti-IRS-1 (B), or anti-IRS-2 (C) antiserum. Bars represent means ± SE from three independent experiments; *, P < 0.05 vs. the indicated control.

Effects of Active MKK Mutants and TNFα on Insulin-Induced Tyrosine Phosphorylation of IR and IRS Proteins

When the effects of selective activation of each MAPK on insulin-induced tyrosine phosphorylation of the IR, IRS-1, and IRS-2 were examined (Fig. 7, A–C), we found that overexpression of LASDSE-MEK1 strongly
inhibited tyrosine phosphorylation of the IR, IRS-1, and IRS-2 (by 52%, 81%, and 63%, respectively), as did exposure to TNFα (by 51%, 68%, and 45%, respectively); EE-MKK6 did not significantly affect tyrosine phosphorylation of the IR or IRS-2, while having a small but significant effect on IRS-1 (a 29% inhibition). Similarly, overexpression of DED-MKK7 had no significant effect on tyrosine phosphorylation of IR.
while significantly inhibiting tyrosine phosphorylation of both IRS-1 and IRS-2, although the effect was not as pronounced (by 37% and 26%, respectively).

Effects of Active MKK Mutants and TNFα on Insulin-Induced p85α (PI3K) Docking and PI3K Activity

The effects of MAPK activation and TNFα on docking of the p85α regulatory subunit of PI3K with IRS (Fig. 8, A and B) and on insulin-induced, IRS-associated PI3K activity (Fig. 9, A and B) were consistent with their effects on tyrosine phosphorylation. To evaluate the influence of these three MKK mutants on overall IR signaling, we determined total phosphotyrosine-phosphorylated protein associated PI3K activity in 3T3-L1 adipocytes infected with these mutants (Fig. 9C). Overexpression of LASDSE-MEK1 and incubation with TNFα each significantly reduced the docking of p85α with IRS-1 (by 83% and 55%, respectively) and IRS-2 (by 72% and 49%, respectively), in turn reducing IRS-associated PI3K activity. Overexpression of DED-MKK7 had a smaller though still significant inhibitory effect on p85α docking and PI3K activities associated with both IRS-1 (by 28% and 30%, respectively) and IRS-2 (by 26% and 34%, respectively), whereas EE-MKK6 significantly affected only p85α docking to IRS-1 (by 29%) and the associated PI3K activity (by 33%). PI3K activity immunoprecipitated by the antiphosphotyrosine antibody was similarly reduced, as observed with IRS-1 or IRS-2, and the reduction was most marked with overexpression of LASDSE-MEK1.

Effects of Active MKK Mutants or TNFα on Akt Expression and Insulin-Induced Akt Phosphorylation

Finally, the effect of MAPK activation on insulin-induced Akt phosphorylation, which is reportedly induced by PI3K lipid products, was investigated (Fig. 10). Overexpression of LASDSE-MEK1 significantly reduced insulin-induced phosphorylation of Akt at Thr-308 (by 92%) and Ser-473 (by 69%), as did incubation with TNFα (by 80% and 66%, respectively); EE-MKK6 and DED-MKK7 also reduced phosphorylation at both Thr-308 (by 58% and 41%, respectively) and Ser-473 (by 24% and 28%, respectively), though not to the degree seen with LASDSE-MEK1 or TNFα (Fig. 10, A and B). Akt expression was not significantly affected by MAPK activation (Fig. 10C).

DISCUSSION

TNFα secreted from adipocytes reportedly impairs insulin activity in peripheral tissues (muscle and fat) as well as in the liver (22, 23, 35, 36). Consistent with...
those findings, treatment with thiazolidinediones, peroxisomal proliferator-activated protein-γ agonists, suppresses TNFα production by adipocytes and normalizes insulin sensitivity in obese insulin-resistant animals (35, 37); and disruption of the gene encoding TNFα or its receptor improves insulin sensitivity in mice (38, 39).

As mentioned in the Introduction, stimulation with TNFα reportedly induces three types of MAPK-ERK, p38, and JNK (16–18). With respect to regulation of glucose transporters, we previously showed that activation of the MEK1-ERK and MKK6/3-p38 pathways up-regulates the expression of GLUT1 but down-regulates that of GLUT4, resulting in increased basal glucose uptake and diminished insulin-sensitive uptake (25). Such regulation of GLUT gene expression by ERK and p38 is thought to be one possible mechanism underlying TNFα-induced insulin resistance. However, effects on GLUT expression are certainly only part of the story, as chronic exposure to TNFα also reduces IRS-1 expression, tyrosine phosphorylation, docking of the p85α regulatory subunit of PI3K, and IRS-associated PI3K activity (22, 23, 40–43). One hypothesis possibly explaining this effect is that ERK plays a key role in TNFα-induced suppression of insulin signaling. Consistent with this idea, treating cells with PD98059, an ERK inhibitor, restores insulin sensitivity in the presence of TNFα (21, 26). On the other hand, it was recently suggested that JNK plays a critical role in TNFα-induced insulin resistance via its association with IRS-1 and the resultant phosphorylation of IRS-1 at serine 307 (27). Serine-phosphorylated IRS-1 is reportedly resistant to being tyrosine-phosphorylated by the insulin receptor (41, 44, 45). Thus, whereas evidence from numerous earlier studies indicates that TNFα induces insulin resistance, the precise mechanism, in particular the pathway via which the signal from the TNFα receptor is transduced, remains unclear.

In the present study, to clarify the individual actions of each MAPK, a constitutively active mutant of the upstream kinase for each MAPK was overexpressed using an adenovirus transfection procedure. The effects of the resultant MAPK activation were comparable to those seen with TNFα stimulation, suggesting them to be within physiological range and that activation of ERK, p38 or JNK can impair insulin signaling. In addition, to confirm that the effects of LASDSE-MEK1 and EE-MKK6 on the regulation of IR or IRS-proteins are truly mediated by the activation of ERK and p38 pathways, respectively, specific ERK and p38 inhibitors were used.

Activation of the MEK1-ERK pathway dramatically suppressed the expressions of IR, IRS-1, and IRS-2. As a result, insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2, docking of the p85α regulatory subunit of PI3K, and PI3K activation were all markedly impaired. When the amounts of immunoprecipitated IRS-1 and IRS-2 were normalized, the PI3K activities associated with equal amounts of IRS-1 and IRS-2
were moderately increased. We speculate that the reductions in IRS-proteins might have increased the individual efficiencies of IRS-1 or IRS-2 molecules in associating with the IR and/or PI3K protein. At any rate, the PI3K activity reduction appears to mainly be attributable to the down-regulation of IR and IRS protein expressions. Taking our earlier report into consideration (25), activation of the MEK1-ERK pathway apparently induces severe insulin resistance by inhibiting expressions of both GLUT4 and insulin-signaling proteins.

Secondly, MKK6-p38 pathway activation only moderately reduced IRS-1 and IRS-2 expressions. When the amounts of immunoprecipitated IRS-1 and IRS-2 were normalized, the PI3K activities associated with equal amounts of IRS-1 and IRS-2 were slightly but not significantly up-regulated (by 10% and 30%, respectively). Consequently, the overall contribution of p38 to TNFα-induced insulin resistance would presumably be smaller than that of ERK. Indeed, treating cells with SB203580, a p38-specific inhibitor, had little effect on TNFα-induced insulin resistance, which suggests that the p38 pathway is not physiologically relevant (46).

Finally, MKK7-JNK pathway activation had no effect on the expression of either IRS-1 or IRS-2 but suppressed tyrosine phosphorylation of both and, in turn, suppressed activation of PI3K and Akt. Consistent with those findings, earlier reports showed JNK to associate with IRS-1 and phosphorylate serine residue-307 (27), thereby inhibiting tyrosine phosphorylation by the IR.

Several previous reports have shown the individual contributions of ERK, p38, and the JNK pathway to TNFα-induced insulin resistance (26, 27, 46–50). However, this study has clearly demonstrated, by overexpressing active mutants of the respective upstream kinases, that ERK, p38 and JNK each have the potential to induce insulin resistance via distinctly different molecular mechanisms. Furthermore, our findings strongly suggest that the ERK contribution is much greater than those of p38 and JNK, based on our observation that ERK markedly suppresses the expressions of IR and IRS-proteins as well as that of GLUT4.

MATERIALS AND METHODS

Materials

Insulin was purchased from Novo Nordisk ( Bagsvaerd, Denmark). BSA, fraction V, was from Intergen (Norcross, GA). 3-Isobutyl-1-methylxanthine and 2-deoxy-D-glucose (2-DG) were from Wako Bioproducts (Osaka, Japan). TNFα was from Genzyme Transgenics Corp. (Cambridge, MA). Recombinant human EGF and human PDGF-BB were from Promega Corp. (Madison, WI) and Life Technologies, Inc. (Gaithersburg, MD), respectively. Inhibitors PD98059 and SB203580 were from Calbiochem (La Jolla, CA). Phosphatidylinositol was from Sigma (St. Louis, MO). Aluminum-backed silica gel thin layer
chromatographic plates were from Merck (Darmstadt, Germany). [\gamma-\text{32P}]ATP and [\gamma-\text{32P}]Juridine triphosphate were from ICN Biomedicals (Irvine, CA), 2-Deoxy-D-[\text{3H}]Glucose, protein A-Sepharose 4 Fast Flow, and an enhanced chemiluminescence detection system were from Amersham Pharmacia Biotech (Arlington Heights, IL). All other reagents from commercial sources were of analytical grade.

**Antibodies**

Specific antibodies against IRS-1 and IRS-2 were prepared as described previously (51). An antibody against Akt was prepared by immunizing rabbits with a synthetic peptide derived from C-terminal amino acids 466–479 of mouse Akt. Anti-p85\alpha antibody and antiphosphotyrosine antibody (4G10) were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-IR\alpha antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody and anti-phospho-JNK (Thr183/Tyr185) were from Cell Signaling Technology (Beverly, MA). Anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody, anti-phospho-Akt (Thr308) antibody and anti-phospho-Akt (Ser473) 4E2 monoclonal antibody were from New England Biolabs, Inc. (Beverly, MA).

**Cell Culture**

3T3-L1 fibroblasts were initially maintained in DMEM supplemented with 10% donor calf serum (Life Technologies, Inc.) under an atmosphere of 90% air/10% CO2 at 37 C. Differentiation was induced 2 d after the cells had reached confluence, by replacing the normal culture medium with DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine, 4 mM 2-Dexpyrididyl, and 10% fetal bovine serum; the medium was changed every other day. With this protocol, more than 90% of the cells differentiated with insulin as described above. Therefore, recombinant adenoviruses were applied at a multiplicity of infection of 200–300 plaque-forming units/cell.

**Construction of MAPK Mutants**

Plasmids containing cDNAs encoding constitutively active MEK1 (LASDSE-MEK1), MKK6 (EE-MKK6), and MKK7 (DED-MKK7) mutants were prepared as follows. LASDSE-MEK1 was constructed by replacing serine-218 with aspartic acid, serine-222 with glutamic acid, and two critical leucines (Leu-11 and Leu-37) in the nuclear export signal sequence of Xenopus MAPKKK with alanines (29). EE-MKK6 was constructed by substituting serine-207 and threonine-211 with glutamic acid (30). DED-MKK7 was constructed by replacing serine-287 with glutamic acid, threonine-291 with glutamic acid (30). DED-MKK7 was constructed by replacing serine-287 with glutamic acid, threonine-291 with glutamic acid, serine-293 with aspartic acid, and two critical leucines (Leu-11 and Leu-37) in the nuclear export signal sequence of Xenopus MAPKKK with alanines (29). EE-MKK6 was constructed by substituting serine-207 and threonine-211 with glutamic acid (30). DED-MKK7 was constructed by replacing serine-287 with glutamic acid, threonine-291 with glutamic acid, serine-293 with aspartic acid.

**Gene Transduction**

The methods of constructing recombinant adenoviruses (52) and infecting 3T3-L1 adipocytes (5, 53) were previously described in detail. Briefly, the expression cosmid cassette pAdexCAvAT was ligated to the cDNA encoding LacZ from *Escherichia coli* or to one of the aforementioned mutants. Thereafter, homologous recombination of the recombinant cosmid cassette and its parental virus genome was carried out, and 3T3-L1 cells were infected with the indicated adenovirus. Experiments were performed 48 h after infection. Therefore, recombinant adenoviruses were applied at a multiplicity of infection of 200–300 plaque-forming units/cell.

**Immunoprecipitation and Immunoblotting**

3T3-L1 adipocytes plated in 12-well tissue culture dishes were first incubated for 3 h in serum-free DMEM at 37 C, and then for an additional 5 min with or without 10−7 M insulin. The cells were then lysed at 4 C with ice-cold PBS (1 ml/well) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate, after which the insoluble material was removed by centrifugation at 15,000 × g for 10 min at 4 C. The cell lysates (1 ml, approximately 1 mg of total protein) were incubated with anti-IR, anti-IRS-1, or anti-IRS-2 antibody (10 μg) for 1 h and precipitated by incubation with 20 μl of protein A-Sepharose for 1 h. The resultant immunocomplexes were washed five times with the lysis buffer and boiled in 30 μl of Laemmli sample buffer containing 10 mM dithiothreitol. The proteins were resolved on 7.5% or 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). After blocking with TBS-T (10 mM Tris, pH 7.6; 150 mM NaCl; and 0.05% Tween-20) containing 3% BSA for 1 h, the membranes were incubated with the appropriate antibodies. The proteins were visualized by enhanced chemiluminescence using horseradish peroxidase-labeled antirabbit or mouse IgG (Amersham Pharmacia Biotech). In some experiments, the band intensities were quantified with a Molecular Imager GS-525 using Screen-CH (Bio-Rad Laboratories, Inc., Richmond, CA).

**Glucose Transport Assay**

3T3-L1 adipocytes in 24-well culture dishes were serum-starved overnight in DMEM containing 0.2% BSA, after which they were incubated for 45 min in glucose-free Krebs-Ringer phosphate buffer [in mM: 137 NaCl, 4.7 KCl, 10 sodium phosphate (pH 7.4), 0.5 MgCl2, and 1 CaCl2 (7)]. Basal and stimulated uptakes of 2-deoxy-D-[\text{3H}]Glucose were then measured as described previously (54).

**P3K Assay**

Isolated adipocytes were prepared, and some were stimulated with insulin as described above. The cells were then solubilized in ice-cold lysis buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 1 mM CaCl2, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 100 μM sodium orthovanadate, after which the lysates were extracted, incubated with anti-IRS-1, anti-IRS-2 or antiphosphotyrosine antibody and precipitated as described above. P3K activities in the immunoprecipitates were assayed as described previously (51).

**RNA Extraction**

Total cell RNA was prepared from 3T3-L1 adipocytes using an Isogen RNA isolation kit (Nippon Gene, Toyama, Japan). RNA concentrations were estimated based on absorbance at 260 nm; 10 μg of RNA from each sample were used for the RNase protection assays described below.

**Preparation of Riboprobes**

Riboprobes were synthesized as described previously (51). To obtain mouse IR, IRS-1, and IRS-2 cDNAs, polymerase chain reaction was performed based on the reported sequences using mouse cDNA libraries. The amplified fragments, which corresponded to nucleotides 2232–2386 of mouse IR cDNA, nucleotides 2200–2350 of mouse IRS-1 cDNA and nucleotides 3270–3420 of mouse IRS-2 cDNA were subcloned into a TA vector. The resultant plasmids were then linearized with Hind III and used for in vitro transcription.

**RNase Protection Assay**

RNase protection assays were carried out according to the manufacturer’s instructions (RPA III; Ambion, Inc., Austin,
Values of $\text{/H11006}$ statistical analysis mined using a Molecular Imager GS-525. Subjected to autoradiography; band intensities were determined using a Molecular Imager GS-525.

Statistical Analysis

Quantitative values were expressed as means $\pm$ SE. Statistical significance was tested using the Mann-Whitney’s $U$ test. Values of $P < 0.05$ were considered statistically significant.

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