Impaired glucose tolerance precedes type 2 diabetes and is characterized by hyperinsulinemia, which develops to balance peripheral insulin resistance. To gain insight into the deleterious effects of hyperinsulinemia on skeletal muscle, we studied the consequences of prolonged insulin treatment of L6 myoblasts on insulin-dependent signaling pathways. A 24-h long insulin treatment desensitized the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) and p42/p44 MAPK pathways toward a second stimulation with insulin or insulin-like growth factor-1 and led to decreased insulin-induced glucose uptake. Desensitization was correlated to a reduction in insulin receptor substrate (IRS)-1 and IRS-2 protein levels, which was reversed by the PI3K inhibitor LY294002. Co-treatment of cells with insulin and LY294002, while reducing total IRS-1 phosphorylation, increased its phosphotyrosine content, enhancing IRS-1/PI3K association. PKD1, mTOR, and MAPK inhibitors did not block insulin-induced reduction of IRS-1, suggesting that the PI3K serine-kinase activity causes IRS-1 serine phosphorylation and its commitment to proteasomal degradation. Contrarily, insulin-induced IRS-2 down-regulation occurred via a PI3K/mTOR pathway. Suppression of IRS-1/2 down-regulation by LY294002 rescued the responsiveness of PKB and MAPK toward acute insulin stimulation. Conversely, adenoviral-driven expression of constitutively active PI3K induced an insulin-independent reduction in IRS-1/2 protein levels. IRS-2 appears to be the chief molecule responsible for MAPK and PKB activation by insulin, as knockdown of IRS-2 (but not IRS-1) by RNA interference severely impaired activation of both kinases. In summary, (i) PI3K mediates insulin-induced reduction of IRS-1 by phosphorylating it while a PI3K/mTOR pathway controls insulin-induced reduction of IRS-2, (ii) in L6 cells, IRS-2 is the major adapter molecule linking the insulin receptor to activation of PKB and MAPK, (iii) the mechanism of IRS-1/2 down-regulation is different in L6 cells compared with 3T3-L1 adipocytes. In conclusion, the reduction in IRS proteins via different PI3K-mediated mechanisms contributes to the development of an insulin-resistant state in L6 myoblasts.

Type 2 diabetes is caused by a progressive decrease in insulin action and gradual development of chronic hyperglycemia. Initial peripheral insulin resistance, i.e. the failure of adipose and muscle tissues to properly dispose of circulating glucose, and the failure of liver to control glucose production, is compensated by increased insulin secretion from pancreatic β-cells, leading to hyperinsulinemia. In the long-term, β-cell failure to compensate for peripheral insulin resistance leads to type 2 diabetes (1). Hyperinsulinemia, in association with increased circulating levels of fatty acids (2), which exacerbate peripheral insulin resistance, is thought to be a major factor contributing to progression to type 2 diabetes.

Activation of the insulin receptor (IR)1 by hormone binding activates a number of cellular responses such as translocation of the glucose transporter GLUT4 to the plasma membrane in muscle and adipocytes (3), hepatic glycogen synthesis (4), lipogenesis (5), and modulation of gene expression (6).

Insulin signaling is initiated by the recruitment of intracellular molecules to the activated receptor and their ensuing tyrosine phosphorylation. These molecules include IRS-1/2/3/4, Cbl, and the adapter proteins Shc (7). Phosphorylated IRSs then engage the phosphoinositide 3-kinase (PI3K) p85/p110 heterodimer by binding the SH2 domains of the p85 adapter to specific pYMMX motifs (8). Once captured by IRS, PI3K becomes activated and produces the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate, which stimulates the serine/threonine kinase cascade PDK1-PKB/Akt-p70 S6 kinase (9). PKB/Akt and p70 S6 kinase are downstream kinases central to insulin action (10), the former controlling GLUT4 translocation, glycosylation, synthesis, and protein synthesis (11), and the latter being a key regulator of the growth promoting action of insulin (12). Other SH2 domain containing proteins, including Grb2, SHP-2, and Nck associate with IRS to mediate insulin responses. In particular, Grb2 links insulin receptor activa-

1 The abbreviations used are: IR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphoinositide 3-kinase; TPCK, N'-tosyl-L-phe-nylalanyl chloromethyl ketone; MAPK, mitogen-activated protein kinase; PKB, protein kinase B; IGF-1, insulin-like growth factor 1; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ds, double stranded; RNAi, RNA interference; siRNA, short interfering double stranded RNA; m.o.i., multiplicity of infection.

**Phosphoinositide 3-Kinase-mediated Reduction of Insulin Receptor Substrate-1/2 Protein Expression via Different Mechanisms Contributes to the Insulin-induced Desensitization of Its Signaling Pathways in L6 Muscle Cells**

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tion to the p42/p44 mitogen-activated protein kinase (MAPK) cascade (7).

The occurrence of insulin-induced peripheral insulin resistance has been attributed to impairment of the tyrosine kinase activity of the insulin receptor, based on biochemical studies performed on skeletal muscle biopsies from type 2 diabetic patients (12–15). However, these findings have been disputed by reports showing normal insulin receptor function, despite downstream metabolic abnormalities (16–18). Recently, the elucidation of the signaling pathways emanating from the activated insulin receptor (10, 19) has paved the way for several studies demonstrating that intracellular signaling molecules also substantially contribute to the insulin-resistant phenotype.

IRS-1 protein levels have been shown to be decreased in 3T3-L1 adipocytes chronically exposed to insulin (20). IRS-1 degradation is triggered by serine/threonine phosphorylation, and is blocked by the PI3K inhibitor LY294002 (21, 22). PI3K may control IRS-1 serine/threonine phosphorylation either via the action of downstream serine/threonine kinases, such as mTOR, GSK-3, and atypical protein kinase Cs, which ultimately phosphorylate IRS-1 (23, 24), or by directly phosphorylating IRS-1 via its intrinsic protein kinase activity (25, 26). Once serine/threonine phosphorylated, IRS-1 degradation occurs via the proteasome degradation pathway (27). In keeping with these studies in cultured cells, is the finding that IRS-1 protein expression is reduced in adipocytes from patients with type 2 diabetes (28) as well as in subjects suffering from insulin resistance (29). Recently, proteasome-mediated IRS-2 degradation has also been demonstrated in insulin/IGF-1-treated 3T3-L1 adipocytes, Fao hepatoma cells, and mouse embryo fibroblasts (30).

Downstream of IRS proteins, other insulin signaling molecules have been reported to be deregulated in insulin-induced insulin resistance or type 2 diabetes. PKB activation is decreased in skeletal muscle and adipose tissue from db/db mice as compared with non-diabetic controls (31). GLUT4 expression is lower in insulin-resistant individuals (32), and in L6 myotubes overexpressing the transporter GLUT4, the insulin-induced GLUT4 translocation to the plasma membrane is reduced after sustained exposure to high glucose and insulin (33). Thus, deterioration of the insulin receptor-signaling pathway at different levels accounts for the evolution from an insulin-resistant state to type 2 diabetes.

Here we evaluate the changes in insulin signaling pathways after a prolonged exposure of L6 muscle cells to insulin and we investigate the underlying molecular mechanisms. We demonstrate that, upon prolonged insulin treatment (mimicking hyperinsulinemia), both the PI3K/PKB and the MAPK signaling cascades are down-regulated and cellular levels of the two major insulin receptor docking proteins, IRS-1 and IRS-2, are decreased. Finally, we show that PI3K/PKB and MAPK down-regulation is causally related to the decrease in IRS-1/2, that PI3K is a key upstream molecule controlling IRS-1/2 degradation via different mechanisms, and that IRS-2 appears to be the functionally most relevant adapter in insulin signal transduction in L6 cells.

EXPERIMENTAL PROCEDURES

Materials—Cell culture solutions and supplements, reagents for SDS-PAGE, and Protein A-Sepharose were from Invitrogen (Carlsbad, CA). Polyvinylidene difluoride membranes for immunoblotting were from Millipore (Bedford, MA). ECL reagents, [γ-32P]ATP (> 5000 Ci/ mmol), and [32P]orthophosphate were from Amerham Biosciences (Uppsala, Sweden). Recombinant human insulin was from Novo Nordisk (Copenhagen, Denmark). IGF-1 and PDGF Bβ were from Calbiochem (La Jolla, CA). Secondary anti-mouse or anti-rabbit antibodies were from Amersham Biosciences (Copenhagen, Denmark). α-Phosphatydilinositol was from Sigma. TLC silica plates were from Merck (Darmstadt, Germany). LY294002, rapamycin, and PD98059 were from Calbiochem (La Jolla, CA) and TPCF3 was from Sigma. Other chemicals were of the highest analytical grade available.

Cell Culture—Rat L6 myoblasts were obtained from the American Type Culture Collection (ATCC, Rockville, MD). L6 were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) calf serum (FCS), penicillin, 100 units/ml, and streptomycin, 100 μg/ml (Invitrogen). Cells were seeded in 6-well plates for Western blotting experiments or in 10-cm diameter plates for immunoprecipitation experiments. 24-h insulin treatments were started at 70–80% confluence in serum-free medium.

Immunoblotting, Immunoprecipitations, PK3 Assays, and 2-Deoxyglucose Uptake—After treatment with insulin or inhibitors, cells were washed with PBS buffer A (138 mM NaCl, 3.7 mM KCl, 0.1 M glycyr, 1 mM sodium-ω-ovanate, 20 μM leupeptin, 18 μM pepstatin, 1/100 Penicillin-Nodiet P-40, 5 mM EDTA, 20 mM NaF, pH 8.0; 0.4 μl/ml plate for 6-well plates and 1 ml plate for 10-cm diameter plates). Lysates were kept on ice for 15 min and the insoluble material was removed by centrifugation at 13,000 × g for 15 min. Protein concentration was determined by the Bio-Rad colorimetric assay (Bio-Rad).

Cell lysis (10 μg of protein) were separated by SDS-PAGE, transferred to Immobilon-P polyvinylidene difluoride membranes, and blocked in 5% (w/v) bovine serum albumin. Membranes were probed with anti-phospho-Ser-473 PKB (New England Biolabs, Beverly, MA), anti-PKB (provided by B. Hemmings, FMI, Basel, Switzerland), anti-phospho-P4K-AP4P4 (anti-PKB), (Promega, Madison, WI), and anti-phospho-Ser-473 PKB (New England Biolabs, Beverly, MA), and anti-phospho-IRβ (240), anti-phospho-MAPK p42/p44 (Promega, Madison, WI), and anti-MAPK p42/p44 (Promega, Madison, WI), and anti-IRβ-subunit (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The proteins were detected by ECL after incubation of the membranes with horseradish peroxidase-conjugated secondary antibodies. Signal intensities were measured with NIH Image after scanning of non-saturated Eastman Kodak Biomax MR-1 films (Sigma).

For immunoprecipitation, lysates were incubated at 4 °C for 30 min with polyclonal antibodies to IRS-1 or to IR β-subunit, followed by addition of protein A-Sepharose and further incubation for 2 h. Immune complexes were washed twice with buffer A and then solubilized in Laemmli sample buffer. If subjected to a PK3 assay, immune complexes were washed twice with buffer A, twice with 0.1 M Tris-HCl, pH 7.4, and twice with lipid kinase buffer (20 mM HEPES, 5 mM MgCl2, pH 7.4) prior to PK3 assay, which was performed on IRS-1 immunoprecipitates as described (26). 2-Deoxyglucose uptake was measured as previously reported (34).

Orphosphate Metabolic Labeling—When at 70–80% confluence, L6 myoblasts (one 10-cm diameter plate/condition) were incubated for 24 h in 1 μCi/ml of phosphatase-free DMEM in the presence of 1 μCi/ml of [32P]orthophosphate and inhibitors as described in figure legends. Cells were labeled for the last 3 h with 0.5 μCi/ml [32P]orthophosphate and IRS-1-associated radioactivity was evaluated in IRS-1 immunoprecipitates by SDS-PAGE separation followed by phosphorimaging of the dried gel.

Generation of Recombinant Adenoviruses—Adenoviruses expressing p110α CAAX and IRS-1 were generated by homologous recombination in Escherichia coli to construct the full-length coding regions of GLUT1, PKB, and IRS-1. Hybridization was performed at 68 °C in ExpressHyb hybridization solution.
**RESULTS**

**Prolonged Insulin Treatment Desensitizes the PI3K/PKB and MAPK Signaling and Decreases Insulin-induced 2-Deoxyglucose Uptake**—To investigate the alterations in intracellular signaling caused by a prolonged insulin treatment in muscle cells, L6 myoblasts were exposed for 24 h to increasing insulin concentrations, followed or not by a second treatment with 1 mM hormone for 10 min.

To evaluate the activation of the PI3K and the MAPK signaling, IRS-1-associated PI3K activity was assayed in IRS-1 immunoprecipitates, and MAPK activation was evaluated by immunoblotting with antibodies to phospho-MAPK. Responsiveness of both PI3K and MAPK to a 10-min exposure to 1 mM insulin was diminished to a similar extent, and in a dose-dependent manner, in cells subjected to a 24-h insulin pre-treatment (Fig. 1, A and B, empty bars), indicating desensitization of the insulin signaling pathways. On the contrary, whereas an IRS-1-associated PI3K activity could be detected after the 24-h insulin treatment, no residual MAPK activity was present (Fig. 1, A and B, full bars). Thus, insulin chronically activates PI3K at a submaximal level, although MAPK activation is transient and becomes undetectable after 4 h insulin stimulation (data not shown).

To determine whether IRS-1-associated PI3K activity relays a downstream signal in intact cells, we next evaluated the activation of PKB by immunoblotting with antibodies to the active MAPK p42/p44. MAPK signal from cells not chronically treated and exposed for 10 min to 1 mM insulin was taken as a 100% (mean ± S.E., n = 3). Bottom, representative anti-pMAPK and anti-MAPK immunoblots. W2, Western blot.

**Fig. 1.** Insulin effect on PI3K and MAPK activities in L6 myoblasts after prolonged exposure to insulin. L6 myoblasts were treated with the indicated concentrations of insulin for 24 h. After 24 h, cells were exposed for 10 min to 1 mM insulin (empty bars) or left untreated (solid bars). A, top: IRS-1-associated PI3K activity was measured in anti-IRS-1 immunoprecipitates using phosphatidylinositol as a substrate (see “Experimental Procedures”; mean ± S.E., n = 3). PI3K activity from cells not pretreated with insulin but exposed for 10 min to 1 mM insulin was taken as a 100%. Bottom, representative anti-p85α immunoblot on IRS-1 immunoprecipitates (IP) showing IRS-1-associated PI3K. B, top: MAPK activation was evaluated by immunoblot analysis on cell lysates with antibodies to active MAPK p42/p44. MAPK signal from cells not chronically treated and exposed for 10 min to 1 mM insulin was taken as a 100% (mean ± S.E., n = 3). Bottom, representative anti-pMAPK and anti-MAPK immunoblots. W2, Western blot.
observed in PI3K/PKB and MAPK signaling was because of insulin-induced IR degradation we quantified the total amount of IR in starved or 24-h insulin-treated L6 myoblasts by immuno blotting with antibodies to IR. As already reported in myotubes (33), the IR levels and insulin-induced IR tyrosine phosphorylation decreased by 50% as compared with untreated cells following a 24-h exposure to 1 μM insulin (Fig. 3). Given that low receptor occupancy can be sufficient to relay a biological effect (39), deregulation of intracellular component(s) of the insulin signaling cascade might also contribute to the observed down-regulation.

Insulin/IGF-1-treated L6 Cells Retain Responsiveness to Activation by FCS, and PDGF Pretreatment Does Not Down-regulate Insulin Signaling—L6 myoblasts express both insulin and IGF-I receptors with a prevalence of IGF-1 receptors (40). Thus, the insulin receptor mainly exists as a hybrid (α/β' subunit) with the IGF-I receptor. To evaluate whether L6 myoblasts respond similarly to a prolonged insulin or IGF-I pretreatment, cells were stimulated with increasing concentrations of either insulin (0–10³ nM range) or IGF-I (0–10⁴ nM range) for 24 h, followed by a 10-min treatment with either 1 μM insulin or 100 nM IGF-I. We observed a down-regulation of PKB and MAPK after prolonged treatment, irrespective of the ligand used for the second exposure (insulin or IGF-I, Fig. 4A). Likewise, a 24-h pretreatment with IGF-I induced a desensitization of PKB and MAPK when cells were subsequently exposed for a further 10 min to either insulin or IGF-I (Fig. 4B). Given the presence of insulin/IGF-1 hybrid receptors in L6 cells and the responsiveness of IGF-1R to insulin at high concentrations, the 24-h exposure to insulin at the highest concentration (i.e. 10³ nM) might lead to PKB and MAPK down-regulation by acting on both receptors. Nevertheless, PKB and MAPK down-regulation was already occurring upon 24 h exposure to 10–10² nM insulin. Time-dependent induction of PKB and MAPK down-regulation was visualized by immunoblotting with antibodies to pPKB Ser-473 and pMAPK. Representative immunoblots from three independent experiments are shown.

Insulin Signaling Desensitization in L6 Cells

**Fig. 2.** Activation of PI3K downstream effector PKB, kinetics of PKB and MAPK down-regulation and [³H]2-deoxyglucose uptake after prolonged exposure to insulin. L6 myoblasts were treated for 24 h with increasing insulin concentrations as indicated. After 24 h, cells were exposed for 10 min to 1 μM insulin. PKB activation was evaluated by immunoblot analysis with antibodies to active PKB. [³H]2-Deoxyglucose uptake was measured as described under “Experimental Procedures.” A, quantification of PKB activation. Empty bars, 10 min exposure to 1 μM insulin; full bars, no second insulin exposure (mean ± S.E., n = 3). PKB activation from cells not chronically treated and exposed for 10 min to 1 μM insulin was taken as 100%. B, representative anti-pPKB immunoblot for the data shown in A. C, time course-dependent down-regulation of PKB and MAPK. L6 myoblasts were exposed for 2, 6, and 18 h to increasing insulin concentrations as indicated. Cells were then left untreated or exposed for 10 min to 1 μM insulin. Time-dependent induction of PKB and MAPK down-regulation was visualized by immunoblotting with antibodies to pPKB Ser-473 and pMAPK. Representative immunoblots from three independent experiments are shown. D, [³H]2-deoxyglucose uptake after 10 min exposure to 1 μM insulin is expressed relative to cells not subjected to 10 min exposure to insulin. (Mean ± S.E. is from three independent experiments. In each independent experiment, triplicate measurements yielded a S.E. < 6% of the mean values.)
nm insulin (with no or little interference with IGF-1R activation) and to 1–10 nm IGF-1 (with no or little interference with IR activation, Fig. 4, A and B). Given that (i) down-regulation is already caused by exposure to low insulin/IGF-1 concentrations, and (ii) prolonged exposure to insulin diminishes acute IGF-1 signaling and vice versa, we suggest that a down-regulation event takes place at a postreceptor level.

Whereas cross-reactivity occurs between insulin and IGF-1 toward their corresponding receptors, FCS and PDGF are not known to interfere with IR or IGF-1R. Consistent with this, MAPK activation by FCS was sustained after a 24-h insulin/IGF-1 exposure (Fig. 4C), indicating that, after prolonged insulin/IGF-1 exposure, the PI3K/PDK-1 and Raf-MEK signaling modules remain responsive to treatment by agonists other than insulin/IGF-1. Because PKB and MAPK are targets of virtually all activated receptor-tyrosine kinases, we evaluated whether...
Insulin signaling down-regulation is specifically achieved by insulin/IGF-1 pre-exposure or if it could also be caused by prolonged exposure to an unrelated receptor-tyrosine kinase agonist. When we exposed cells for 24 h to PDGF and subsequently treated acutely with either insulin or PDGF, we observed homologous desensitization of PKB and MAPK (complete and ~50%, respectively) after PDGF treatment, but no down-regulation of insulin signaling (Fig. 4D). This indicates that down-regulation of insulin signaling strictly depends on a prior perturbation of the proximal insulin/IGF-1 receptor signaling pathway.

Desensitization of Insulin Signaling Is Associated with a PI3K-dependent Decrease in IRS-1 and IRS-2 Protein Levels—Previous studies on Zucker fatty rats and ob/ob mice, an hyperinsulemic and insulin-resistant rodent models, respectively, show decreased IRS-1 expression levels in skeletal muscle (41, 42). Likewise, IRS-1 was found to be down-regulated in adipose tissue from diabetic patients (28) and in 3T3-L1 adipocytes treated chronically with insulin (43).

Therefore, we evaluated the protein levels of IRS-1 and IRS-2 in 24-h insulin-treated L6 myoblasts. Both IRS-1 and IRS-2 decreased after a 24-h exposure to insulin in a dose-dependent manner, reaching 30% of untreated controls with 1 μM insulin (Fig. 5, A and B). The decrease in IRS-1 was post-transcriptional, because we did not observe decreased expression of the IRS-1 mRNA after insulin treatment, although GLUT-1 mRNA was induced, confirming the insulin-induced transcriptional effects (Fig. 5C (44)). We also evaluated the protein levels of p42/p44 MAPK and PKB. p42/p44 expression was unaltered by prolonged insulin treatment (Fig. 5A). On the contrary, PKB expression increased up to 2.4-fold in a concentration-dependent manner (Fig. 5, A and B).

Insulin-induced degradation of IRS-1 is mediated by the proteasome degradation pathway (27, 45) following serine/threonine phosphorylation (46). Using pharmacological inhibitors, we attempted to determine which kinase(s) might lead to IRS-1 serine/threonine phosphorylation. Co-treatment of cells with insulin and LY294002 resulted in the inhibition of IRS-1 degradation and in a lowering of its apparent molecular mass, indicating a decreased serine/threonine phosphorylation. Likewise, LY294002 prevented insulin-induced IRS-2 degradation (Fig. 6A). The inhibitory action of LY294002 throughout the 24-h treatment was confirmed by immunoblot analysis with antibodies against active PKB (Fig. 6B). That LY294002 blocks insulin-induced IRS-1 down-regulation indicates that PI3K regulates IRS-1 serine/threonine phosphorylation, either directly, or via activation of downstream kinase(s) and/or inhibition of phosphatases. To assess the role of PI3K-activated kinases in regulating IRS-1 down-regulation, 24-h insulin-stimulated L6 cells were simultaneously treated with the anti-proliferative agent TPCX, which inhibits both PKB and p70 S6 kinase by disrupting PDK1 signaling (47). In contrast to LY294002, TPCX, although inhibiting both PKB and p70 S6 kinase activation (Fig. 6C), was ineffective in blocking insulin-induced IRS-1 down-regulation (Fig. 6C).

To evaluate the potential role of p42/p44 MAPK and mTOR, which have been reported to phosphorylate IRS-1 (24, 48), we incubated L6 myoblasts with or without 1 μM insulin for 24 h in the presence of either 50 μM LY294002, 50 nM rapamycin or 10 μM PD98059. In the absence of insulin, LY294002, but not rapamycin or PD98059, induced a mobility shift toward a lower apparent molecular mass, indicating that basal IRS-1 phosphorylation is chiefly controlled by PI3K. Moreover, LY294002, but not rapamycin or PD98059, blocked IRS-1 degradation (Fig. 6D). On the contrary, treatment of cells with either LY294002 or rapamycin, irrespective of insulin co-treatment, led to an induction of IRS-2, indicating that a basal activity of the PI3K-mTOR pathway suffices to trigger a constitutive IRS-2 degradation pathway. This observation is in agreement with the work of Simpson et al. (49) who, by overexpressing the phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase PTEN in PTEN-null breast cancer cells, observed a feedback up-regulation of IRS-2 mRNA and protein level.

To gain further insight into the kinase(s) involved in insulin-induced IRS-1 degradation, 24-h insulin-treated L6 cells were labeled with [32P]orthophosphate in the presence of the above inhibitors and IRS-1 phosphorylation was quantitated in IRS-1 immunoprecipitates. LY294002 and rapamycin, but not PD98059, induced a decrease in IRS-1 phosphorylation (Fig. 7A, left). Because LY294002 (but not rapamycin) also inhibited the insulin-induced IRS-1 decrease (Fig. 6, A and D) it is PI3K inhibition that yields the maximal inhibitory effect on IRS-1 phosphorylation (Fig. 7A). Moreover, in 24-h insulin-treated L6 cells, LY294002 addition (but not addition of other protein kinase inhibitors nor of MG132, a proteasome inhibitor, see legend to Fig. 7) resulted in increased IRS-1 tyrosine phospho...
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FIG. 7. In 24-h insulin-treated cells, LY294002 attenuates IRS-1 phosphorylation, increases IRS-1 phosphotyrosine content, and increases IRS-1-associated p85α. A, left: IRS-1 was immunoprecipitated from 32P-labeled L6 myoblasts treated for 24 h with insulin in the presence of 0.1% (v/v) MeSO (−), 50 μM LY294002 (LY), 50 nM rapamycin (R), or 10 μM PD98059 (PD) as indicated. The IRS-1-associated radioactivity was visualized by phosphorimaging after separation with a 7.5% SDS-PAGE. Right, IRS-1 relative phosphorylation was calculated by dividing the incorporated radioactivity by the IRS-1 expression level (C, α-IRS-1 immunoblot, lanes 5–8). A result representative of two independent experiments is shown. B, IRS-1 was immunoprecipitated from L6 myoblasts treated with insulin in the presence of 0.1% (v/v) MeSO (−), 50 μM LY294002 (LY), 50 nM rapamycin (R), 10 μM PD98059 (PD), 200 nM GF109 (GF), or 10 μM MG132 (MG) as indicated. IRS-1 tyrosine phosphorylation (upper blot) and IRS-1-associated PI3K adapter p85α (lower blot) are shown. A representative blot of four independent experiments is shown.

FIG. 6. Differential effects of LY294002, TPCK, rapamycin, and PD98059 on IRS-1 and IRS-2 insulin-induced down-regulation. A, L6 myoblasts were incubated for 24 h with increasing insulin concentrations in the presence of 50 μM LY294002 (+) or 0.1% (v/v) MeSO (−). IRS-1 and IRS-2 expression levels were evaluated in total cell lysates by immunoblotting with the respective antibodies. B, immunoblotting with antibodies against pPKB confirmed the inhibitory effect of LY294002 on the PI3K/PKB signaling pathway. Cells were incubated for 24 h with insulin and LY294002 as indicated and subjected to a further 10 min stimulation with 1 μM insulin before lysis. C, L6 myoblasts were incubated for 24 h without or with 1 μM insulin in the presence of 0.1% (v/v) MeSO (−), 5 μM or 17 μM TPCK, 50 μM LY294002 (LY), or 50 nM rapamycin (R) as indicated. The IRS-1 expression level was evaluated by immunoblotting with antibodies to IRS-1. The inhibitory effect of TPCK, LY, and R was verified by band shift analysis of p70 S6 kinase as well as by immunoblotting to pPKB. Equal loading is demonstrated by immunoblotting to total PKB (performed after strip of the immunoblot against the phosphorylated form). D, L6 myoblasts were incubated for 24 h without or with 1 μM insulin in the presence of 0.1% (v/v) MeSO (−), 50 μM LY294002 (LY), 50 nM rapamycin (R), or 10 μM PD98059 (PD) as indicated. IRS-1 and IRS-2 expression levels were evaluated by immunoblotting of total lysates. The dots indicate cross-reactive bands. The immunoblots shown are representative of at least two independent experiments.

rulation and increased IRS-1-associated p85α (Fig. 7B). Thus, although PI3K inhibition during a 24-h insulin treatment blocks phosphatidylinositol 3,4,5-trisphosphate-mediated downstream signaling in L6, the IRS-1/PI3K complex remains potentially active as demonstrated by increased IRS-1/PI3K association, higher IRS-1 tyrosine phosphorylation, and lower IRS-1 serine/threonine phosphorylation level.

To confirm the role of PI3K in controlling IRS-1/2 down-regulation, L6 myoblasts were infected at increasing multiplicity of infection with adenovirus expressing a constitutively active PI3K catalytic subunit (p110CAAX wt) or, as a control, a kinase-dead mutant (p110CAAX KR, Fig. 8A). Expression of p110CAAX wt (but not KR) activated the PI3K downstream targets PKB (Fig. 8B) and p70 S6 kinase (not shown) (50), and induced a decrease in IRS-1 and IRS-2 protein expression, thus confirming that IRS-1/2 down-regulation can be induced in a receptor-independent manner and is controlled by PI3K.

Insulin-induced Desensitization of the PI3K/PKB and MAPK Pathways Is Reversed by a Blockade of IRS-1/2 Down-regulation—To demonstrate a direct link between desensitization of insulin signaling upon prolonged exposure to insulin and decreased levels of IRS molecules, L6 myoblasts were exposed for 24 h to insulin in the presence of 50 μM LY294002, thus inhibiting down-regulation of IRS1/2. After 24 h, the inhibitor was removed and the cells were stimulated with 1 μM insulin, as indicated in Fig. 9. A 24 h pretreatment with 10^3 nM insulin down-regulated PKB and MAPK (Fig. 9, lanes 1–4; see also Figs. 1 and 2). However, PKB could be maximally activated by a 10-min exposure to 1 μM insulin after a 24-h incubation with...
Fig. 8. Expression of a catalytically active p110κ CAAX down-regulates IRS-1 and IRS-2 proteins. A, L6 myoblasts were infected with adenovirus expressing p110κ CAAX wt and kinase dead (KR). Transcription of the p110κ CAAX mRNA was visualized by Northern blot. B, L6 myoblasts were incubated overnight in DMEM without serum and with increasing multiplicity of infection of adenovirus expressing p110κ CAAX wild type (wt) or kinase-dead. The cells were then kept in DMEM, 10% FCS (v/v) for 36 h followed by overnight starvation prior to lysis and immunoblotting with antibodies to IRS-1, IRS-2, and active PKB (phospho-Ser-473) as indicated. The blots shown are representative of two independent experiments. C, quantification of IRS-1 and IRS-2 expression levels in uninfected cells and infected cells infected at a m.o.i. of 100 (mean ± S.E., n = 3).

10^2 or 10^3 nM hormone, provided that LY294002 was added (Fig. 9). Likewise, a transient maximal activation of MAPK could be induced after 24 h treatment with 10^2 nM insulin and LY294002, and a lower activation could still be observed when 10^3 nM insulin was administered. The concomitant occurrence of (i) inhibition of IRS-1/2 down-regulation by PI3K inhibition, and (ii) activation of PKB and MAPK by acute insulin stimulation after prolonged insulin/LY294002 treatment, indicates that the desensitization of insulin signaling depends on the decreased protein levels of IRS-1/2 proteins.

RNAi-mediated Knockdown Establishes That IRS-2 Is the Major Adapter Accounting for Insulin-induced Desensitization of the PI3K/PKB and MAPK in L6 Cells—To elucidate the relative contribution that the decrease in IRS-1 versus IRS-2 plays in the down-regulation of insulin signaling, we took advantage of the recently described RNAi approach, whereby a protein can be selectively knocked down by transfection of siRNA (51). Double stranded siRNAs designed to selectively knockdown IRS-1 or IRS-2 in L6 cells reduced their expression level by >80% (Fig. 10B). Insulin stimulation of cells knocked down for IRS-1 led to an activation of PKB and MAPK comparable with that of cells not exposed to siRNA. On the contrary, elimination of IRS-2 rendered both PKB and MAPK almost unresponsive to insulin, indicating that the major adapter molecule linking the activated insulin receptor to downstream PKB and MAPK activation is IRS-2 in L6 cells (Fig. 10A). Because by knockdown of IRS-2, we observed a decrease in IRS-1 levels (Fig. 10B) it could not be ruled out that the impaired activation of PKB and MAPK was because of the concomitant decrease in IRS-1. To test whether physiological levels of IRS-1 may overcome the lack of IRS-2, L6 cells in which RNAi to IRS-2 had been performed were infected at a 10–150 m.o.i. of an adenovirus expressing IRS-1. Under these experimental conditions, IRS-1 expression was restored, with over-expression at the highest multiplicity of infection. However, upon insulin stimulation no rescue of either PKB or MAPK activities was observed (Fig. 10C), indicating that even a fully functional IRS-1 cannot overcome, for these biological responses, the absence of IRS-2 in L6 cells.

**DISCUSSION**

Insulin resistance results from the inability of insulin-responsive tissues to respond properly to the hormone. This state is associated with hyperinsulinemia, and when increased insulin secretion becomes insufficient to maintain glucose homeostasis, chronic hyperglycemia and type 2 diabetes occur.

To investigate the molecular alterations induced by hyperinsulinemia in muscle cells, we treated L6 cells for 24 h with different hormone concentrations followed by a second 10-min acute hormone treatment. These conditions mimic a hyperinsulinemic state, whereby a temporarily increased insulin secretion is superimposed upon an already elevated insulin level.

In this system, PI3K recruitment to IRS-1 and activation of PI3K/PKB and MAPK pathways by acute insulin stimulation were reduced in a dose-dependent manner by the preceding 24-h insulin treatment, with a 50% down-regulation of both pathways at 10^4 nM hormone pretreatment. The responses of the two pathways to the sole 24-h insulin exposure differed, MAPK activity being absent and the PI3K/PKB activation reaching a submaximal level (30–50% of the maximal insulin-stimulated activity) at all hormone concentrations tested. The 24-h insulin-induced down-regulation of PI3K/PKB resulted in...
a decreased acute insulin-induced $[3H]$2-deoxyglucose uptake. This reduction is likely to be because of a defective insulin-induced GLUT4 transloca
tion at the plasma membrane (33).

Given the debate as to whether down-regulation of insulin receptor number and/or reduced kinase activity is responsible for determining the insulin-resistant state (see Introduction), we evaluated the amount and extent of tyrosine phosphorylation of the insulin receptor (Fig. 3). Blotting of immunoprecipi
tated insulin receptors with antibodies to the receptor demonstrated a reduction of $\sim50\%$ in both receptor content and receptor tyrosine phosphorylation after a 24-h exposure to in
sulin. Given that low receptor occupancy and activation can relay a biological signal, we investigated whether intracellular downstream targets might also be involved in the down-regula
tion of insulin-induced intracellular responses.

L6 cells possess a higher number of IGF-1 receptor molecules than IR molecules (52). Therefore, because of the presence of a high proportion of IR/IGF-1R hybrid receptors, the reciprocal effects of insulin and IGF-1 on PKB and MAPK were tested by prolonged incubation with insulin or IGF-1 followed by a sec
donate exposure to either insulin or IGF-1. We observed PKB and MAPK down-regulation after acute IGF-1 or insulin treatment, irrespective of the agonist used in the 24-h pretreatment. This suggests that heterologous desensitization occurs between insulin and IGF-1. On the contrary, FCS could still activate MAPK after a prolonged insulin treatment (Fig. 4C). These observations imply that the PI3K/PKB and MAPK down-regu
eration depends on a signaling event downstream of, but proximal to, the insulin/IGF-1 receptor.

Several post-receptor defects in insulin resistance have been reported, including proteasome-mediated degradation of IRS-1, decreased IRS-2 mRNA levels, decreased GLUT4 protein expression levels (27, 30, 32, 53), as well as desensitization of IGF-1 and lysophosphatidic acid-mediated MAPK activation via $\beta$-arrestin-1 down-regulation (54). Thus, we tested whether the insulin-induced insulin signaling down-regulation might depend on alterations in the levels of IRS molecules and/or the downstream kinases PKB and MAPK. We observed an insulin
induced down-regulation of IRS protein levels that parallels the down-regulation of IRS-1 and IRS-2. IRS-1 down-regulation could be blocked by PI3K inhibition with LY294002, but not by PDK1, mTOR, or MAPK inhibition. Of particular interest is the observation that insulin-induced IRS-1 degrada
tion was not blocked by T92, an inhibitor of PDK1 action. As P3K-activated PDK1 phosphorylates and activates several downstream kinases, including PKB, p70 S6 kinase, SGK, RSK, and atypical protein kinase Cs (55), the involvement of these kinases in IRS-1 phosphorylation in L6 cells can be excluded.

In 3T3-L1 adipocytes, proteasome-mediated IRS-1 degrada
tion is controlled by a rapamycin-sensitive pathway (46). On the contrary, in our system as well as in Chinese hamster ovary/IR/IRS-1 cells (45), insulin-induced IRS-1 degradation was not affected by rapamycin, indicating that kinases up

![Fig. 10. IRS-2 is the adapter accounting for insulin-induced activation of PKB and MAPK in L6 cells. IRS-1, IRS-2, or both were knocked down by RNA interference. RNAi was performed by calcium phosphate transfection of dsRNAs. 24 h post-transfection, cells were starved overnight and treated or not for 10 min with 1 $\mu$M insulin as indicated prior to cell lysis. A, cell lysates were then subjected to immunoblotting with antibodies to IRS-1, IRS-2, PKB Ser-473, and active MAPK as indicated. Equal loading is demonstrated by immuno

blotting to total MAPK, total PKB (performed after stripping the immunoblot against the phosphorylated form), and p85 PI3K adapter subunit. The blots shown are representative of at least four independ
ten ones. B, quantification of the expression level of IRS-1 (left graph) and IRS-2 (right graph) following RNAi to IRS-1, IRS-2, and IRS-1 + IRS-2. Expression levels with no addition of siRNA is taken as 100% (mean $\pm$ S.E., n = 4). C, to assess the relevance of the partial decrease of IRS-1 following RNAi to IRS-2, after knockdown of IRS-2 cells were infected by an adenovirus expressing IRS-1 (Ad IRS-1) at the indicated multiplicity of infection. After overnight starvation, cells were left un
untreated or treated with 1 $\mu$M insulin for 10 min. Immunoblotting with antibodies to IRS-1, IRS-2, PKB phospho-Ser-473, and active MAPK was performed as indicated.
stream of mTOR, possibly PI3K itself, or kinases independent of mTOR are required in L6 and Chinese hamster ovary cells to elicit IRS-1 degradation. Similar to IRS-1, IRS-2 levels were decreased upon prolonged insulin treatment in a dose-dependent manner. In contrast to IRS-1 down-regulation, which was only dependent on PI3K, both LY294002 and rapamycin blocked insulin-induced IRS-2 down-regulation. Furthermore, exposure of starving cells to LY294002 or rapamycin induced an increase in the IRS-2 expression level (without affecting IRS-1 expression), thus a basal PI3K activity suffices to mediate IRS-2 down-regulation, via mTOR. Together, the above observations suggest that PI3K is the chief molecule controlling insulin-induced degradation of both IRS-1 and IRS-2. Whereas insulin-dependent regulation of IRS-2 levels occurs in part by transcriptional repression of the IRS-2 gene (56), we found that 32P labeling of insulin-treated L6 cells, in the presence of different inhibitors (LY294002, PD98059, or rapamycin), resulted in a minimal 32P incorporation into IRS-1, yet a higher tyrosine phosphorylation, only in LY294002-treated cells. This indicates that the PI3K serine kinase activity directly phosphorylates IRS-1 on serine/threonine residues, provoking its degradation. In support of this is our observation that immunoprecipitated IRS-1 from insulin-treated L6 is phosphorylated in an in vitro kinase assay in a PI3K-dependent manner. On the contrary, although mTOR inhibition caused approximately a 50% reduction in IRS-1 32P incorporation, this was not sufficient to prevent IRS-1 from being degraded, indicating that mTOR-dependent phosphorylation sites do not participate (or do not suffice for) in IRS-1 degradation.

We obtained further confirmation of the involvement of PI3K in IRS-1/2 down-regulation by expressing an agonist-independent, constitutively active PI3K consisting of the catalytic subunit p110α fused at its C terminus to the farnesylation signal of H-Ras. This moiety targets the protein to the plasma membrane (p110α CAAX), activating it (57). In the absence of insulin, expression of p110α CAAX wild type (but not kinase-dead p110α CAAX K502R) was sufficient to activate PKB, in a multiplicity of infection-dependent manner and induced a decrease in both IRS-1 and IRS-2 as observed in 3T3-L1 adipocytes (58). To causally link insulin-induced down-regulation of PI3K/ PKB and MAPK pathways and degradation of IRS proteins, we explored the possibility of blocking insulin-induced IRS degradation by inhibiting PI3K. After a 24-h treatment with insulin in the presence of LY294002, IRS-1 and IRS-2 expression levels were preserved. Subsequent insulin stimulation (in freshly changed medium to remove LY294002 and recover PI3K activation) restored PKB stimulation and greatly improved MAPK responsiveness after the 102 nM insulin treatment. We were unable to significantly regain MAPK activation after 103 nM insulin treatment. This is likely because of the fact that at high concentrations insulin induces a persistent dissociation of the Grb2/SOS complex, which can only be reversed by insulin withdrawal (59) and might also explain the more rapid kinetics of MAPK down-regulation as compared with PKB shown in Fig. 2C. Having demonstrated the involvement IRS-1 and -2 down-regulation we next sought to determine the relative contribution of each isoform by selective RNA interference knockdown of each isoform. Whereas IRS-1 knockdown had no effect on the insulin-stimulated activation of PKB and MAPK, we found that knockdown of IRS-2 induced an almost total loss of insulin activation of PKB and MAPK, thus defining IRS-2 as the chief molecule directing insulin downstream signaling, at least in L6 cells.

In summary, our data indicate that prolonged insulin treatment of L6 muscle cells mimics the effects of hyperinsulinemia, leading to down-regulation of both PI3K/PKB and MAPK signaling pathways and glucose uptake via a decrease in IRS-1/2 docking molecules. We also show that PI3K is the key molecule controlling the decrease in IRS-1/2 as down-regulation of IRS-1/2 is effectively prevented by PI3K inhibition but induced by expression of a constitutively active PI3K. Finally, we provide evidence for the existence of a causal link between PI3K-elicited IRS-1/2 decrease and down-regulation of the PI3K/PKB and MAPK pathways, with a prominent role played by IRS-2 as shown by RNAi experiments. Our data suggest that, in L6 cells, insulin-induced degradation of IRS molecules is driven by distinct mechanisms. Indeed, for IRS-1 a PI3K-dependent pathway, with PI3K itself acting as a IRS-1 serine/threonine kinase, appears to be involved, whereas IRS-2 degradation is controlled by a PI3K-mTOR-dependent mechanism. Moreover, tissue-specific mechanisms may control IRS degradation, as IRS-1 degradation in adipocytes appears to be mTOR-dependent (46) but mTOR-independent in L6 and Chinese hamster ovary/IR/IRS cells (45). Because degradation of IRS proteins is promoted by serine/threonine phosphorylation (21) and provides a molecular link to insulin resistance (60), it will be of importance to determine next the relative contribution of each serine/threonine kinase among the several proposed, not the least PI3K itself, toward IRS serine/threonine phosphorylation.

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Phosphoinositide 3-Kinase-mediated Reduction of Insulin Receptor Substrate-1/2 Protein Expression via Different Mechanisms Contributes to the Insulin-induced Desensitization of Its Signaling Pathways in L6 Muscle Cells

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