Insulin Activates RSK (p90 Ribosomal S6 Kinase) to Trigger a New Negative Feedback Loop That Regulates Insulin Signaling for Glucose Metabolism*

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Background: RSK is a downstream effector of the Ras/ERK pathway and shares high homology with S6K.

Results: RSK1 inhibition improved insulin signaling and insulin action on glucose metabolism in myotubes and hepatocytes by preventing IRS-1 Ser-1101 phosphorylation.

Conclusion: RSK1 promotes insulin resistance by phosphorylating IRS-1 Ser-1101.

Significance: RSK1 might be a new mediator of insulin resistance in hyperinsulinemia or diabetic states.

We previously demonstrated that the mTORC1/S6K1 pathway is activated by insulin and nutrient overload (e.g., amino acids (AA)), which leads to the inhibition of the PI3K/Akt pathway via the inhibitory serine phosphorylation of IRS-1, notably on serine 1101 (Ser-1101). However, even in the absence of AA, insulin can still promote IRS-1 Ser-1101 phosphorylation by other kinases that remain to be fully characterized. Here, we describe a new negative regulator of IRS-1, the p90 ribosomal S6 kinase (RSK). Computational analyses revealed that Ser-1101 within IRS-1 falls into the consensus motif of RSK. Moreover, recombinant RSK phosphorylated IRS-1 C-terminal fragment on Ser-1101, which was prevented by mutations of this site or when a kinase-inactive mutant of RSK was used. Using antibodies directed toward the phosphorylation sites located in the activation segment of RSK (Ser-221 or Ser-380), we found that insulin activates RSK in L6 myocytes in the absence of AA overload. Inhibition of RSK by using either the pharmacological inhibitor BI-D1870 or after adenoviral expression of a dominant negative RSK1 mutant (RSK1-DN) showed that RSK selectively phosphorylates IRS-1 on Ser-1101. Accordingly, expression of the RSK1-DN mutant in L6 myocytes and FAO hepatic cells improved insulin action on glucose uptake and glucose production, respectively. Furthermore, RSK1 inhibition prevented insulin resistance in L6 myocytes chronically exposed to high glucose and high insulin. These results show that RSK is a novel regulator of insulin signaling and glucose metabolism and a potential mediator of insulin resistance, notably through the negative phosphorylation of IRS-1 on Ser-1101.

The insulin receptor substrate-1 (IRS-1)5 has been recognized as an insulin signaling nexus and, as such, a master regulator of insulin sensitivity as it integrates signals from many signaling pathways. On one hand, activation of the insulin receptor will phosphorylate IRS-1 on tyrosine (Tyr) residues, promoting the metabolic effects of insulin, mostly through the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. On the other hand, IRS-1 can be phosphorylated on serine residues (Ser) to uncouple PI3K from IRS-1, which down-regulates insulin signaling through the PI3K/AKT pathway (1,2). Several Ser/Thr kinases have been found to phosphorylate multiple Ser residues in IRS-1 in response to growth factors or inflammatory stimuli. These include the mammalian target of rapamycin (mTOR) and the p70 S6 kinase (S6K1) as well as the c-Jun NH2-terminal kinase (JNK) and the inhibitor of nuclear factor-κB (IKK) (reviewed in Refs. 2, 3). We first showed that prolonged insulin stimulation combined with nutrient overload, such as an excess of amino acids (AA), promotes serine phosphorylation of IRS-1 through chronic activation of the rapamycin-sensitive mTOR complex 1-S6K1 (mTORC1/S6K1) (4). Subsequent studies revealed that mTORC1 and/or S6K1 phosphorylate specific serine residues on IRS proteins (rodent/human) such as Ser-265/270 (5), Ser-302/307 (6), Ser-307/312 (7), Ser-632/636 (8), and Ser-1097/1101 (9). Moreover, some of these IRS-1 sites, notably Ser-1101 and Ser-636, were differentially regulated by mTOR and S6K1 (10, 11). We have further reported that Ser-1101 was an important target of S6K1 and that S6K1 overactivation contributes to Ser-1101 hyperphosphorylation in liver and muscle of several

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5 The abbreviations used are: IRG, insulin receptor substrate; 2-DG, 2-deoxyglucose; AA, amino acid; RSK, p90 ribosomal S6 kinase; mTOR, mammalian target of rapamycin; S6K1, p70 S6 kinase; CTKD, C-terminal kinase domains; NTKD, N-terminal kinase domains.
animal models of obesity as well as human muscle during nutrient excess (9). However, we have previously noted that rapamycin-insensitive kinases are also involved in the insulin-dependent serine phosphorylation of IRS-1 in the absence of nutrient overload (4). The identity of potential serine kinase(s) and whether Ser-1101 could be one of their potential targets is still unknown.

The p90 ribosomal S6 kinase (RSK) constitutes a family of four members that are activated by the ERK-MAP kinase pathway, where RSK1–3 are the most abundant and are ubiquitously expressed, whereas RSK4 is weakly expressed in a few tissues (12, 13). The structure of RSK is comprised of two kinase domains at the N- and C-terminal kinase domains (NTKD and CKTD, respectively), interspaced by a linker region (14, 15). RSK is activated through a complex process in which ERK first phosphorylates both threonine 573 (Thr) of the CKTD and Thr-359/Ser-363 in the linker region. The CKTD then auto-phosphorylates Ser-380 in the linker region to promote recruitment of phosphoinositide-dependent kinase-1 (PDK1) which in turn phosphorylates Ser-221 of the NTKD which then becomes capable of phosphorylating its substrates (16). The biological role of RSK, based on the identity of its known substrates, is mostly ascribed to regulation of development, cell motility, and survival, as well as cell proliferation and growth (16–18). RSK promotes cell growth by directly regulating protein synthesis (19) or via its ability to modulate the mTORC1 pathway (17, 20, 21). Moreover, the NTKD is similar to S6K1 with 57% amino acid identity, suggesting that RSK could potentially phosphorylate similar substrates (13). However, whether RSK may be implicated in the regulation of insulin sensitivity and glucose homeostasis remains unexplored.

Here, we describe a new negative feedback loop whereby insulin activates RSK, which promotes IRS-1 phosphorylation on Ser-1101, independently of the mTOR/S6K1 pathway. This in turn restricts insulin signaling and glucose metabolism in skeletal muscle and hepatic cells.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—DMSO, fish gelatin, and rapamycin were from Sigma-Aldrich. BI-D1870 was from the MSI/WTB complex at the University of Dundee, UK. PF-4708671 was from Symansis (Timaru, NZ). The insulin was from Eli Lilly (Toronto, ON, Canada). The anti-IRS1, anti-G6Pase, anti-PGC1α, and anti-RSK antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-phospho-RSK Ser-221 and Ser-380 were from Abcam (Cambridge, MA). The anti-phospho-IRS-1 Ser-1101 and Ser-636/9; the anti-phospho-S6K1 Thr-389, the anti-phospho-GSK3 Ser-21/9, the anti-GSK3, the anti-phospho-Foxo Ser-256, the anti-Foxo, the anti-phospho-mtor Ser-2448, and the anti-AKT Ser-473 were from Cell Signaling Technologies (Danvers, MA). The anti-tubulin was from Sigma-Aldrich. The p85 antibody was from Millipore (San Francisco, CA). The anti-PEPCK was from Cayman Chemical (Ann Arbor, MI). The RSK1 dominant negative (RSK1-DN) and LacZ adenoviruses were already described (22).

**Cell Culture**—L6 myoblasts were grown in α-MEM (Invitrogen) supplemented with 10% FBS and differentiated into myotubes in α-MEM with 2% FBS as previously described (27). HepG2 cells were grown in DMEM (Invitrogen). L6 and HepG2 cells were serum deprived for 4 h prior to the experiment, and 100 nM insulin was used to stimulate the cells during the last hour of deprivation. FAO hepatocytes were maintained in RPMI media (Invitrogen). FAO cells were serum-deprived overnight and stimulated with the indicated concentration of insulin.

**Western Analyses**—Western blots were performed as described (23). Briefly, equal amounts of protein were separated by SDS–PAGE (9%) and transferred onto nitrocellulose membrane. Membranes were blocked in 5% fish gelatin diluted in PBS, pH 7.4, 0.1% Tween (PBS-T), and incubated overnight with the respective antibodies diluted in 1% fish gelatin in PBS-T. Immunoprecipitations were performed as described with minor modifications (24). Total cells lysates (500 μg) were pre-cleared with protein A-Sepharose and immunoprecipitated with the relevant antibodies coupled to protein A-Sepharose.

2-Deoxyglucose (2-DG)—2-DG uptake procedures were used as previously described (25). In brief, cells were incubated for 8 min in HEPES-buffered saline containing 10 μM unlabeled 2-DG and 10 μM 2-deoxy-[3H]glucose (0.5 μCi/ml). The reaction was terminated by washing three times with ice-cold 0.9% NaCl (w/v). Cell-associated radioactivity was determined by lysing the cells with 0.05 N NaOH, followed by liquid scintillation counting, and normalized to the protein concentration.

**Protein Phosphotransferase Assays**—Transfected HA-tagged wt or kinase inactive (KD) RSK1 (K112/464R) was immunoprecipitated from cells starved overnight and treated with DMSO or PMA (100 ng/ml) for 30 min, as previously described (PMID: 23401857). Immunoprecipitations were washed three times in lysis buffer and twice in kinase buffer (25 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 5 mM β-glycerophosphate). Kinase assays were performed with bacterially purified GST- and GST-IRS-1 (wt or Ser1101A (SA)) fusion proteins as substrates, under linear assay conditions. Assays were performed for 10 min at 30 °C in kinase buffer, and all samples were subjected to SDS-PAGE followed by immunoblotting with IRS-1 phosphospecific antibodies. A similar procedure was used for the RSK1 phosphotransferase assay. Cells were serum deprived for 4 h and treated with 100 nM insulin for the last 45 min prior to cell lysis. Saturating amounts of antibody selective for the RSK1 isoform were incubated overnight with 500 μg of proteins. Immunoprecipitated RSK1 was incubated in a tube with GST-S6 in kinase buffer supplemented with 100 μM ATP and with or without RSK inhibitor (BI-D1870) 10 μM, S6K inhibitor (PF-4708671) 10 μM, or PMA (50 ng/ml) for 10 min at 30 °C. Inhibitors were added after the immunoprecipitation. All samples were subjected to SDS–PAGE followed by immunoblotting with anti-phospho-S6 Ser-235/236 antibody.

**Glucose Production**—FAO cells were incubated 16 h in serum-free medium, with or without insulin (100 nM). The cells were washed three times with PBS and incubated with phenol- and glucose-free DMEM medium supplemented with 20 mM sodium L-lactate and 2 mM sodium pyruvate for 5 h with or without insulin. Cell supernatants were collected, and glucose
concentration was measured with the Amplex-Red Glucose assay kit according to the manufacturer’s instructions (Invitrogen). Cells were lysed with 50 mM NaOH, and protein concentration was determined using a BCA protein assay kit to normalize glucose production.

Quantification and Statistical Analyses—Western blot was quantified with theQuantity One software version 4.6.9 (Bio-Rad). One- and two-way ANOVA with Bonferroni or Tukey post hoc and t-tests were performed with GraphPad Prism version 5.0a for Mac (GraphPad Software).

RESULTS

A Rapamycin-insensitive Kinase Phosphorylates IRS-1 at Normal AA Concentrations—As previously shown (4), insulin stimulates the phosphorylation of IRS-1, mainly through a rapamycin-sensitive kinase under conditions of nutrient overload, such as an excess of AA (see the IRS-1 molecular shift prevented by rapamycin under 2× AA conditions, Fig. 1A). However, at low to normal amino acid concentrations (0–1× AA), rapamycin only had minor effects on the insulin-induced higher molecular shift of IRS-1, suggesting that mTORC1/S6K1 is not responsible for the mobility shift. These results also indicate that other kinase(s) might phosphorylate IRS-1 in the absence of AA overload (see 0–1× AA conditions, Fig. 1A). Along the same line, the phosphorylation of RSK is not affected by different amino acid concentrations or rapamycin (Fig. 1A).

RSK1 Phosphorylates IRS-1 on Ser-1101—Upon analysis of the amino acid sequence of IRS-1, we noted that the peptidic environment around Ser-1101 is conserved throughout evolution and corresponds to the type 1 motif of phosphorylation present in many RSK substrates (RXRXX-pS/T-XXX, see figure legend for details; Fig. 1, B and C). We then searched within the human IRS-1 sequence for the presence of this consensus motif and found 5 putative serine residues of IRS-1 present in the RSK phosphorylation motif (Fig. 1D).

These analyses suggest that RSK might represent a new kinase that phosphorylates IRS-1 upon insulin treatment. Therefore, we used the predicted phosphorylation of Ser-1101 as a marker to validate the capacity of RSK to phosphorylate IRS-1 (see Fig. 1D). A non-radioactive kinase assay that relies on an antibody that specifically detects the phosphorylation of IRS-1 Ser-1101 was designed to assess the capacity of RSK to phosphorylate this serine residue in vitro (26). Recombinant wild-type or a S1101A-mutated C-terminal region of IRS-1 fused to a GST tag (C-ter IRS-1 wt and SA, respectively) was used as substrate (Fig. 1E) (9). HA-RSK1 kinase was immunoprecipitated from HEK293 cells that were stimulated or not with phorbol 12-myristate 13-acetate (PMA), a classical RSK activator (27). In basal conditions, no phosphorylation of the C-ter IRS-1 proteins was detected. However, in cells stimulated with PMA, the immunoprecipitated HA-RSK1 was able to phosphorylate the wt C-ter IRS-1 protein (Fig. 1E). This signal was specific as no phosphorylation could be detected when the C-ter IRS-1 SA mutant was used as a substrate. Moreover, the phosphorylation of the Ser-1101 residue appears to rely on an active RSK1 kinase per se, as a kinase dead (KD) mutant of HA-RSK1 could not phosphorylate Ser-1101 residue in the C-ter IRS-1 proteins, even in PMA-stimulated cells. These data indicate that RSK1 phosphorylates IRS-1 in vitro and that phosphorylation of Ser-1101 is achieved through a direct interaction between RSK1 and IRS-1.

Next, we sought to determine if RSK could also phosphorylate IRS-1 Ser-1101 in more typical insulin-targeted cells. L6 (rat myocytes) and HepG2 (human hepatocytes) cell lines were serum starved for 4 h and treated with 100 nM insulin. The activation of RSK was then assessed by Western blotting using antibodies directed against the phospho-Ser-221 or the phospho-Ser-380 residues of RSK, the last steps in the mechanism leading to the activation of RSK (16). We found that RSK is activated by insulin in a time-dependent manner in L6 myocytes (Fig. 2A) and in HepG2 hepatic cells (Fig. 2B). Moreover, RSK was activated by insulin with a kinetic similar to that of mTOR and S6K1, as revealed by phosphorylation of S6 protein in muscle cells (Fig. 2A) or mTOR and S6K1 proteins in hepatocytes (Fig. 2B).

We also determined that the RSK1 isoform is activated by insulin in L6 myocytes (Fig. 2, C and D). A non-radioactive kinase assay was performed to assess RSK’s ability to phosphorylate the Ser-235/6 residue of a recombinant GST-S6 in vitro after insulin treatment. Again, RSK1 activity was increased by insulin since insulin increased Ser235/236 phosphorylation of RSK1, and this activity was inhibited by a RSK inhibitor (BI-D1870). We also confirmed that S6K, a protein with an amino acid sequence similar to RSK, was not co-immunoprecipitated since a S6K1 inhibitor (PF-4708671) did not interfere with the phosphorylation of GST-S6 protein. These results show that RSK1 is activated in response to insulin stimulation in L6 myocytes, which is in agreement with a previous study showing that RSK could be activated by insulin in the epiphenolecric muscle of rats (28).

RSK Phosphorylates IRS-1 Ser-1101 Independently from mTORC1/S6K1—To distinguish the role of RSK and mTOR in the phosphorylation of Ser-1101 and Ser-636/9, we also treated L6 cells during the last hour of deprivation with BI-D1870, a pharmacological inhibitor of RSK (29) or the mTORC1 inhibitor, rapamycin. We used 10 μM BI-D1870 since this dose was required to inhibit RSK Ser-221 phosphorylation as well as phosphorylation of the RSK substrate S6 (Fig. 3A). It is also the dose required to reduce phosphorylation of IRS-1 on Ser-1101 by RSK (Fig. 3A). In vehicle-treated L6 cells (DMSO), stimulation with insulin significantly increased the phosphorylation of both Ser-1101 and Ser-636/9 (Fig. 3B). However, treatment with BI-D1870 selectively and significantly inhibited the phosphorylation of Ser-1101 without blocking the phosphorylation of Ser-636/9 residues (Fig. 3B). These results are in agreement with our bioinformatics analyses, which did not predict Ser-636/9 phosphorylation by RSK (see Fig. 1D). On the other hand, rapamycin treatment significantly blunted only the phosphorylation of IRS-1 Ser-636/9 residues in insulin-treated L6 cells under normal amino acid conditions as we previously observed (4) (Fig. 3B, lower panel).

The finding that rapamycin treatment failed to reduce the insulin-induced Ser-1101 phosphorylation of IRS-1 in L6 myocytes not exposed to nutrient stress (i.e. incubated with normal AA concentrations) suggests that the mTORC1/S6K1 pathway is not acting downstream of RSK to phosphorylate this site.
FIGURE 1. The IRS-1 scaffold protein presents putative residues that could be phosphorylated by the RSK kinase. A, differentiated L6 myotubes were incubated in increasing concentrations of amino acids (0X AA, 1X AA, 2X AA) for 60 min. Rapamycin was added to a final concentration of 25 nM over 60 min or, alternatively, equivalent volumes of DMSO (vehicle). Insulin was added for the last 45 min at a final concentration of 100 nM. Cells were then harvested and processed for SDS-PAGE and Western blot using the anti-IRS-1 polyclonal antibody, anti-phospho-S6K1 Thr-389 or anti-phospho-RSK Ser-221. Tubulin levels are presented as loading control.

B, WebLogo frequency plot of the target sequences in known RSK substrates, and the evolutionary conservation around IRS-1 Ser-1101. Bt, Bos taurus; Dr, Danio rerio; Hs, Homo sapiens; Mm, Mus musculus; Xt, Xenopus tropicalis. C, phosphorylation site alignment of a selection of known RSK substrates (human sequences).

D, human IRS-1 sequence was probed for the presence of RSK consensus sequence of phosphorylation (RXRX(S/T)) using ExPaSy tools (see “Experimental Procedures” for details). Putative serines phosphorylated by RSK are underlined with a solid line and highlighted in bold. The Ser-1101 residue is highlighted by a red line, whereas the residues Ser-636/9 that are phosphorylated downstream of the mTOR pathway and not recognized by RSK are shown with a dashed line.

E, HEK293 cells were transfected with the indicated constructs. After 24 h, cells were serum starved for 18 h and stimulated with or without PMA (50 ng/ml). Cells were lysed, and HA-RSK1 was immunoprecipitated from 200 μg of clarified lysate. Beads were washed and incubated with 100 ng of wt or Ser1101A (SA) mutant GST-IRS-1 C-ter in RSK kinase buffer supplemented with 200 μM ATP for 10 min at 30 °C. Proteins were then separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies. The relative amount of GST-IRS-1 C-terminal proteins used in kinase assays was controlled by loading 1 μg of each protein on SDS-PAGE followed by Coomassie staining.
However, previous studies have implicated RSK in the activation of the mTORC1/S6K1 pathway by inhibiting the tuberous sclerosis complex (TSC) (20, 30); thus promoting indirectly the phosphorylation of the mTORC1-scaffold protein Raptor, which then stimulates the activation of mTORC1 and/or via the phosphorylation of IRS-1 (20, 30); thus promoting indirectly the activation of mTORC1/S6K1 pathway by inhibiting the tuberous sclerosis complex (TSC) (20, 30); thus promoting indirectly downstream insulin signaling in muscle cells using Akt Ser-473 phosphorylation as a molecular readout. BI-D1870 treatment did not result in the expected increase in Akt phosphorylation despite the drug’s effect to blunt inhibitory IRS-1 phosphorylation on Ser-1101 (Fig. 3D). However, it has been previously reported that BI-D1870 partially inhibits insulin action through inhibition of Akt phosphorylation in 3T3-L1 cells, suggesting some off-target action of this inhibitor when used at 10 μM (31). We thus moved toward a genetic approach to inhibit RSK activity, using a dominant negative mutant of RSK1 (RSK1-DN) that was expressed in L6 myocytes using an adenoviral vector (22). As expected, in LacZ-expressing control cells, insulin could not increase the phosphorylation levels of this residue (Fig. 4A, white bars). However, under similar conditions, Ser-636/9 IRS-1 remained phosphorylated by insulin (Fig. 4A).

### Figure 2: RSK1 is activated in insulin-targeted cell lines

**A** L6 myotubes, and **B** HepG2 cells were incubated with 100 nM insulin for the indicated time. Following stimulation, cells were harvested and processed for SDS-PAGE and Western blot with the indicated antibodies. Tubulin levels are presented as loading control. Equal volumes of DMSO (vehicle) were added as control (n = 3). C, L6 cells were serum deprived and treated with 100 nM insulin for the last 45 min. Following cell lysis, saturating amounts of antibody selective for the RSK1 isoform were incubated overnight with 500 nM or 56K inhibitor (PF-4708671) 10 μM in a tube with kinase buffer supplemented with 100 μM ATP for 10 min at 30 °C. SDS-PAGE and anti-phospho-S6 Ser-235/236 GST-S6 and with or without RSK inhibitor (BI-D1870) 10 μM or S6K inhibitor (PF-4708671) 10 μM in a tube with kinase buffer supplemented with 100 μM ATP for 10 min at 30 °C. SDS-PAGE and anti-phospho-S6 Ser-235/236 were used to assess the level of RSK activity. n-a: no antibody, TCL: total cell lysate. **B** graphs show the mean ± S.E. of densitometry analyses of the Western blot (n = 4). *p < 0.05; A.U., arbitrary units.

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**Relative kinase activity**

|        | L6 cells | HepG2 cells |
|--------|----------|-------------|
| Insulin (100nM) | 0 5 15 30 45 90 180 min | 0 15 30 45 90 180 min |
| pRSK S380 | - - - + + + + | - - - + + + + |
| pRSK S221 | - - - + + + + | - - - + + + + |
| RSK | - - - + + + + | - - - + + + + |
| pIRS-1 S1101 | - + + + + + | - + + + + + |
| IRS-1 | - + + + + + | - + + + + + |
| pS6 S235/236 | - + + + + + | - + + + + + |
| S6 | - + + + + + | - + + + + + |
| pAKT S473 | - + + + + + | - + + + + + |
| Akt | - + + + + + | - + + + + + |
| Tubulin | + + + + + + | + + + + + + |

**D** graphs show the mean ± S.E. of densitometry analyses of the Western blot (n = 4). *p < 0.05; A.U., arbitrary units.
**Inhibition of RSK Activity Improves the Metabolic Effects of Insulin**—We have previously shown that IRS-1 phosphorylation on Ser-1101 contributes to the inhibition of insulin signaling at the level of PI3K/Akt (9). Thus we evaluated the association of the p85 subunit of PI3K to IRS-1 in muscle cells expressing either RSK1-DN or LacZ. Saturating amounts of antibody were used to immunoprecipitate IRS-1 followed by Western blot analysis. The data show that insulin stimulation increased the association of p85 with IRS-1 in control cells (LacZ, basal versus insulin; Fig. 4B), and this response is further increased in myocytes expressing RSK1-DN. Moreover, RSK1-DN expression was found to improve insulin-induced Akt phosphorylation, which was statistically significant for Ser-473 but not for Thr-308 site, as compared with LacZ-infected controls (Fig. 4C).

We next assessed whether the up-regulation of insulin signaling to PI3K/Akt by expression of RSK1-DN have a functional impact on muscle glucose metabolism by measuring insulin-mediated 2-deoxy-D-[3H]glucose uptake in L6 myocytes. Three days prior to experimentation, L6 myocytes in differentiation media were infected with adenoviruses encoding either RSK1-DN or LacZ as a control. Cells were then serum starved for 4 h and treated with 100 nm insulin for the last 45 min, and glucose uptake was measured (25). As shown in Fig. 4D, insulin induced a 1.7-fold increase in glucose uptake, which was further increased to 2.5-fold by the expression of the RSK1-DN construct compared with basal cells expressing LacZ. These results are consistent with the hypothesis that RSK activity is inhibitory to insulin's metabolic action in skeletal muscle.

To determine whether RSK also controls insulin action in liver, we then evaluated whether RSK inhibition enhances insulin-mediated suppression of gluconeogenesis in FAO hepatic cells. Three days after infection with either the LacZ or RSK1-DN adenoviruses, hepatic cells were serum deprived and then stimulated with the indicated concentrations of insulin for 16 h followed by incubation in a medium containing lactate and pyruvate as the sole source of carbon and increasing insulin concentrations. The glucose produced from these gluconeogenic substrates was then determined. In control LacZ-expressing cells, we observed a dose-dependent inhibition of glucose production by insulin (Fig. 5A). However, in FAO cells expressing the RSK1-DN construct, the suppressive action of insulin on glucose production was significantly potentiated at several
Inhibition of RSK1 activity improves insulin sensitivity in L6 cells. A, L6 myocytes were infected with an adenovirus encoding a dominant negative RSK1 mutant (RSK1-DN) or the control LacZ gene after 4 days of differentiation. After 7 days of differentiation, cells were serum deprived for 4 h and treated with 100 nM insulin for the last 45 min prior to cell lysis. Equal amounts of proteins were separated on SDS-PAGE and processed for Western blot analyses using the indicated antibodies. Tubulin levels were used as loading controls. Graph shows the mean S.E. of densitometry analyses of the Western blot normalized for the loading controls (n = 5). *, p < 0.05; A.U., arbitrary units. B, on the day of the experiment, L6 cells were serum deprived for 4 h and treated with 100 nM insulin for the last 45 min. Cells were then harvested, and equal amounts of total cell lysate were subjected to immunoprecipitation with an anti-IRS-1 antibody. As a control, irrelevant IgG was used. Western blot analyses using the indicated antibodies were performed. Total cell lysates (TCL) are shown to control the relative expression of each protein. The expression of either IRS-1 or p85 PI3K was not affected by expression of RIK1-DN (lower panel, TCL fractions). Equal amounts of irrelevant IgG were used as a control, and, as expected, IRS-1 was not immunoprecipitated under these conditions nor could we detect p85 in Western blot analyses. C, Western blot of pAkt Ser-473 and Thr-308. Actin levels were used as loading controls. Graph shows the mean ± S.E. of densitometry analyses of the Western blot normalized for the loading controls (n = 6). *, p < 0.05, A.U., arbitrary units. D, L6 myocytes were infected with an adenovirus encoding a dominant negative RSK1 mutant (RSK1-DN) or the control LacZ gene at day 4 of differentiation. After 7 days of differentiation, 2-deoxyglucose uptake was evaluated by incubating cells with 2-[^3H]deoxyglucose for 8 min. Myocytes were then lysed, and protein concentrations were evaluated to normalize radioactive counts. Results are presented in fold over basal-LacZ conditions and are the mean ± S.E. of three independent experiments. A.U., arbitrary units.

doses, indicating an improvement of hepatic insulin sensitivity upon RSK inhibition.

To examine whether improvement of insulin action in hepatic cells expressing RSK1-DN was linked to increased insulin signaling, we next measured the association of the p85 subunit of PI3K with IRS-1. As depicted in (Fig. 5B), insulin stimulation increased the amount of p85 subunit in IRS-1 immunoprecipitates from control cells (lacZ, basal versus insulin). In cells infected with RSK1-DN, we found that the association of p85 with IRS-1 was further increased upon insulin stimulation as compared with LacZ-expressing controls, suggesting that RSK1 inhibition improves insulin signaling to PI3K. The expression of either IRS-1 or p85 PI3K were not affected by expression of RSK1-DN (lower panel, TCL fractions). This improvement was also associated with an increase in insulin-induced pAkt on both Ser-473 and Thr-308. Moreover, RSK1 down-regulation increased phosphorylation of FoxO1 Ser-256 and GSK3 Ser-21/9, two important targets of Akt, in both the basal and insulin-stimulated state (Fig. 5C). We also measured the expression of gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) as well as a co-activator of Foxo1, PPARγ coactivator-1α (PGC1α). These results show that only G6Pase is decreased in RSK1-DN-infected cells, while PEPCK and PGC1α expression levels are similar between experimental conditions (Fig. 5D). These data indicate that, as observed in muscle cells, RSK1 restricts the metabolic effects of insulin in hepatic cells through the regulation of insulin signaling and by modulating G6Pase expression.

These results prompted us to evaluate the implication of RSK1 in the context of insulin resistance. Previously, Huang et al. reported that a sustained exposure of 24 h to a glucose/insulin-enriched medium (High G/I) significantly decreased insulin-induced glucose uptake in L6 myocytes by down-regu-
lating the IRS-1/PI3K signaling pathway (Fig. 6A) (32). We took advantage of this experimental paradigm to promote insulin resistance in L6 myocytes and test whether this could be prevented by RSK inhibition. As expected, incubation with the High G/I medium strongly interfered with the capacity of insulin to stimulate glucose uptake in control cells expressing LacZ (Fig. 6B). However, we found that RSK1-DN expression induced a significant 1.6-fold improvement of glucose uptake, consistent with a role for RSK in the promotion of insulin resistance in myocytes exposed to High G/I medium. We observed also that even with High G/I medium RSK1-DN was able to reduce IRS-1 phosphorylation on Ser-1101 in both basal and insulin conditions (Fig. 6C). This was linked to improved insulin signaling as revealed by enhanced Akt phosphorylation on both Ser-473 and Thr-308 residues upon insulin stimulation of RSK1-DN-expressing cells as compared with LacZ-infected controls (Fig. 6, C and D). These data suggest that RSK1 plays a role in the development of muscle insulin resistance following prolonged hyperinsulinemia and hyperglycemia, as commonly observed in insulin-resistant and glucose-intolerant subjects.

**DISCUSSION**

Insulin resistance is a hallmark of obesity-linked type 2 diabetes. The mechanisms leading to insulin resistance are currently viewed as an intricate network of molecular steps where signaling proteins like IRS-1 integrate signals provided by inflammation and nutrient-sensing pathways to generate a pathophysiologic response. For example, dysregulation of mTORC1/S6K1 signaling caused by hyperinsulinemia and nutrient overload in obese animals has been shown to increase IRS-1 serine phosphorylation and to down-regulate the IRS-1/PI3K/Akt signaling axis, which, in turn, interferes with the metabolic actions of insulin in skeletal muscle and liver (9, 33). However, this pathway appears to be dominant mostly in the context of nutrient excess, particularly when branched chain AA concentration is increased (4, 9, 34–36). It is still to be determined if IRS-1 may be phosphorylated by other kinase(s) in the context of normal nutrient levels. As shown in this report, IRS-1 can also be serine phosphorylated by prolonged insulin exposure, independently from nutrient status and mTORC1/
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S6K1 activation. We thus decided to explore the role of other Ser/Thr kinases in the regulation of IRS-1 signaling and their impact on glucose metabolism.

Herein we describe a new mechanism of regulation of insulin signaling through phosphorylation of the IRS-1 scaffold protein by the Ser/Thr kinase RSK. Indeed, we found that RSK is a novel mediator of IRS-1 serine phosphorylation, leading to inhibition of insulin’s metabolic actions in muscle and liver cells. Since we have previously shown that Ser-1101 in IRS-1 is a key site of negative regulation of insulin signaling for glucose metabolism (9), and that this site falls into the consensus motif of RSK, we explored the ability of this Ser/Thr kinase to phosphorylate this site in various cell types. We found that RSK can phosphorylate Ser-1101 of IRS-1 in HEK293 cells but not when a mutant IRS-1 SA mutant was transfected in these cells. We further showed that insulin increases RSK phosphorylation and activates RSK1 kinase in typical insulin target cells and that IRS-1 Ser-1101 was phosphorylated under these conditions. Using both a RSK inhibitor and a RSK1-DN mutant, we confirmed that RSK activation by insulin phosphorylates Ser-1101. We have further shown that RSK-induced IRS-1 Ser-1101 phosphorylation does not rely on nutrient excess and, accordingly, is independent of the mTORC1/S6K1 pathway. Insulin-induced RSK activation is therefore a novel autologous mechanism by which the hormone’s metabolic actions can be regulated through a negative feedback loop at the level of IRS-1, leading to attenuation of PI3K/Akt signaling. Our data further show that expression of the RSK1-DN mutant in muscle and hepatic cells increased insulin-stimulated glucose uptake and augmented the hormone’s sensitivity for suppression of glucose production, respectively. Both effects were linked to an increased association of the PI3K p85 subunit to IRS-1 suggesting that insulin signaling was improved through reduced inhibitory Ser-1101 phosphorylation of IRS-1. As expected, this translates into an increased phosphorylation of Akt in both muscle and hepatic cells. Moreover, phosphorylation of Foxo1 and GSK3, two important targets of Akt controlling glucose metabolism, were increased in FAO cells. Since glucose production was reduced in RSK1-DN-infected cells, we measured the expression of gluconeogenic enzymes. Interestingly, only G6Pase protein expression was decreased by the RSK1-DN, while PEPCK expression was similar between LacZ and infected cells. Foxo1 has been reported to induce higher activation of G6Pase than that of PEPCK, and this could be due to the presence of only one insulin response sequence for Foxo1 in the latter gene in comparison to three insulin response sequences in the G6Pase promoter (37–39). Additionally, overexpression of Foxo1 increases the expression of the catalytic subunit of G6Pase in response to CAMP and dexamethasone, and not the expression of PEPCK (40). However, more studies are needed to understand the role of RSK on hepatic gluconeogenic enzyme expression, specifically regarding insulin regulation of gluconeogenic enzyme expression and function in insulin-resistant conditions.

RSK is a downstream effector of the ERK/MAP kinase pathway, and previous studies have shown a potential role for ERK in mediating IRS-1 serine phosphorylation in both inflammatory and nutrient stress conditions. Indeed, ERK has been reported to phosphorylate IRS-1 on Ser-612 and Ser-632 and this was proposed to modulate the association of the p85 subunit of PI3K to IRS-1 (3). Furthermore, elevated Ser-636 phosphorylation of IRS-1 (corresponding to the Ser-632 in rodents) has been correlated to higher ERK activity in primary cultures of skeletal muscles from type 2 diabetic patients (41). Interestingly, cross-breeding ERK1−/− mice with genetically obese...
ob/ob mice was found to improve their insulin sensitivity in association with improved Akt phosphorylation suggesting that the ERK pathway is implicated in obesity-linked insulin resistance (42). However, it remains to be clarified if ERK itself is the principal mediator of inhibitory serine IRS-1 phosphorylation in these in vitro and in vivo studies, or if downstream kinases such as RSK could be also involved. Indeed, although ERK phosphorylates Ser-636 (43), it is not expected to phosphorylate Ser-1101 since this site is not followed by a proline, and ERK is a proline-directed kinase. On the other hand, we have demonstrated that Ser-636 is not a target of RSK, since its inhibition did not affect insulin-mediated Ser-636 phosphorylation in muscle cells. Taken together, these results suggest that ERK-mediated Ser-636 phosphorylation is independent of RSK, but that RSK could be a downstream mediator of Ser-1101 phosphorylation upon activation of the ERK/MAP kinase pathway. More studies will be needed to determine whether the other 4 Ser residues within IRS-1 (Fig. 1D), which were also identified as potential RSK target sites by our bioinformatic analysis, are also phosphorylated by RSK in vivo and whether they can alter PI3K/Akt signaling and glucose metabolism.

Our finding that RSK-mediated autologous inhibition of insulin signaling is likely involved in the physiological regulation of insulin’s metabolic actions does not exclude that it may also be implicated in the development of insulin resistance. Indeed, we found that interfering with RSK1 activity not only potentiated the physiological response to insulin but also improved insulin sensitivity in a well-known model of insulin resistance induced by chronic exposure to high glucose high insulin in L6 myocytes. These data are consistent with the hypothesis that RSK promotes insulin resistance through increasing IRS-1 Ser-1101 phosphorylation thereby attenuating the activation of PI3K/Akt signaling, a negative feedback mechanism that is shared by S6K1 to promote insulin resistance upon nutrient excess (9).

Whereas our results suggest a new role for the RSK pathway in the control of insulin’s metabolic actions, our conclusions are limited to the RSK1 isoform, and we cannot exclude a role of other RSK isoforms, as the approaches used to interfere with RSK activity (e.g. pharmacological inhibition BI-D1870 and expression of the RSK1-DN mutant) cannot discriminate the potential roles of other RSK isoforms. Indeed, exploration of the BioGPS database revealed that RSK1, RSK2, and RSK3 are expressed in skeletal muscle while RSK1 is the main isoform expressed in liver from both human and mice (13). With regards to RSK2, Chang et al. (44) previously reported that its activity is decreased in muscles of obese and insulin-resistant mice. This was measured after a single time point (15 min) following in vivo insulin injection. Moreover, ERK1/2 activation was also reduced in the same animals, which is in contrast with others studies that reported increased ERK1/2 MAPK activity in obese animals (45–47) and with the observation that ERK1−/− mice are more insulin sensitive and protected from high-fat diet-induced obesity (48). The conclusions of the present study may seem at odds with a previous study reporting that RSK2−/− mice develop insulin resistance and are glucose intolerant (49). However, these mice are also lipodystrophic, which likely explains their secondary insulin resistance through lipotoxicity. It should also be noted that despite their lower body weight and whole-body insulin resistance, RSK2−/− mice actually showed enhanced insulin-stimulated Akt phosphorylation in skeletal muscle, which supports a negative role for this RSK isoform in the control of insulin’s metabolic signaling (50). Further analysis of the role of both RSK1 and RSK2 isoforms in the regulation of insulin action, and glucose metabolism in muscle and liver is therefore warranted. Selective deletion of individual RSK isoforms in those tissues will likely be needed to confirm their specific metabolic functions in vivo as well as their role in the development of insulin resistance in obese and hyperinsulinemic states.

Taken together, our results demonstrate that RSK is involved in the negative regulation of IRS-1 through its ability to directly phosphorylate Ser-1101, independently from nutrient excess and from mTORC1/S6K1 activation. Using a RSK1-DN mutant further allowed us to demonstrate a functional role for RSK1 as a negative regulator of glucose metabolism in muscle and liver cells, through IRS-1 Ser-1101 phosphorylation and inhibition of PI3K/Akt signaling. Our findings that RSK1 mediates insulin resistance in HG/Hi-treated muscle cells further suggest that RSK is a new potential target for the treatment of insulin resistance, at least in vitro. Future studies in animal models lacking individual RSK isoforms in key metabolic tissues will be needed to fully understand the role of each member of this family of Ser/Thr kinases in the regulation of insulin action in physiological and pathological states.

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